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Abstract:

Background - Patients with congenital heart disease (CHD) and heterotaxy show high postsurgical morbidity/mortality, with some developing respiratory complications. While this 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, typically low with PCD. Eighteen patients exhibited CD characterized by abnormal ciliary motion and nNO below or near the PCD cut off values. Patients with CD >6 years old show increased respiratory symptoms similar to that 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 two mutations in *DNAH11*, the only PCD gene known to cause hyperkinetic beat. Amongst PCD patients, two had known PCD causing *CCDC39* and *CCDC40* mutations.

Conclusions - Our studies show 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.

Key words: genomic studies; heart defects, congenital; heterotaxy; nitric oxide; primary ciliary dyskinesia

Introduction

Heterotaxy is characterized by randomized variation in the patterning of left-right asymmetry of visceral organs in the thoracic and abdominal cavities. As left-right asymmetries in the cardiovascular system is 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 post-operative morbidity and mortality[1], often with prolonged clinical courses associated with respiratory and multiple serious complication[2]. A retrospective review of 96 patients with congenital heart disease (CHD) operated at Children's Hospital in Boston showed 11 of 16 patients dying during the one-month follow-up period were heterotaxy patients[3]. Another study analyzing surgical outcomes showed 4.8% post-operative mortality for CHD patients with heterotaxy vs. 2.4% for CHD without heterotaxy[4]. Our retrospective study of CHD patients undergoing cardiac surgery at Children's National Medical Center showed 84 heterotaxy patients had greater post-surgical mortality, increased post-surgical respiratory complications, and more complicated post-surgical course as compared to 634 CHD patients without heterotaxy undergoing surgeries with comparable RACHS-1 scores[5]. These findings suggest 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 as 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 more than 500 proteins[6]. Cilia are found from single cell organisms such as *Chlamydomonas* to man 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 due to immotile cilia or abnormal ciliary motion[8]. Consequently, PCD patients often exhibit newborn respiratory distress and develop sinopulmonary disease, including bronchiectasis. As 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 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]. While 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 amongst over 300 PCD patients examined, with 1/3 of the heterotaxy patients also exhibiting CHD[13]. As 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/congenital heart disease.

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 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 CNMC, NIH, University of Pittsburgh

and University of North Carolina using 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 old. Medical and family history was obtained using information from a questionnaire and medical chart review. Blood and/or saliva samples were obtained for genetic analysis. Cardio-thoraco-abdominal organ situs was assessed utilizing data obtained by echocardiography, catheterization, and chest and abdominal radiographs and sonographs (see Supplemental Tables 1,2). Cardiac situs was delineated using Van Praagh segmental classification[14].

Nasal Nitric Oxide Measurements

Nasal NO (nNO) measurements were made using a chemiluminescence nitric oxide analyzer (CLD88 SP, ECOPHYSICS AG) with established protocols[15] (see Supplemental Methods and Supplemental Figure 2). For participants >6 years old, measurements were made using velum-closure technique according to ATS/ERS guidelines. In patients <6 years old, NO measurements were made using tidal breath sampling[16]. Some patients with CD and low nNO were reevaluated with a sweat chloride test to exclude cystic fibrosis (Supplemental Table 1).

Nasal Tissue Sampling and Ciliary Motion Analysis

Nasal tissue was collected using Rhino-Probe (Arlington Scientific, Springville, UT) curettage of the inferior nasal turbinate. Exclusion criteria included severe bleeding diathesis or condition 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 100X oil objective under DIC optics. Movies were recorded at 200 frames/sec at room temperature using a Phantom v4.2 camera (Vision Research, NJ) and digital recordings were evaluated by a blinded panel of co-investigators (ML, MK,RF,CL,HO).

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 carried out using standard protocols (http://www.nimblegen.com/products/lit/SeqCap_UsersGuide_Service_v3p0.pdf). Exome 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 using AB Bioscope, with further analysis using CLC Genomics Workbench v4.0.3. Single-nucleotide variants and small insertions/deletions (1-10bp) were identified by SNP/DIP detection program at bases with quality scores >20 (99% accuracy), coverage >5x and variant allele frequency >20%. NCVs were validated by Sanger sequencing.

Statistical Analysis

The Welch's 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 vs. no-CD heterotaxy patients, healthy controls, or PCD patients; CD-A vs. CD-B; no-CD vs. healthy control) in each age group, and a *P*-value < 0.01 was considered statistically significant. If nNO measurements in the CD group was 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 Chi-square 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's 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's exact test and the Kruskal-Wallis test, respectively, and a *P*-value <0.025 was considered statistically significant for each

test based on the Bonferroni correction. Pairwise comparisons were performed using the Fisher's 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

48 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 1) 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 Supplementary 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; Supplemental Table 1)[11,17]. Thoracic laterality defects and other disorders were also recorded (see Supplemental Table 2). Patients were further grouped as perioperative for those hospitalized for surgical procedures, or non-perioperative for patients that are either seen in the Adult Congenital Heart Disease Clinic or undergoing in hospital diagnostic evaluation (see Supplemental Methods) (Table 1). In addition, we also recruited 18 PCD patients without congenital heart disease as disease controls and 25 normal 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 vs. healthy controls ($p>0.5$) (Supplemental Figure 1). 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 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 comprised of forward and reverse strokes sweeping in a planar motion synchronously across the respiratory epithelium (Figure 2D;Supplementary Movie 1)[18]. In PCD patients, a wide range of ciliary motion defects were observed, including immotility, stiff/dyskinetic ciliary motion, and other motion defects (Supplemental Movie 2 for PCD patient 9028; Supplemental Table 6).

Many of the heterotaxy patients also exhibited aberrant ciliary motion (Table 1; Supplemental Movies 3-6). This included immotile cilia, cilia with stiff/dyskinetic beat (Supplemental Movie 3), incomplete stroke (ciliary beat with decreased amplitude) (Supplemental Movie 4 and Figure 2E), wavy stroke (Supplemental Movies 5; Figure 2F), or asynchronous beat (Supplemental Movie 6). 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 (EM) 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, EM analysis of 9 PCD patients

showed 7 with a variety of ultrastructural defects typically seen with PCD (Supplemental Table 6).

Assessment of Nasal Nitric Oxide

We measured nasal NO (nNO), which is typically low in PCD patients. As nNO increased with age, we grouped the nNO measurements into three groups: <1 year old, 1-6 years old, and >6 years old. Newborns exhibit nNO just a few nl/min at birth. At 1-6 years old, nNO levels rise to over 100 nl/min[16]. In healthy adults, nNO values are usually over 200 nl/min[19]. PCD patients 1-6 years have nNO values <50 nl/min, and those >6 years have nNO values <100 nl/min[20]. For patients <1 year old, nNO cut off values have not been established, given nNO values are usually low but dynamically increasing.

For controls, we sampled 25 healthy adults, which 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 cut off values (Tables 1,2; Figure 3). We noted no difference in NO levels in perioperative vs. nonperioperative patients (Supplementary Table 3). 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 yr)**:** 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; $P<0.001$). Amongst the 9 patients with CD, 5 patients had nNO of 64.2±28.7 nl/min, below the 100 nl/min PCD cut off value, and are referred to as CD type A or CD-A (Table 2). Four had nNO of 134.8±19.6nl/min, just above the PCD cut off 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 from the PCD patients (*P*<=0.001) (Table 2).

Patients 1-6 yrs (median 2.5 yr)**:** 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 cut off value and thus was categorized as CD-A (Table 2). The other three with ciliary motion defects exhibited 58.3±3.2 nl/min, just above the 50 nl/min PCD cut off 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) vs. no CD (79.8±34.3) (*P*=0.12), but the CD patient nNO measurements were significantly different from that 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 old and perioperatively at 5.5 months of age (see below). The assessment at 1.4 years old yielded 34 nl/min nNO, within the PCD range (Table 1).

Patients <1 yr of age (median 8 months): Five heterotaxy patients in this age group with ciliary motion defects had mean nNO of 6.1 ± 2.7nl/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 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 old.

Analysis of Laterality Defects, Operative Status, and Gender

The prevalence of CD was greater in the cohort with both cardiovascular and abdominal/lung laterality defects (57%; Cohort II) vs. those with cardiovascular laterality defects only (25%; Cohort I) (*P*<0.05) (Figure 1), but it was not different between the perioperative vs. nonperioperative patients (Supplementary Table 4). In the >6 year old group, CD (type A and B)

was observed in 8 nonperioperative vs. 1 perioperative patient (Table 1). 50% of the CD patients were male, as compared to only 28% males amongst patients with no CD, but this difference was not significant given the small sample size (P=0.14; Supplementary Table 4).

Analysis of Respiratory Symptoms

Analysis of the prevalence of respiratory symptoms showed 24 of the 43 (56%) patients have history of at least one chronic respiratory manifestation. This was not significantly different between the CD vs. non-CD cohort with 10 (56%) of the 18 CD vs.14 (56%) of the 25 no-CD patients reporting at least one respiratory symptom (P>0.1) (Table 3; Supplementary Table 5). Comparison of the number of perioperative vs. non-perioperative patients with respiratory symptoms also showed no difference (Supplemental Table 3). Amongst the 9 patients >6 years with at least one respiratory manifestation, 5 patients with CD had more symptoms when compared to the 4 no-CD patients (median are 4 and 2, respectively, P = 0.024). Sequela associated with PCD (neonatal respiratory distress, bronchiectasis, or recurrent otitis media) were present in 5 of the 8 CD patients > 6 yrs 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 Supplemental Materials and Methods)[21-23]. Sequencing analysis was carried out 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 42X was obtained encompassing 99-100% of the bases in 12 of the 14 PCD genes (Supplementary Table 7). 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 (SNV), 34 of which validated by Sanger sequencing (Table 4, Supplemental Table 8).

Four PCD patients were found to have two known PCD causing mutations (Supplemental Table 8). 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 (Supplemental Table 6). 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 (Supplemental Table 6). In patients 1174, two novel *DNAH5* mutations were also found in trans, and consistent with a role in disease, EM analysis showed outer dynein arm defects (Supplemental Table 6). Sequencing analysis of the 13 heterotaxy patients with CD revealed one individual (9002) heterozygous for the *DNAI1* founder mutation, IVS1+2_3insT, known to cause PCD[24]. Another patient (9026) had three novel mutations, 2 in *DNAH5* and 1 in *DNHA11*. Patient 9003 exhibited two *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; Supplemental Table 9), followed by heterotaxy patients with CD yielding 0.769 NCV/subject, then heterotaxy patients with no-CD with 0.417 NCV/subject (Figure 4; Supplemental Table 9). 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 when compared to controls (Figure 4; Supplemental Table 9). This is not accountable by differences in racial composition, as Fisher's exact test for race-ethnicity between

the control subjects vs. heterotaxy patients with CD yielded p=0.33 (Table 4; Supplemental Table 8).

Discussion

We showed 18 of 43 (42%) of patients with CHD associated with heterotaxy have CD. The abnormal ciliary motion is similar to those seen with PCD, including dyskinetic, stiff or wavy ciliary beat. The latter may be comparable to the whip-like 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 cut off values. Post-surgical trauma cannot account for the finding of CD, since 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. As PCD and heterotaxy each have a prevalence of 1 in 15,000 to 20,000 live births, this is not likely by chance, but may reflect the common requirement for motile cilia in mucociliary clearance and left-right patterning.

Ciliary Dysfunction in Heterotaxy vs. PCD Patients

While we observed heterotaxy patients with CD have low nNO, their mean nNO values were higher than that of PCD patients, even amongst CD-A patients. Heterotaxy patients with CD also have higher CBFs than PCD patients. Moreover, none of the 11 heterotaxy patients with CD examined by EM 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 mutations in PCD genes (*Dnahc5*, *Dnaic1, Dnahc11)* can cause CHD with heterotaxy[11, 27, 28]. However, these may be under represented

amongst heterotaxy patients, as 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% amongst 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 as compared to healthy controls. We noted 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, while four PCD patients (three unrelated) had 2 known PCD causing *CCDC39 or CCDC40* mutations. As PCD is a recessive disorder, PCD causing mutations are expected to be homozygous or compound heterozygous. Consistent with this, three PCD patients were homozygous or compound heterozygous for PCD causing *CCDC39 or CCDC40* mutations. In contrast, no heterotaxy patients had two known PCD mutations.

For 5 heterotaxy patients with CD and two 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 PCD or CD in heterotaxy patients may arise from two mutations, one in each of two 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 vs. PCD patients. This genetic model is supported by studies in *Chlamydomonas* using stable diploids, which has shown noncomplementation of unlinked genes required for ciliogenesis[30, 31].

Heterotaxy patient 9003 had two *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). While 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 1/3 exhibiting abnormal spleens[29].

Respiratory Diseases in Heterotaxy Patients with Ciliary Dysfunction

Respiratory symptoms and disease were increased in heterotaxy patients with CD over 6 years old. 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.

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Conflict of Interest Disclosures: None

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Table 1. Cilia analysis and NO measurements

		Age	Nasal NO (nl/min)	Cilia Analysis Motion*	CBF(Hz)	Cohort†	Cilia‡ Function	EM‖
> 6 years perioperative								
9011	Asp	12 yr	85	e	4.9	II	CD-A	Y
9013		7 yr	115	a	4.7	I	no-CD	Y
9016		19 yr	216	a	3.9	I	no-CD	Y
9046		9 yr	238	a,j	n/a	II	no-CD	N
nonperioperative								
9003	Polysp	26 yr	31	b,d,e	16	II	CD-A	Y
9020	?	46 yr	90	b,d	7.7	I	CD-A	Y
9032	Normal	19 yr	80	d	3.6	I	CD-A	Y
9037	Normal?	18 yr	35	b,c	9.5	II	CD-A	Y
9008	Normal	10 yr	138	b,g	4.2	I	CD-B	N
9026	Polysp	18 y	161	d	6.8	II	CD-B	Y
9027	Left Iso	6.5yr	123§	b,j	6.2	II	CD-B	N
9031	Normal	15 yr	117	b	7.2	II	CD-B	N
9009		30 yr	205	d	5.1	I	no-CD	Y
9019		44 yr	392	a	7.5	I	no-CD	N
9033		29 yr	270	d	5.1	I	no-CD	Y
9035		28 yr	695	a	4.7	II	no-CD	Y
9036		25 yr	194	a	4.9	I	no-CD	Y
9038		50 yr	398	a	3.2	I	no-CD	N
9042		12 yr	256	a	7.1	I	no-CD	N
1-6 years perioperative								
9010	?	2 yr	59	d,f	4.9	I	CD-B	N
9017	Normal	5.9 yr	55	d,f	8.7	II	CD-B	Y
9014		3 yr	64§	a	3	I	no-CD	Y
9022		2.5 yr	56	a	8.1	I	no-CD	ND
9024		2 yr	56	a	8.8	II	no-CD	Y
nonperioperative								
9004	Asplen	1.4 yr	34§	j	n/a	II	CD-A	N
9006	Unkno	2.5 yr	61	d,e	6	II	CD-B	Y
9001		5 yr	141	a	7.1	II	no-CD	Y
9007		18 mo	86§	a.g	10.7	II	no-CD	Y
< 1 year perioperative								
9004	Asplen	5.5mo	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 days	9.2	e	n/a	II	CD	Y
9005		24 days	11.7	a	8.7	II	no-CD	N
9025		10 days	10	a	8.5	II	no-CD	N
9039		18 days	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		0.8mo	14	a	9.7	I	no-CD	Y
nonperioperative								
9002	Aplenia	17 days	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 days	20	a	7.2	II	no-CD	Y
9041		8 days	10	a	6	I	no-CD	Y

*a=Normal, b=Stiff/Dyskinetic, c=Immotile, d=Incomplete Stroke, e=Wavy Stroke, f=Asynchronous; g=Excessive mucus present, j=Few/no cilia seen

†Cohort I= cardiovascular situs abnormalities only; Cohort II= cardiovascular with thoracic and/or abdominal situs abnormalities;

‡CD=Ciliary Dysfunction; CD-A=Ciliary Dysfunction with nNO below PCD cut off, CD-B=Ciliary Dysfunction with reduced nNO value bordering PCD cut off

‖cilia EM obtained (Y) or not adequate/not available (N).

§nNO remeasured.

Table 2. Nasal NO measurements in heterotaxy patients*

Age	Heterotaxy Patients				Healthy Controls	PCD
	All with CD (n=18)	CD-A (n=6)	CD-B (n=12)	no-CD (n=25)		
< 1 year (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§					
1-6 years (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*=0.008‖ *P*<0.001§		*P*<0.001‖ *P*<0.001§	*P* = 0.097†		
≥6 years (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*<0.001§	*P*<0.001‡ *P*=0.003‖ *P*=0.001§	*P*=0.002‡ *P*<0.001‖ *P*<0.001§			

*nNO in nl/min.
†Adapted from Chawla et al.[16]
‖,§Welch's t-test comparison of CD with no-CD‡, with controls‖, with PCD patients§.
#CD-A vs. CD-B comparison
**no-CD vs. healthy control comparison.

Table 3. Respiratory manifestations in heterotaxy patients*

		Age	Gender	Recurrent otitis media	Recurrent lower respiratory illnesses	Neonatal respiratory Distress	Chronic wet cough	Chronic nasal congestion	Chronic sinusitis	Respiratory Insufficiency/ Tracheotomy	Bronchiectasis
> 6 years perioperative											
CD-A	9011	12 yr	F	N	N	N	N	N	N	N	N
	9013	7 yr	F	N	Y	N	N	N	N	N	N
	9016	19 yr	F	N	N	N	N	N	N	N	N
	9046	9 yr	F	Y	N	N	N	N	N	N	N
nonperioperative†											
CD-A	9003	26 yr	F	**Y**	**Y**	Y	Y	N	Y	N	Y
CD-A	9020	46 yr	F	**Y**	**Y**	N	Y	N	N	N	N
CD-A	9032	19 yr	M	N	N	N	N	N	N	N	N
CD-A	9037	18 yr	M	Y	N	Y	Y	Y	Y	N	N
CD-B	9008	10 yr	M	N	N	N	N	N	N	N	N
CD-B	9026	18 y	F	N	N	N	N	N	N	N	N
CD-B	9027	6.5yr	F	N	Y	Y	Y	Y	N	N	N
CD-B	9031	15 yr	M	Y	Y	N	N	Y	Y	N	N
	9009	30 yr	F	N	N	N	N	N	N	N	N
	9019	44 yr	M	N	Y	N	Y	N	Y	N	N
	9033	29 yr	M	N	N	N	N	N	N	N	N
	9035	28 yr	F	N	N	N	N	N	N	N	N
	9036	25 yr	M	N	N	N	N	N	Y	N	N
	9038	50 yr	F	N	Y	N	N	N	Y	N	N
	9042	12 yr	F	N	N	N	Y	Y	N	N	N

*Y=with symptoms; N=without symptoms
Black vs. gray highlighting denote respiratory symptoms in CD vs. no-CD patients, respectively.
†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	Afric Amer	**CD**	***DNAH11***	4520A>C	Q1507P‡
			DNAH11	9397G>A	E3133K‡
9004	Asian	**CD**	***TXNDC3***	1630G>A	A544T‡
9006	White	**CD**	***CCDC39***	626C>G	A209G‡
9008	Afric Amer	**CD**	None		
9011	White	**CD**	None		
9015	White	**CD**	***LRRC50***	1294G>A	E432K
9017	Afric Amer	**CD**	None		
9018	Afric Amer	**CD**	None		
9026	Afric Amer	**CD**	***DNAH11***	9203A>G	E3068G‡
			DNAH5	11140A>G	I3714V
			DNAH5	638C>A	P213Q‡
9027	White	**CD**	None		
9031	White	**CD**	None		
9037	Afric Amer	**CD**	***DNAI1***	1579T>G	S527A‡
9005	Afric Amer	no-CD	None		
9007	Afric Amer	no-CD	None		
9009	White	no-CD	None		
9012	Afric Amer	no-CD	None		
9016	White	no-CD	None		
9019	Afric Amer	no-CD	***DNAH5***	6710A>G	N2237S
9024	Afric Amer	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	V393M
9035	White	no-CD	None		
9040	Afric Amer	no-CD	None		
9068	White	no-CD	None		

*Ciliary dysfunction (CD).
†Each allele listed, with homozygous mutation listed twice.
‡*DNAI1* founder mutation[24]

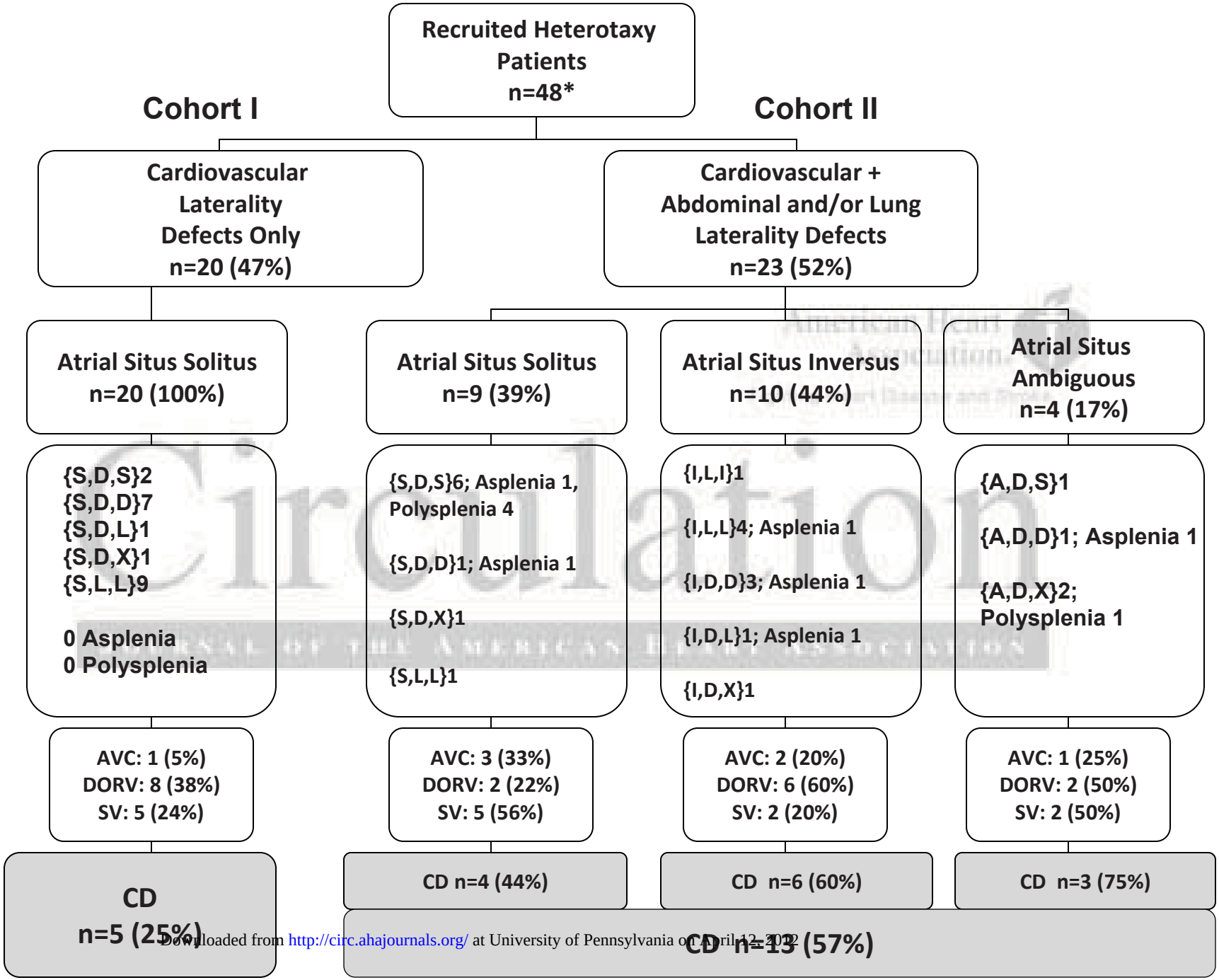
Figure Legends:

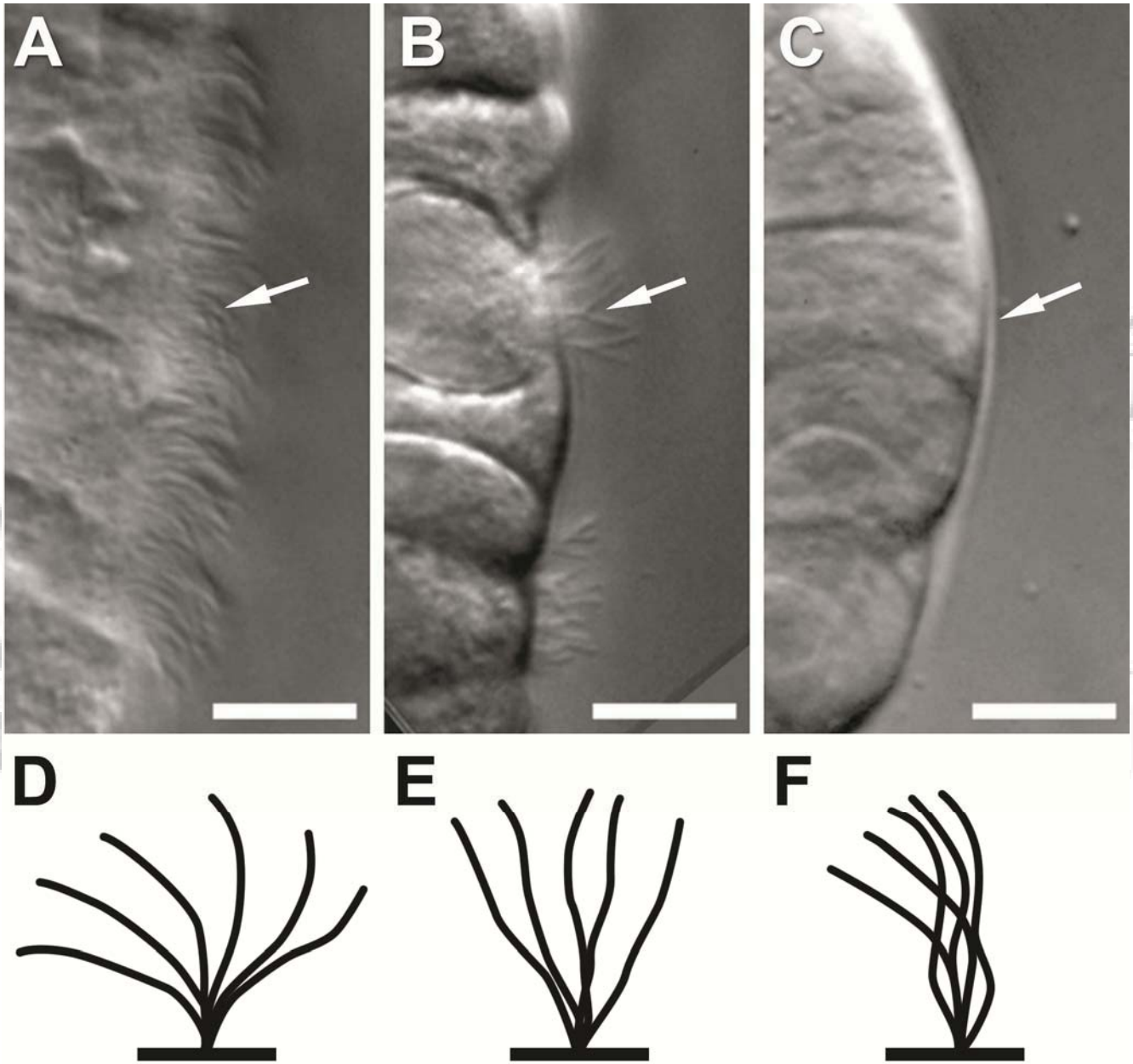
Figure 1. Laterality and Cardiovascular Defects in Heterotaxy Patients**.** Patients recruited for study, with 5 failing to complete study. AVC:atrioventricular canal, DORV:Double Outlet Right Ventricle,SV:single ventricle, CD:ciliary dysfunction.

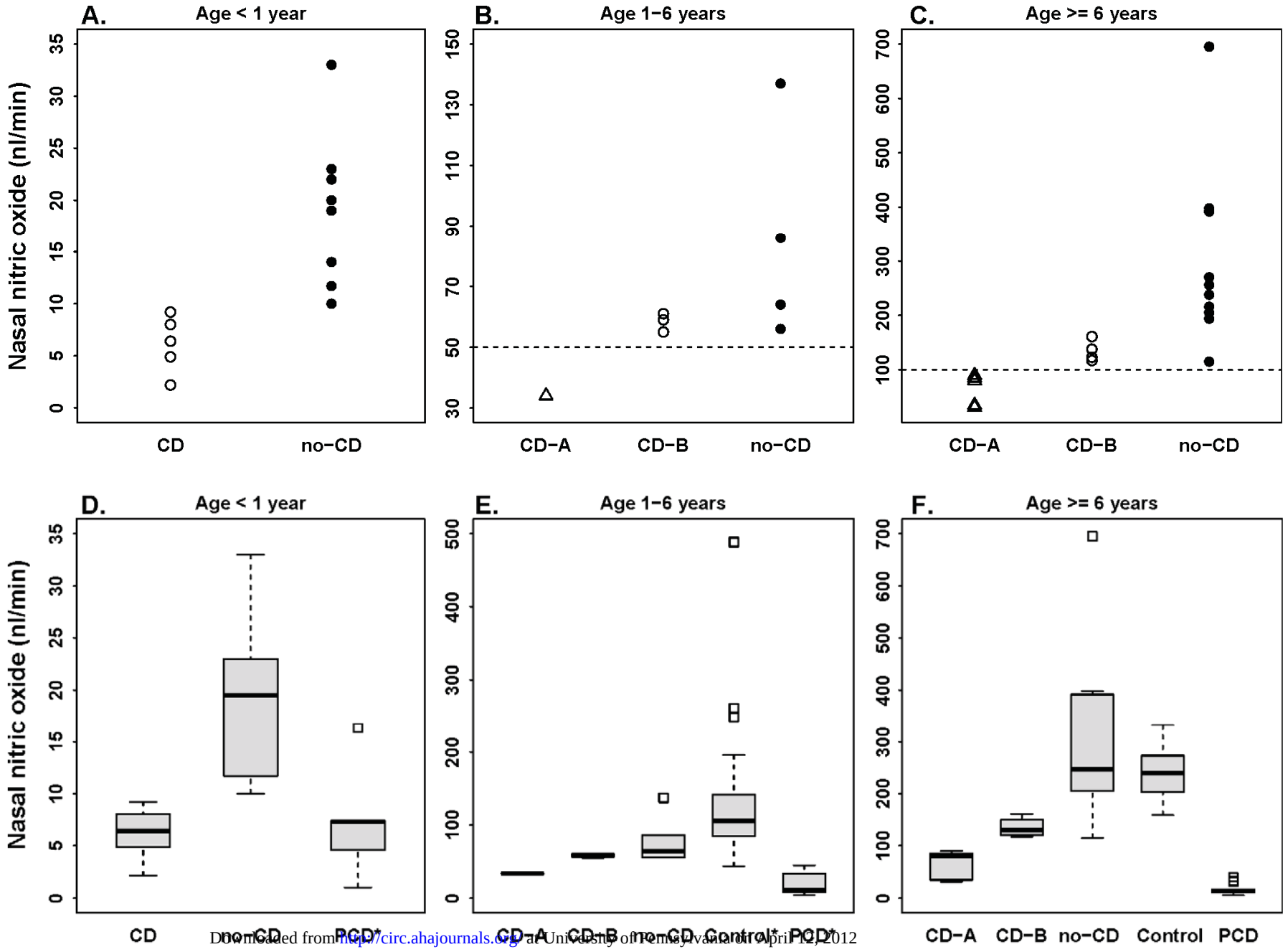
Figure 2. Ciliary motion in nasal epithelia from heterotaxy and PCD patients. **(A)** Abundant cilia (arrow) were observed in patient 9033 exhibiting normal ciliary motion. **(B)** Paucity of cilia (arrow) in 9027 with CD. **(C)** No cilia found in nasal epithelia (arrow) of 9004. **(D)** Healthy control showing normal motion with full forward and recovery strokes (Supplementary Movies1). **(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 (Supplementary Movie5). Scale bar=10 μm.

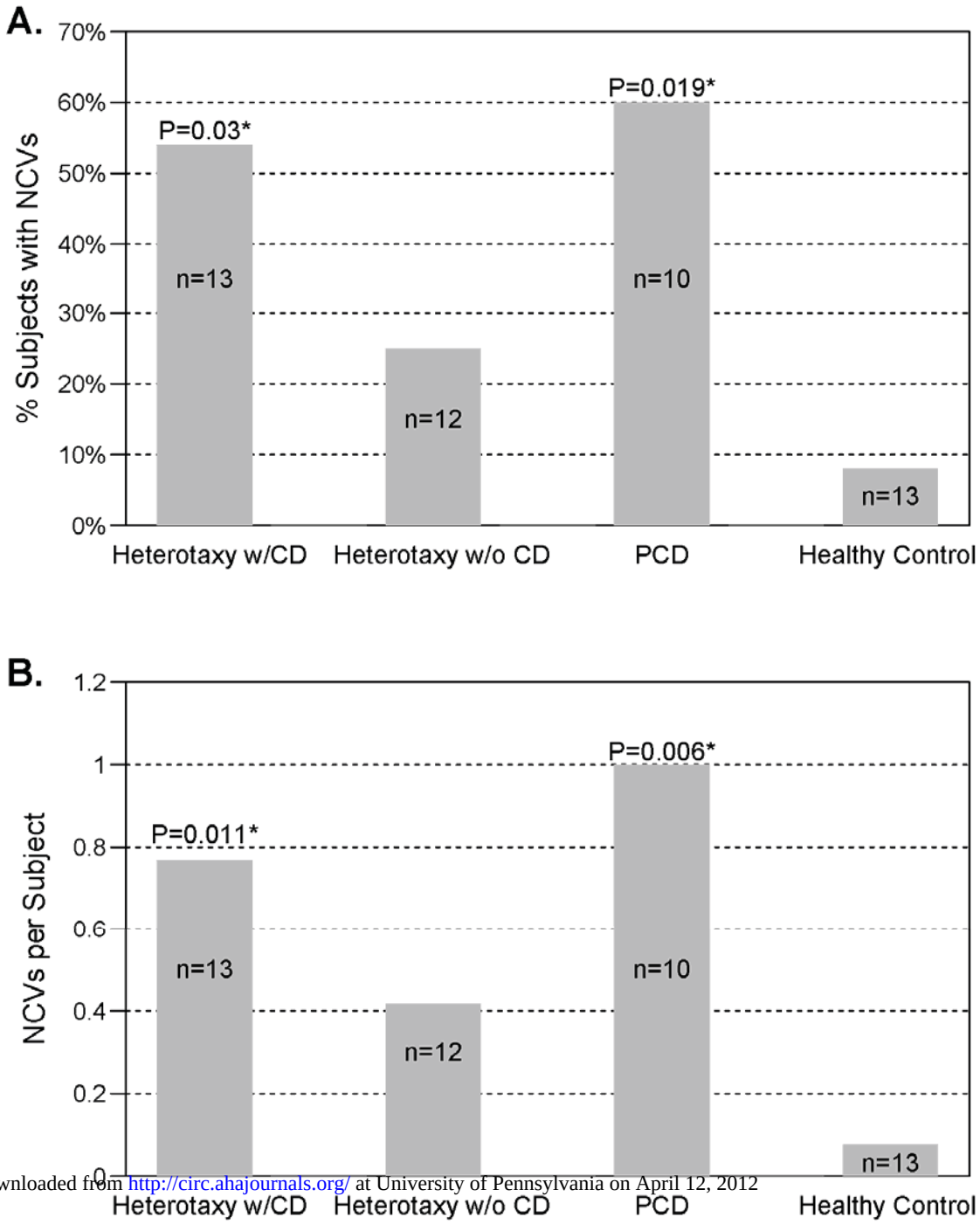
Figure 3. Nasal nitric oxide measurements in heterotaxy patients. (**A-C**) nNO measurements from each patient are plotted in three age groups. **(D-F)** Box plots show interquartile range (IQR) from 25-75th percentile, with median denoted by bold line. Whiskers denote minimum-maximum value not more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

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. Asterisk refers to p values obtained with comparison to controls. Statistical analysis was carried out with Fisher's exact test **(A)** or Kruskal Wallis followed by Wilcoxon rank sum test **(B)** with p-value <0.025 considered significant based on Bonferroni correction.









SUPPLEMENTAL MATERIAL

A. SUPPLEMENTAL METHODS

Patient Recruitment

CHD patients with heterotaxy and some PCD patients were recruited from Children's National Medical Center (CNMC). Other PCD patients and healthy control subjects were recruited from the NIH, University of North Carolina, and University of Pittsburgh. All centers used the same testing protocols devised by the Mucociliary Clearance Consortium headed by Dr. Michael Knowles. A total of 48 CHD patients were recruited into this study. This included those with CHD and abnormal or discordant situs in either pulmonary, gastrointestinal, splenic, hepatobiliary and/or cardiovascular systems. Thus CHD patients with polysplenia or asplenia, pulmonary or bronchial isomerism, midline or left-sided liver, malrotation and midline or right-sided stomach were included. Also included were CHD patients with cardiovascular situs anomalies only, such as dextrocardia or mesocardia, and patients with interrupted IVC, atrial situs inversus/ambiguous/isomerism, atrioventricular discordance, superior/inferior ventricles, and ventricular-arterial discordance.

Nineteen of the CHD patients were perioperative, evaluated during their inpatient surgical hospitalization (n=19). Most of these patients were evaluated when they had little or no symptomatic cardiopulmonary issues – either prior to surgery or just prior to being discharged. Surgical patients (perioperative) recruited into the study were all subjected to a rigorous institutional preoperative clearance protocol, which included multiple clinical visits, laboratory evaluations and anesthesiology clearance. Patients on medications that may affect NO levels were excluded (such as those on sildenafil, quinidine, procainimide, nitrates, a-blockers, protease inhibitors, and/or ketoconazole). Twenty nine patients were nonperioperative, with 11 being nonsurgical hospitalization for diagnostic evaluation, and 18 were patients seen in the Adult CHD Clinic at CNMC. Five patients were excluded from the final analysis, given failure to complete the ciliary function tests.

We also recruited 18 PCD patients greater than 6 yaers of age that served as disease controls, 10 of which were subjects from the University of North Carolina Mucociliary Consortium. In addition, 25 healthy adult controls were recruited from the NIH and the University of Pittsburgh. These PCD and healthy control subjects were similarly assessed for nNO levels and nasal scrapes were conducted for subsequent assessment of ciliary motion by videomicroscopy.

Classification of Cardiac Situs

The cardiac situs was delineated using the Van Praagh segmental approach, which entails classification of the heart according to atrial, ventricular and arterial segments[1]. Using this classification system, the atrial and arterial segments are classified either as solitus (S) for normal situs, inversus (I) for mirror-image symmetric situs, and ambiguous (A) when it is neither (S) nor (I). The ventricular segment is defined as dextral (D) when the right ventricle

(RV) is right-sided relative to the left-ventricle (LV), while levo (L) is the mirror image. Thus the normal heart is designated {S,D,S} in the Van Praagh classification: solitus atria, D-looped ventricles, and solitus great artery positioning. In contrast, in patient 9033, an adult with levo (or L-loop, "corrected") transposition of the great arteries (L-TGA), the Van Praagh classification yields {S,L,L}, with solitus atria, L-looped ventricles, and left-anterior positioning of the aorta yielding physiologically corrected transposition.

nNO Measurements

Nasal nitric oxide (nNO) measurements were made by inserting a NO inert sampling line into one nostril while the contralateral nostril was left open and measurements made using a chemiluminescence nitric oxide analyzer (CLD 88 SP, ECO PHYSICS AG, Duerten, Switzerland) at a constant rate of 0.3 liters/min [2-4]. Standardized nNO production in nl/min was calculated as the product of measured levels in parts per billion (ppb) and the sample flow rate (L/min). Ambient levels of NO were noted to not exceed 8 ppb.

For participants over six years of age, measurements were made using velum-closure technique via exhalation from a deep inspiration through a fixed resistor (disposable, cardboard cylinder with 1 mm opening; DirectMed Inc., Glen Cove, NY) for 20-40 seconds or in children, via a party favor blowout toy with a comparable expiratory resistance. Both types of resistor required slight puffing of the cheeks to develop a mouth pressure > 5 cm H_2O, a pressure sufficient to close the soft palate. The maneuvers were performed according to ATS/ERS guidelines [5, 6].

In patients under 6 years of age, NO measurements were made using tidal breath sampling [7, 8]. For velum closure measurements, the mean nNO value was obtained from the average of 5 steady-state peak recordings from each nare, while the plateau method was used

to determine nNO levels obtained by tidal breathing (Supplemental Figure 1). For a few patients with repeat nNO measurements, the higher of the two values were retained. Contraindication to nNO measurement included the presence of anatomic abnormality of the nose or sinuses (e.g. complete sinus blockage or turbinatectomy), acute upper airway infection, significant epistaxis within the prior week, or lower airway infection with uncontrollable coughing. In the latter instances, the participant was re-evaluated after resolution of these symptoms. In addition, as cystic fibrosis (CF) can have similar clinical manifestations including low nNO, patients with abnormal ciliary motion, low nNO, and a clinical history of recurrent respiratory tract infections were also evaluated with a sweat chloride test to exclude CF.

Statistical Analysis

The Welch's t–test for unequal variances was used to compare nNO measurements after logarithmic transformation of the data. To control for type I error, we applied Bonferroni correction for multiple comparisons (CD vs. no-CD heterotaxy patients, healthy controls, or PCD patients; CD-A vs. CD-B; no-CD vs. healthy control) in each age group. A P-value < 0.01 was considered statistically significant. If the CD group is statistically significantly different from no-CD, healthy controls, or PCD in nNO measurements, then we also compare the CD-A and CD-B subgroups with these groups. The data are presented as mean ± SD, and displayed graphically by box plots. The chi-squared 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's exact test to compare the frequency of individual symptoms between the CD and no-CD patients. All tests were two-tailed.. Analyses were performed with SAS 9.1 (SAS Institute, Cary, NC).

For analysis of the DNA sequencing data, we examined the differences in the presence of NCVs and in the number of NCVs among the four groups (heterotaxy patients with CD, heterotaxy patients without CD, PCD patients, and control subjects) and a p-value <0.025 was considered statistically significant for each comparison based on the Bonferroni correction. The Fisher's exact test and the Kruskal Wallis test were used for the comparisons of the presence and the number of NCVs in the four groups, and both yielded significant results (P=0.021 and P=0.024, respectively). Then pairwise comparisons were carried out using the Fisher's exact test and the Wilcoxon rank-sum test to test the differences in the proportions and in the mean numbers of NCVs between any two subgroups.

Ciliary Ultrastructural Analysis by Electron microscopy

Electron microscopy was performed on nasal epithelial samples fixed in 2% glutaraldehyde, 2% paraformaldehyde, 0.5 % tannic acid in 0.05 M sodium phosphate buffer. Samples were postfixed in 1% osmium tetroxide, and processed for thin section electron microscopy as previously described using a JEM 1200EX electron microscope (JEOL USA) with an AMT XR-60 digital camera (Advanced Microscopy Techniques Corp)[9].

Sequence capture and SOLiD Sequencing Analysis

Sequence capture and library preparation for SOLiD sequencing were carried out using standard protocols (http://www.nimblegen.com/products/lit/SeqCap_UserGuide_Delivery_v3p2.pdf), with modifications developed by the Baylor Human Genome Sequencing Center for adding on SOLiD adapters prior to sequence capture to facilitate subsequent SOLiD library preparation for sequencing on the SOLiD3+ or SOLiD4 sequencing system (Applied Biosystems) (http://www.hgsc.bcm.tmc.edu/documents/Preparation_of_SOLiD_Capture_Libraries.pdf).

The SOLiD-ready sequence captured DNA library were clonally amplified by emulsion PCR, and approximately 60 million beads were deposited for each sample onto an octet SOLiD slide configuration for cycled ligation sequencing. Some of the samples were bar coded and sequenced as a pool on one full slide. Color space reads were mapped to the hg19 reference genome using gapped alignment algorithm with stringent filtering criteria with maximum of 2 mismatches allowed. The 14 PCD genes analyzed included several motor dyneins and other proteins localized to the cilia (*DNAH5, DNAI1, DNAH11, DNAI2, TXNDC3, RSPH9, RSPH4A, CCDC39, CCD40*) and a number of non-axonemal proteins required for cilia structure and function (*LRRC50, KTU, RPGR, OFD1, DNAL1*). [10-12]

Supplemental Table 1. Cardiovascular anatomy and situs delineation in heterotaxy subjects

	Age	Gender	Weight (kg)	Cardiac Position[A]	Van Praagh[B]	Complete Cardiovascular Anatomy[C]	Procedure (RACHS-1)[D]
> 6 years							
perioperative							
9011	12 yr	F	51	L	{I,D,D}	Unbalanced AVCD, DORV, single RV morphology (LV hypoplasia), PA, D-malposed aorta	
9013	7 yr	F	21	D	{S,L,L}	L-TGA, ventricular inversion, PA, VSD, ASD, PVs to RA, RSVC to RA, IVC to RA	Right Ventricle-Pulmonary Artery conduit replacement (3)
9016	19 yr	F	58	M	{S,L,L}	DORV w/ straddling MV, small LV, L-TGA, LPA stenosis	Pulmonary valvotomy, obliteration of PA stump (2)
9046	9 yr	F	22	M	{I,D,X}	DORV, VSD, anterior aorta, pulmonary atresia, Rt aortic arch with mirror image branching	Bidirectional Glenn (2)
nonperioperative							
9003	26 yr	F	55	L	{S,D,S}	Normal, ipsilateral descending aorta and IVC (no interruption)	
9020	46 yr	F	68	L	{S,L,L}	DORV, L-TGA, inlet VSD, PS	
9032	19 yr	M	70	L	{S,L,L}	L-TGA	
9037	18 yr	M	55	D	{I,L,I}	Azygous continuation of interrupted IVC	
9008	10 yr	M	36	D	{S,D,D}	Truncus arteriosus, VSD, ASD, LSVC	
9026	18 y	F	65	D	{A,D,X}	HLHS, Bilateral SVCs with unbalanced AVC, Interrupted IVC, Rt aortic arch	
9027	6.5yr	F	31	L	{A,D,S}	TOF, L SVC drains into L atrium, L atrial isomerism, Rt aortic arch, hypoplastic PAs with collaterals	
9031	15 yr	M	75	D	{I,L,L}	Atrial situs inversus, TGA	
9009	30 yr	F	54	L	{S,D,D}	DORV, PA, VSD, D-TGA, ASD	
9019	44 yr	M	75	D	{S,D,S}	Isolated dextrocardia	
9033	29 yr	M	80	L	{S,L,L}	L-TGA	
9035	28 yr	F	60	L	{S,D,S}	VSD, PDA, secundum ASD	
9036	25 yr	M	65	L	{S,L,L}	L-TGA, Ebstein's anomaly	
9038	50 yr	F	60	L	{S,L,L}	L-TGA	
9042	12 yr	F	75	L	{S,L,L}	L-TGA, VSD, Severe MR	
1-6 years							
perioperative							
9010	2 yr	M	9.0	L	{S,D,D}	DORV, PS, D-TGA, VSD	
9017	5.9 yr	M	19	D	{I,L,L}	Atrial & ventricular inversion, DORV, L-TGA, PA, VSD, Left SVC to LA/mRA, Left IVC to LA/mRA, Rt aortic arch	
9014	3 yr	F	10	D	{S,D,X}	Superior/inferior ventricles, TGA, TS, PA, Hypoplastic RV, Rt aortic arch, small LSVC	Right blalock shunt (3)
9022	2.5 yr	F	13	L	{S,D,D}	DORV, Interrupted IVC w azygos continuation into R SVC, L SVC to coronary sinus	Extracardiac fenestrated Fontan (3)
9024	2 yr	F	11	D	{I,L,L}	Inverted atria, Interrupted IVC with Az cont, PAPVR:isomerism, unbalanced AVC, L-malposed Aorta	Extracardiac Fontan (3)
nonperioperative							
9004	1.4 yr	F	10	L	{S,D,D}	DORV, unbalanced AVCD, PS, LSVC to LA, RSVC to RA, D-TGA, RV hypoplasia, Rt aortic arch	
9006	2.5 yr	M	15	D	{I,D,D}	DORV, PA, common atrium, PAPVR, atrial inversus	
9001	5 yr	F	19	L	{S,D,S}	Interrupted IVC with AZ continuation to RSVC	
9007	18 mo	F	9.0	L	{I,D,L}	DORV, unbalanced AVCD, superior/inferior ventricles, PS, bilateral SVCs, Rt aortic arch	
< 1 year							
perioperative							
9015	9 mo	F	5.0	L	{S,D,S}	Unbalanced AVC, HLV, LSVC to LA, ASD, small aortic arch, CoA, LPA stenosis	Bidirectional Glenn (2)
9018	3 mo	F	4.0	L	{S,D,S}	unbalanced AVC, PS	
9023	7 mo	F	4.0	D	{I,L,L}	DORV, L-malposition, Rt aortic arch, CoA, TAPVR, LSVC, VSD, ASD	Left sided Glenn with repair of TAPVR (3)
9043	14 days	M	2.4	L	{A,D,X}	DORV, hypoplastic L ventricle, D-TGA, pulmonary atresia, common atrium, ambiguous atrial situs, TAPVR	
9005	24 days	F	2.4	L	{S,D,S}	Interrupted IVC with AZ continuation to SVC, VSD, ASD, interrupted aortic arch	Arch repair, end side coA reconstruct anastomosis, ASD and VSD closure (5)
9025	10 days	F	2.5	M	{I,D,D}	Ventriclar inversion, D-TGA, atrial situs inversus, LSVC to CS	Blalock Taussig shunt (3)
9039	18 days	F	2.5	L	{S,L,L}	L-TGA	Arterial switch (3)
9044	9 mo	F	7.0	L	{SDD}	DILV, Single ventricle, D-TGA, CoA, AS	Bidirectional Glenn (2)
9045	1.5 mo	M	5.0	L	{S,D,D}	DORV, LSVC to CS, VSD, PA with ductal dependent branched pulmonary arteries in continuity, secundum ASD	Rastelli (3)
9049	0.8 mo	M	3.6	L	{S,D,L}	DORV, superior-inferferior single ventricle, crisscross A-V valves, VSD, tricuspid stenosis, RV hypoplasia	Pulmonary Artery Band (3)
nonperioperative							
9002	17 days	M	3.0	M	{S,D,S}	Interrupted IVC with AZ continuation, Rt aortic arch, LSVC to CS	
9021	3 mo	M	4.5	D	{S,L,L}	L-TGA	
9029	5 mo	M	5.0	L	{A,D,D}	Rt Atrial Isomerism, Rt aortic arch w/ mirror imaging branching, DORV, TGA, Hypoplastic RV, B/L SVC, TAPVR	
9040	15 days	F	3.0	L	{S,D,X}	DORV, D-malposed aorta, unbalanced CAVC, PS, primum ASD	
9041	8 days	F	4.5	L	{S,D,X}	DORV, HLH, MA, Interrupted aortic arch type B, L aortic arch, secundum ASD, VSD	

CD-A	CD-B	No-CD

A: L=Levocardia, M=Mesocardia, D=Dextrcardia; B: Van Prague Classification (Van Praagh 1977); C: AVC = AV canal defect, TGA = Transposition of the great Arteries (Levo- or Dextra-), DORV = Double oulet right ventricle, DILV = Double inlet left ventricle, RV = Right ventricle, MV = Mitral valve, LV = Left ventricle, PA = Pulmonary Atresia, VSD = Ventricular septal defect, ASD = Atrial septal defect, R (L) SVC = Right (Left) superior vena cava, IVC = Inferior vena cava, TV = Tricuspid valve, HLHS = Hypoplastic left heart syndrome, PS = Pulmonic stenoisis, LA (mLA) = Left atrium (morphologic left atrium), RA (mRA) = Right atrium (morphologic right atrium), Az = Azygous vein, T(P)APVR = Total (Partial) anomalous pulmonary venous return, CoA = Coarctation of aorta, LPA = Left pulmonary artery, CS = Coronary sinus, MA = Mitral atresia; D: Risk Adjustment in Congenital Heart Surgery[13].

Supplemental Table 2. Thoracic laterality defects and other disorders

		Age	Gender	Other disorders	Bronchial Laterality	Thoracic Radiologic Findings[A]
>6 Years						
perioperative						
	9011	12 yr	F	none	Normal	R effusion
	9013	7 yr	F	Von Willebrand disease	Normal	B/L effusion
	9016	19 yr	F	none	Normal	L effusion
	9046	9 yr	F	none	Inversus	none
nonperioperative						
*	9003	26 yr	F	none	Normal	LLL infiltrate
	9020	46 yr	F	none	Normal	R basilar effusion, atelectasis
	9032	19 yr	M	none	Normal	none
*	9037	18 yr	M	none	Normal	none
	9008	10 yr	M	protein-losing enteropathy, renal agenesis	Normal	B/L effusion, atelectasis
	9026	18 y	F	none	Left Isomerism	none
*	9027	6.5yr	F	none	Left Isomerism	B/L infiltrates
	9031	15 yr	M	none	Normal	none
	9009	30 yr	F	renal insufficiency, anemia	Normal	B/L effusions
	9019	44 yr	M	none	Normal	none
	9033	29 yr	M	none	Normal	none
	9035	28 yr	F	none	Normal	none
	9036	25 yr	M	none	Normal	none
	9038	50 yr	F	none	Normal	none
	9042	12 yr	F	none	Normal	none
1-6 years						
perioperative						
	9010	2 yr	M	none	Normal	diffuse L atelectasis
	9017	5.9 yr	M	none	Inversus	L infiltrates, R effusion
	9014	3 yr	F	none	Normal	small R effusion
	9022	2.5 yr	F	none	Normal	atelectasis
	9024	2 yr	F	none	Normal	none
nonperioperative						
	9004	1.4 yr	F	none	Normal	none
	9006	2.5 yr	M	protein-losing enteropathy	Inversus	B/L effusions
*	9001	5 yr	F	none	Normal	none
	9007	18 mo	F	none	Normal	none
<1 year						
perioperative						
	9015	9 mo	F	undiagnosed, thumb deformity	Normal	RUL atelectasis, hypoinflation
	9018	3 mo	F	none	Normal	perihilar pulmonary edema
	9023	7 mo	F	undiagnosed	Normal	B/L effusions
	9043	14 days	M	undiagnosed, vertebral anomalies	Inversus	none
	9005	24 days	F	none	Normal	none
	9025	10 days	F	none	Inversus	none
	9039	18 days	F	none	Normal	none
	9044	9 mo	F	none	Normal	increased pulm blood flow
	9045	1.5 mo	M	none	Normal	none
	9049	0.8 mo	M	none	Normal	increased pulm blood flow
nonperioperative						
	9002	17 days	M	none	Normal	none
	9021	3 mo	M	none	Normal	none
	9029	5 mo	M	none	Right Isomerism	none
	9040	15 days	F	none	Normal	none
	9041	8 days	F	chromosome 8,10 duplication	Normal	none

CD-A | CD-B | No-CD

A: R=Right, L=Left, B/L=Bilateral, LLL=Left lower lobe, RUL=Right upper lobe

*Sweat chloride testing negative for cystic fibrosis. 9003=8.3mmol/L;9037=24.5mmol/L;9027= 9mmol/L; 9001=16mmol/L

Supplemental Table 3. Characteristics of perioperative and nonperioperative patients[A]

	Heterotaxy Patients (n=43)		
	Perioperative (n=19)	**Non-perioperative (n=24)**	**P-value**
CD (A and B)	7 (37%)	11 (46%)	0.76
CD-A	1 (5%)	5 (21%)	0.20
CD-B	6 (32)	6 (25%)	0.74
Male	5 (26%)	11 (46%)	0.22
Cohort II	9 (47%)	14 (58%)	0.55
Respiratory Symptoms	10 (53%)	15 (63%)	0.55

	Patients with Age < 1 year (n=15)		
	Perioperative (n=10)	**Non-perioperative (n =5)**	P-value
CD (A and B)	4 (40%)	1 (20%)	0.60
nNO	13.4 ± 9.1	18.5 ±10.6	0.35
nNO in CD patients	6.1 ± 3.2	6.4 (-)	-

	Patients with Age 1-6 years (n=9)		
	Perioperative (n=5)	**Non-perioperative (n =4)**	**P-value**
CD (A and B)	2 (40%)	2 (50%)	1
nNO	58.0 ± 3.7	79.5 ± 43.8	0.38
nNO in CD patients	57.0 ± 2.8	47.5 ± 19.1	1

	Patients with Age ≥ 6 years (n=20)		
	Perioperative (n=4)	**Non-perioperative (n=15)**	**P-value**
CD (A and B)	1 (25%)	8 (53%)	0.58
nNO	163.5 ± 74.9	212.3 ± 174.7	0.80
nNO in CD patients	85.0 (-)	96.9 ± 46.9	-

[A]nNO values represent mean ±SD.

Supplemental Table 4. Characteristic findings in heterotaxy patients

	Heterotaxy Patients (n=43)		
	CD[A] (n=18)	No CD (n=25)	*P*
Gender, n (%)			
male	9 (50%)	7 (28%)	0.14
female	9 (50%)	18 (72%)	
Laterality Defects, n (%)			
cardiac/lung/abdominal (cohort II)	13 (72.2%)	10 (40%)	0.037
cardiovascular only (cohort I)	5 (27.8%)	15 (60%)	
Operative Status, n (%)			
perioperative	7 (38.9%)	12 (48%)	0.55
non-perioperative	11 (61.1%)	13 (52%)	

A: CD-A (n=6) and CD-B (n=12) patients were pooled since the percentages were not significantly different between the two groups by the Fisher's exact test.

Supplemental Table 5. Respiratory manifestations in heterotaxy patients[A]

		Age	Gender	Recurrent otitis media	Recurrent lower respiratory illnesses	Neonatal respiratory Distress	Chronic wet cough	Chronic nasal congestion	Chronic sinusitis	Respiratory Insufficiency/ Tracheotomy	Bronchiectasis
> 6 years perioperative											
CD-A	9011	12 yr	F	N	N	N	N	N	N	N	N
	9013	7 yr	F	N	Y	N	N	N	N	N	N
	9016	19 yr	F	N	N	N	N	N	N	N	N
	9046	9 yr	F	Y	N	N	N	N	N	N	N
nonperioperative[B]											
CD-A	9003	26 yr	F	Y	Y	Y	Y	N	Y	N	Y
CD-A	9020	46 yr	F	Y	Y	N	Y	N	N	N	N
CD-A	9032	19 yr	M	N	N	N	N	N	N	N	N
CD-A	9037	18 yr	M	Y	N	Y	Y	Y	Y	N	N
CD-B	9008	10 yr	M	N	N	N	N	N	N	N	N
CD-B	9026	18 y	F	N	N	N	N	N	N	N	N
CD-B	9027	6.5yr	F	N	Y	Y	Y	Y	N	N	N
CD-B	9031	15 yr	M	Y	Y	N	N	Y	Y	N	N
	9009	30 yr	F	N	N	N	N	N	N	N	N
	9019	44 yr	M	N	Y	N	Y	N	Y	N	N
	9033	29 yr	M	N	N	N	N	N	N	N	N
	9035	28 yr	F	N	N	N	N	N	N	N	N
	9036	25 yr	M	N	N	N	N	N	Y	N	N
	9038	50 yr	F	N	Y	N	N	N	Y	N	N
	9042	12 yr	F	N	N	N	Y	Y	N	N	N
1-6 years perioperative											
CD-B	9010	2 yr	M	N	N	N	N	N	N	N	N
CD-B	9017	5.9 yr	M	N	N	N	N	N	N	N	N
	9014	3 yr	F	N	N	N	N	N	N	N	N
	9022	2.5 yr	F	N	N	N	Y	N	N	N	N
	9024	2 yr	F	N	N	N	N	N	N	N	N
nonperioperative											
CD-A	9004	1.4 yr	F	N	N	Y	N	N	N	N	N
CD-B	9006	2.5 yr	M	N	N	N	N	N	N	N	N
	9001	5 yr	F	Y	Y	N	Y	N	Y	N	N
	9007	18 mo	F	N	N	N	N	N	N	N	N
< 1 year perioperative											
CD-B	9015	9 mo	F	N	Y	N	N	N	N	Y	N
CD-B	9018	3 mo	F	N	N	Y	N	N	N	N	N
CD-B	9023	7 mo	F	N	N	Y	N	N	N	Y	N
CD-B	9043	14 days	M	N	N	N	N	N	N	N	N
	9005	24 days	F	N	N	Y	N	N	N	N	N
	9025	10 days	F	N	N	Y	N	N	N	N	N
	9039	18 days	F	N	N	N	N	N	N	N	N
	9044	9 mo	F	N	N	N	N	Y	N	N	N
	9045	1.5 mo	M	N	N	N	N	N	N	N	N
	9049	0.8 mo	M	N	N	Y	N	N	N	N	N
nonperioperative											
CD-B	9002	17 days	M	N	N	Y	N	N	N	N	N
	9021	3 mo	M	N	N	Y	N	N	N	N	N
	9029	5 mo	M	N	N	Y	N	N	N	N	N
	9040	15 days	F	N	N	N	N	N	N	N	N
	9041	8 days	F	N	N	N	N	N	N	N	N

A: Y=with symptoms; N=without symptoms

Red vs. yellow highlight denote respiratory symptoms in CD vs. no-CD patients, respectively.

B: Wilcoxon rank sum test comparison of the number of respiratory manifestations in symptomatic (≥1 respiratory manifestation) nonperioperative CD vs. no-CD patients > 6 yrs show P=0.024.

Supplemental Table 6. Ciliary function and EM ultrastructure analyses of PCD patients

ID	Type	Age	NO levels*	Ciliary Motion†	Cilia Ultrastructure‡
9028	PCD	6	5.73	b,d	CA and RS defects, Some IDA defects
9098	PCD	9	12.5	b,d	Not available
564	PCD	59	11.8	b,d	IDA defect
1621	PCD	7	7.0	e	CA defect
388	PCD	11	6.6	b,d	ODA/IDA defects
1322	PCD	8	12.5	b	Normal
1174	PCD	35	32.1	b,d	ODA defects
1848	PCD	14	17	d	Normal
1929	PCD	52	33.2	b,d	CA and RS defects
1401	PCD	2	8.3	b	CA/IDA defects

*All subjects are >6 yrs of age. PCD cut off values for nNO is <100 nl/min for this age group.
†Ciliary motion scoring same as for heterotaxy subjects in Table 1; b=stiff/dyskinetic, d=incomplete stroke, e=wavy stroke.
‡IDA: inner dynein arm, ODA: outer dynein arm, CA: central appratus, RS: radial spokes

Supplemental Table 7. Sequencing coverage in the 14 PCD genes

Gene	Average Coverage	Percentage of bases covered
DNAI1	54.3X	100.00%
DNAI2	47.5X	100.00%
DNAH5	41.7X	99.70%
DNAH11	42.6X	99.90%
DNAL1	51.2X	98.70%
TXNDC3	29.8X	99.80%
LRRC50	44.8X	99.80%
OFD1	22.4X	98.80%
CCDC39	25.2X	99.70%
CCDC40	40.1X	99.65%
KTU	21.2X	88.20%
RSPH4A	36.9X	99.90%
RSPH9	38.9X	100.00%
RPGR	34.6X	77.30%

Supplemental Table 8. Novel Sequence Variants in PCD Genes in PCD Patients and Healthy Controls.

Patient	Ethnicity	Type	Gene	Base Change*	Amino Acid
9028	White	PCD	***CCDC39***	1072delA†	T358fs
			CCDC39	1072delA†	T358fs
9098	White	PCD	***CCDC39***	1072delA†	T358fs
			CCDC39	1072delA†	T358fs
564	White	PCD	***CCDC40***	248delC†	A83fs
1621	White	PCD	***RSPH9***	G373C	V125L‡
388	White	PCD	*None*		
1322	White	PCD	*None*		
1174	White	PCD	***DNAH5***	G4116C	Q1372H‡
			DNAH5	G10616A	R3539H‡
			DNAH11	C2569T	R857X
1848	White	PCD	*None*		
1929	White	PCD	*None*		
1401	White	PCD	***CCDC40***	248delC†	A83fs
			CCDC40	A1312T	K438X‡
1001	Asian	Control	*None*		
1005	Asian	Control	*None*		
1006	White	Control	*None*		
1017	White	Control	*None*		
1023	Asian	Control	*None*		
1026	White	Control	*None*		
1028	White	Control	*None*		
1029	White	Control	***DNAI2***	C109G	L37V‡
1030	Afric Amer	Control	*None*		
1031	White	Control	*None*		
1032	White	Control	*None*		
1033	Afric Amer	Control	*None*		
1034	Afric Amer	Control	*None*		

*****Mutations listed are for each allele of the diploid genome, and thus heterozygous mutation is listed once, while homozygous mutation is listed twice.
†*CCDC39* and CCDC40 mutations in red are known to cause PCD[11].
‡Predicted damaging base on Polyphen and SIFT.

Supplemental Table 9. Novel Coding Variants in Heterotaxy and PCD Patients*

Patient Type	Total No. Subjects	No. with NCVs (%)†	No. NCVs	NCVs Per Subject‡
Heterotaxy with CD	13	7 (54%) p=0.019	10	0.769 p=0.011
Heterotaxy without CD	12	3 (25%)	5	0.417
PCD	10	6 (60%) p=0.03	10	1.000 p=0.006
Healthy Controls	13	1 (8%)	1	0.077

*p-value <0.025 was considered statistically significant for each comparison based on the Bonferroni correction. The Fisher's exact test and the Kruskal Wallis test were used for the comparisons of the presence and the number of NCVs in the four groups, and both yielded significant results (*P*=0.021 and *P*=0.024, respectively).

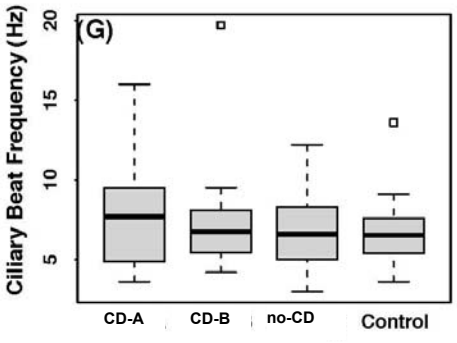
†Using Fisher's exact test, comparing all four groups yielded P=0.021, suggesting at least one pair of two groups are statistically significantly different. Then pairwise tests of two-group combination yielded the following:

- **P=0.030** for comparing heterotaxy with CD vs. healthy controls;
- P=0.22 for comparing heterotaxy with CD vs. Heterotaxy without CD;
- P=1 for comparing heterotaxy with CD vs. PCD
- **P=0.019** for comparing PCD vs. healthy controls;
- P=0.19 for comparing PCD vs. heterotaxy without CD;
- P=0.32 for comparing heterotaxy without CD vs. healthy controls.

‡Using The Kruskal Wallis test, we examined for differences in the mean number of NCVs per subject in a global comparison amongst all four groups, which yielded P=0.024. This indicated at least one pair of two groups have statistically significant different mean number of variants. Then pairwise Wilcoxon rank-sum test was carried out for two group combinations, which yielded the following:

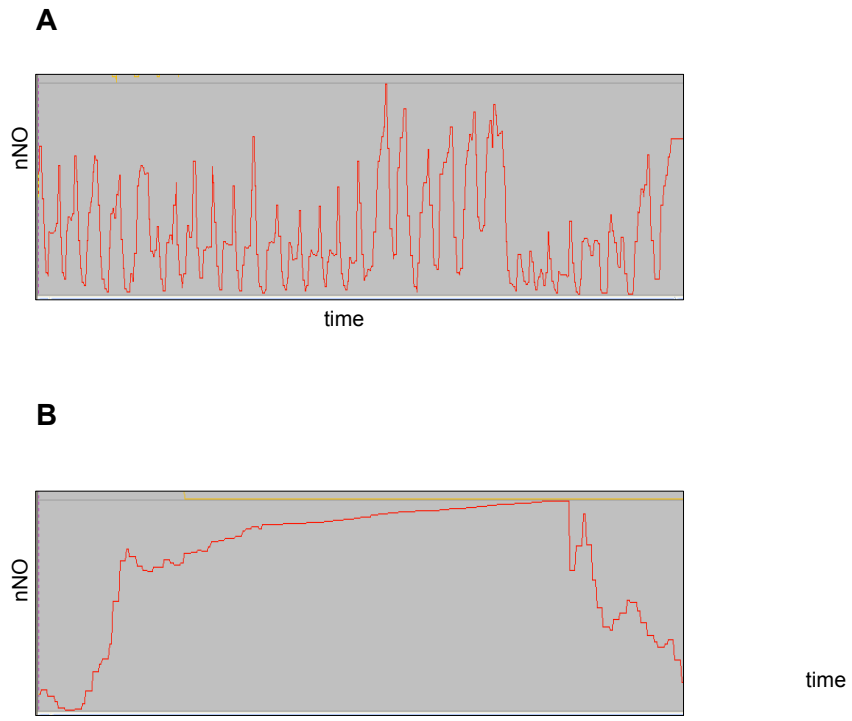
- **P=0.011** for comparing heterotaxy with CD vs. healthy controls;
- P=0.23 for comparing heterotaxy with CD vs. Heterotaxy without CD;
- P=0.51 for comparing heterotaxy with CD vs. PCD
- **P=0.006** for comparing PCD vs. healthy controls;
- P=0.12 for comparing PCD vs. heterotaxy without CD;
- P=0.23 for comparing heterotaxy without CD vs. healthy controls.

Supplemental Figure 1. Ciliary beat frequency measurements in heterotaxy patients



Ciliary function was assessed by measuring cilia beat frequencies (CBF) using the digital movies obtained by videomicroscopy. The distribution of measurements are shown in box plots. Note the broad distribution of CBFs observed amongst the CD-A patients.

Supplemental Figure 2. Nasal nitric oxide tracings with tidal breathing vs. resistor method



A. Steady peaks and troughs during online nasal nitric oxide measurement in a patient under 6 years of age. The mean of the readings at five consecutive peaks was taken and repeated on the contralateral nare.

B. Plateau reading obtained during online nasal nitric oxide measurement in a patient older than 6 years of age while exhaling through a cardboard resistor. The mean of 2 recordings (minimum 3-second plateaus) was taken and repeated on the contralateral nare.

SUPPLEMENTAL MOVIE LEGEND

Videomicroscopy of nasal epithelia cilia motion associated with the respiratory epithelia. Movie A corresponds to real time, while movie B has been slowed to 15% of real time.

Supplemental Movie 1A,B: Videomicroscopy of nasal epithelia from a healthy individual showing rapid synchronous motion.

Supplemental Movie 2A,B: Videomicroscopy of nasal epithelia from PCD patient 9028 showing immotile cilia and cilia with stiff/dyskinetic motion.

Supplemental Movie 3A,B: Videomicroscopy of nasal epithelia from CHD patient 9031 showing stiff/dyskinetic stroke.

Supplemental Movie 4A,B: Videomicroscopy of nasal epithelia from CHD patient 9026 with heterotaxy showing incomplete stroke.

Supplemental Movie 5A,B: Videomicroscopy of nasal epithelia from CHD patient 9011 with heterotaxy showing wavy stroke.

Supplemental Movie 6A,B: Videomicroscopy of nasal epithelia from CHD patient 9024 with heterotaxy showing asynchronous motion.

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