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Founder mutation in *RSPH4A* identified in patients of Hispanic descent with Primary Ciliary Dyskinesia

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Abstract

Primary ciliary dyskinesia (PCD) is a rare, autosomal recessive, genetically heterogeneous disorder characterized by ciliary dysfunction resulting in chronic oto-sino-pulmonary disease, respiratory distress in term neonates, laterality (situs) defects, and bronchiectasis. Diagnosis has traditionally relied on ciliary ultrastructural abnormalities seen by electron microscopy. Mutations in radial spoke head proteins occur in PCD patients with central apparatus defects. Advances in genetic testing have been crucial in addressing the diagnostic challenge. Here, we describe a novel splice-site mutation (c.921+3_6delAAGT) in *RSPH4A*, which leads to a premature translation termination signal in nine subjects with PCD (seven families). Loss-of-function was confirmed with quantitative ciliary ultrastructural analysis, measurement of ciliary beat frequency and waveform, and transcript analysis. All nine individuals carrying c.921+3_6delAAGT splice-site mutation in *RSPH4A* were Hispanic with ancestry tracing to Puerto Rico. This mutation is a founder mutation and a common cause of PCD without situs abnormalities in patients of Puerto Rican descent.

Keywords

Cilia; Kartagener syndrome; sequencing; *RSPH4A*

Primary ciliary dyskinesia (PCD; MIM# 244400) (http://www.ncbi.nlm.nih.gov/Omim/) is a rare, autosomal recessive, genetically heterogeneous disorder manifested by ciliary dysfunction, which causes respiratory distress in term neonates, laterality (situs) defects,

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otitis media, sinusitis, bronchiectasis, and male infertility (Barbato et al., 2009; Leigh et al., 2009; Noone et al., 2004; Zariwala et al., 2013; Zariwala et al., 2007). The test to confirm the diagnosis of PCD has traditionally been based on identifying ciliary ultrastructural abnormalities using electron microscopy. However, we now know that at least one-third of PCD patients have normal ciliary ultrastructure based on a characteristic clinical phenotype, (Knowles et al., 2012; Zariwala et al., 2013) diminished production of nasal nitric oxide (nNO) (<100 nl/min versus ~300 nl/min for controls), and recent advances in genetic testing (Barbato et al., 2009; Knowles et al., 2012; Leigh et al., 2009; Noone et al., 2004; Zariwala et al., 2013).

Definition of the molecular (genetic) pathophysiology of PCD is challenging because of the genetic heterogeneity and the complexities of cilia structure and function. Motile cilia in the respiratory tract extend from the cellular membrane from the basal body, which is a special derivative of the centriole (Zariwala et al., 2007). An outer ring of 9 microtubular doublets connected by nexin links surround a central pair of microtubules (9+2 axonemal structure) (Figure 1A). Inner and outer dynein arms extend from the central pair to the outer doublets and contain ATPases that provide motive force for bending of the cilia (Zariwala et al., 2007). Radial spokes, similar to spokes on a bicycle wheel, extend from the outer doublets to the central pair and provide support for the axonemal structure. Mutations affecting any part of ciliary biogenesis, structure, or activity can cause impaired function, which can manifests as inadequate mucociliary clearance, upper and lower airway disease, and dysmotile sperm. The specialized motile cilia at the embryonic node does not have the central pair of microtubules (9+0 axonemal structure); therefore, altered left-right placement of organs during embryogenesis does not occur with radial spokes mutations, but does occur with mutations in components of the 9 microtubular doublets or dynein arms (Barbato et al., 2009; Leigh et al., 2009; Zariwala et al., 2013; Zariwala et al., 2007).

Genetic testing is now becoming useful to address the diagnostic challenge in PCD. There are currently 19 genes known to harbor PCD-causing mutations, accounting for two-thirds of PCD cases (Horani et al., 2012; Knowles et al., 2013; Kott et al., 2012; Wirschell et al., 2013; Zariwala et al., 2013). The majority of diagnosed PCD cases reflect defects in the outer dynein arm (ODA) and result from mutations in *DNAH5* (MIM# 603335) and *DNAI1* (MIM# 604366) (Leigh et al., 2009; Zariwala et al., 2007). There is emerging recognition that mutations in genes encoding cytoplasmic proteins involved in assembling axonemal components (dynein axonemal assembly factors; DNAAF) lead to loss of both outer and inner dynein arms of cilia (Horani et al., 2012; Kott et al., 2012; Zariwala et al., 2013). Most cases of PCD with defects in the inner dynein arms (IDA) also have microtubular disorganization and reflect mutations in *CCDC39* (MIM# 613798) and *CCDC40* (MIM# 613799) (Antony et al., 2012). Mutations in radial spoke head genes *RSPH4A* (MIM# 612647) and *RSPH9* (MIM# 612648) occur in PCD patients with normal IDA and ODAs, but approximately half of the cilia have defects in the central apparatus (Castleman et al., 2009; Zietkiewicz et al., 2012).

We performed genetic studies in patients evaluated at UNC and collaborating sites under the auspices of the Genetic Disorders of Mucociliary Clearance Consortium (GDMCC) (http://rarediseasesnetwork.epi.usf.edu/gdmcc/index.htm), which is studying rare genetic disorders of mucociliary clearance. Study subjects were evaluated by a detailed history, physical exam, chest radiographic studies, and measurements of nasal nitric oxide (nNO) (Noone et al., 2004). Electron microscopy (EM) of nasal epithelial samples obtained by scrape biopsy of the inferior turbinate was performed, and images were evaluated at UNC through a standardized protocol and blinded review of more than 20 cilia images per subject (Knowles et al., 2012; Noone et al., 2004). Ciliary beat frequency and waveform were assessed and analyzed by standard methods (Knowles et al., 2012; Zhou et al., 2009).

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Thirty-six PCD patients were selected to test for *RSPH4A* (NM_001010892.2) mutations. All patients had clinical phenotype consistent with PCD, low nNO (<100 nl/min), and EM images that were normal and/or had a subset of cilia with central apparatus abnormalities (Supp. Table S1). Ages ranged from 2 to 63 years and one third of patients were male. Situs abnormalities were present in 5 patients. Twenty-two had normal ciliary EMs, 12 had central apparatus abnormalities with normal dynein arms, and 2 did not have EMs. The average nasal NO value was 26.9 nl/min +/− 23.1 (mean+/−).

Genomic DNA was extracted either from peripheral blood, lymphoblastoid cell lines or buccal swabs (Noone et al., 2004; Zariwala et al., 2001). Mutation profiling was carried out for all 6 coding exons and splice junctions in *RSPH4A*, using gene specific primers from the flanking introns (Supp. Table S2). 10–100 ng of genomic DNA was used for amplification using the reagents and cycle conditions as previously reported (Zariwala et al., 2001; Knowles et al., 2012). Reagents and thermal cycler used were from Life Technologies (Carlsbad, CA). Successfully amplified products were subjected to bidirectional Sanger sequencing using gene specific primers and Prism BigDye primer Cycle Sequencing Ready Reaction kit (Life Technologies, Carlsbad, CA) and analyzed as previously reported (Zariwala et al., 2001; Knowles et al., 2012). Mutation profiling of *RSPH4A* identified biallelic mutations in 8 unrelated families (10 subjects) without known consanguinity (Table 1). This included four mutant alleles, of which three were novel (Table 1, Supp. Figure S1) and all are reported to the mutation specific database (www.lovd.nl/RSPH4A). Interestingly, one of the novel alleles (c.921+3_6delAAGT) was seen in a homozygous state in 6 families (8 subjects), all of whom were of Hispanic ethnicity from Puerto Rico. Another individual (#501–1623) of Hispanic ethnicity was compound heterozygous for the identical c. 921+3_6delAAGT mutation and the novel c.1732_1733delG mutation (Supp. Figure S1). Two unrelated individuals (#668–2033, #235–1147) of non-Hispanic descent (White) carried a novel c.116C>A mutation on one allele, and #668–2033 carried the known c. 1162+2_5del mutation on the other allele (Zietkiewicz et al 2011). Despite full gene sequencing, the second mutant allele was not identified on #235–1147 which could be due to (1) the presence of a large deletion not identified by the Sanger sequencing, (2) deep intronic mutation affecting splicing that would require transcript analysis, or (3) the presence of biallelic mutations in another gene. Segregation analysis in all 8 families with biallelic mutations was consistent with recessive inheritance (Supp. Figure S2). It is pertinent to mention that screening for mutations in RSPH4A has not been completed for; hence, the fraction of PCD patients carrying mutation in this gene has not been calculated.

The functional significance of the splice-site mutation c.921+3_6delAAGT at intron 2 was assessed by studies of RNA splicing. Lymphoblastoid cell lines from carrying mutation were grown, harvested and subjected to reverse transcriptase-PCR (RT-PCR) as reported previously (Knowles et al., 2012). Gene specific primers (Supp. Table S2) were designed to encompass region of mutation and *cyclophilin* was used as a housekeeping control for amplification to demonstrate the integrity of the cDNA (Knowles et al., 2013). Amplification and sequencing conditions were same as described previously (Knowles et al., 2012).

Analysis from an individual (#135–853) who is homozygous for c.921+3_6delAAGT mutation revealed an out-of-frame deletion of exon 2 (r.687_921del) leading to a premature translation termination signal (Supp. Figure S3).

All ten individuals with biallelic mutations in *RSPH4A*, including Hispanic c. 921+3_6delAAGT homozygotes, had situs solitus (Table 1), which is congruent with other PCD patients reported to have radial spoke head mutations in *RSPH4A* and *RSPH9*, and defects of the central apparatus in a subset of motile cilia (Castleman et al., 2009; Stannard

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et al., 2004; Zietkiewicz et al., 2012). The lack of laterality defects is presumed to reflect the fact that the 9+0 embryological nodal cilia is unaffected by the central apparatus defects seen in *RSPH4A* mutations; thus, normal lateralization during embryogenesis is preserved (Papon et al., 2010; Zariwala et al., 2013; Zariwala et al., 2007).

The clinical phenotypes in our patients with biallelic mutations in *RSPH4A* were similar to PCD patients with "classic" dynein arm defects, including a high prevalence of neonatal respiratory distress, otitis media, sinusitis, and bronchiectasis, as well as low nNO (mean 20.6 nl/min; range 10 to 42.7 nl/min) (Noone et al., 2004; Zariwala et al., 2013). Additionally, the clinical phenotype of our patients with *RSPH4A* mutations are also compatible with previously reported patients carrying *RSPH4A* (*RSPH9*) loss-of-function mutations, including seven UK-Pakistani families, and 4 Eastern-European (Polish) families with PCD, i.e. these patients had chronic oto-sino-pulmonary disease beginning early in life (Castleman et al., 2009) (Zietkiewicz et al., 2012).

Quantitative ciliary ultrastructural analysis of our patients with biallelic mutations in *RSPH4A* were particularly interesting, as ~50% of the cilia had normal ultrastructure (Figure 1A), which is much lower than seen in normal subjects (>85%) (Papon et al., 2010). In the ~ 50% of cilia with central apparatus defects, the most common abnormality was absence of the central pair (Figure 1B + 1C) (18% of cilia) with complete absence in 9% and electron dense material in the central region in 9%. Translocation of an outer doublet into the central region, with (Figure 1D) or without a central pair (Figure 1E), being present was seen in 12% of ciliary cross sections. Less common abnormalities included a single microtubule in place of the central pair (Figure 1F) (5%), eccentric ring of outer doublets (3%), off-center central pair (Figure 1G) (3%), multiple extra microtubules in the center of the cilia with a normal central pair (Figure 1I + 1J) (6%), and multiple single microtubules outside the ring of outer doublets (Figure 1K) (1%). Other findings included 8 outer doublets without a central pair or translocation (Figure 1L) and 8 outer doublets with 4 single microtubules or 2 doublets in the center (2%). Taken together, the prevalence of central apparatus defects in patients with *RSPH4A* mutations (50%) is much greater than seen in normal cilia from normal subject (<15%) (Papon et al., 2010).

We assessed ciliary beat frequency (CBF) and waveform by high-speed videomicroscopy at room temperature in nasal epithelial samples from six individuals with biallelic mutations in *RSPH4A* (Zhou et al., 2009). The mean CBF was slightly lower (4.7 Hz +/− 1.2) than normal (7.3 Hz +/− 0.3) from multiple measurements in 10 random fields. However, there were focal regions with much slower CBF. Similar abnormalities were seen in waveform and dyskinesia in samples from all six subjects. When the cilia were viewed from a lateral perspective, there was a decrease in the range of motion, compared to normal, and a lack of coordinated movement (dyskinesis) with adjacent cilia (Supp. Videos S1 and S2). When viewed from the top, the cilia exhibited "rotational" movement (Supp. Videos S2 and S3). These abnormalities have been reported by others in studies of patients with ultrastructural defects in the central apparatus, and mutations in radial spoke head genes (Castleman et al., 2009; Chilvers et al., 2003; Stannard et al., 2004; Zietkiewicz et al., 2012). With intact dynein arms, the velocity of the cilia is less affected by the central apparatus defect; instead, the cilia waveform mirrors the circular movement seen in nodal cilia, which also lack the central apparatus.

All individuals carrying the c.921+3_6delAAGT mutation identified themselves as Hispanic ethnicity from Puerto Rico. Detailed family history did not reveal any known parental consanguinity. Before the discovery and colonization of the Caribbean by Spanish Explorers, the Taíno Indians inhabited Hispaniola, Puerto Rico, and the eastern portion of Cuba (Lalueza-Fox et al., 2001). European colonization and the African slave trade resulted

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in a genetic admixture reflecting Taíno Indian, European, and African American ancestry. Mitochondrial DNA analysis confirmed the historical records and discovered that the predominant maternal ancestry in Puerto Rico traces to the Taíno Indians (61.3%) (Martinez-Cruzado et al., 2005). The geographic isolation of the island of Puerto Rico has permitted Taíno ancestry to be very prevalent, which may select for rare recessive disorders like PCD. Thus, the c.921+3_6delAAGT splice site mutation in *RSPH4A* is likely a Taíno Indian mutation, rather than a Hispanic mutation from the Spanish colonization. This is supported by the segregation analysis for patient #501–1623, a compound heterozygote, with segregation of the c.921+3_6del mutation to the maternal ancestry from Puerto Rico, while her father identifies as White (non-Hispanic).

In summary, the c.921+3_6delAAGT splice site mutation in *RSPH4A* is a founder mutation that is a common cause of PCD without situs abnormalities in patients of Hispanic Puerto Rican descent. The functional consequences of this splice site mutation reflect full loss-of-function in respiratory cilia, based on the RNA splice defect leading to a premature termination signal. Clinical features of respiratory disease and low nNO are also similar to that seen in patients with “classic” dynein arm defects. This splice site mutation is also associated with similar defects in ciliary ultrastructure and abnormal ciliary beat frequency and waveform, as reported in other PCD patients with ultrastructural abnormalities of the central apparatus and loss-of-function mutations in *RSPH4A*. Genetic testing for this founder mutation is warranted to assess for PCD in patients who identify as Hispanic, particularly those from Puerto Rico, with clinical features consistent with PCD but without situs abnormalities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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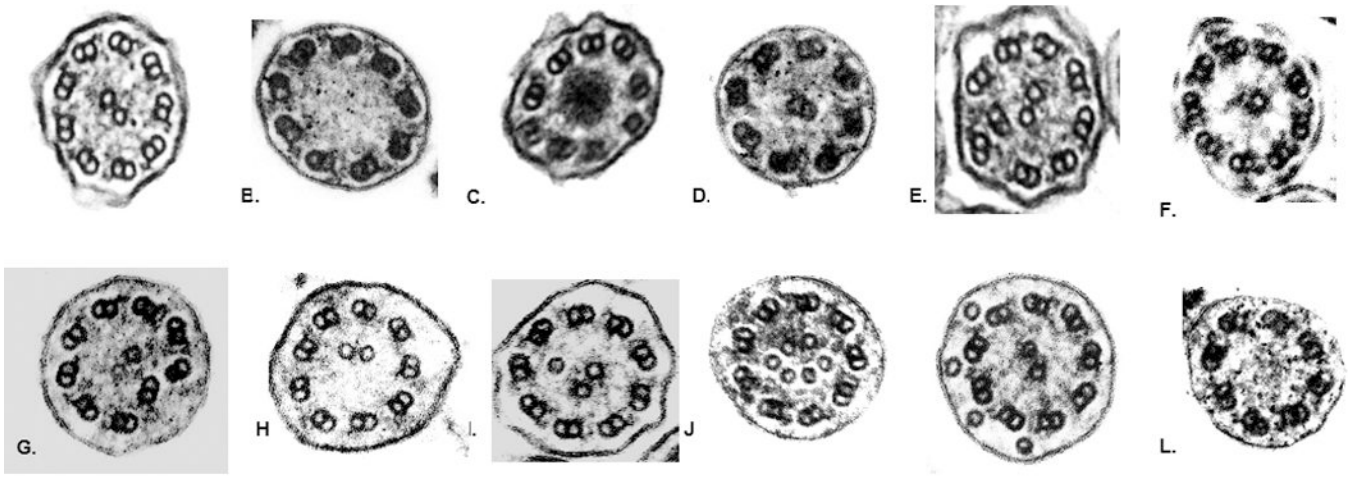

	(A) Normal Axonemal Structure	(B, C) Absence of Central Pair or Electron Dense Material	(D, E) Translocation of Outer Doublet to Cilia Center	(F) Single microtubule in center	(G) Eccentric Outer Doublets	(H) Off-Center Central Pair	(I, J) Extra central microtubules (9D +CP +1S, 9D+4S, 9D+CP+2S)	(K) Extra single microtubules outside doublet ring	(L) Other Ultrastructure Abnormalities
Mean	50%	18%	12%	5%	3%	3%	6%	1%	2%
Range	34-60%	10-27%	3-23%	1-11%	1-6%	1-6%	2-11%	0-7%	0-6%

* Other: 8 outer doublets with or without a normal central apparatus; 7 outer doublets with a central pair and 2 single microtubules in the center; 8 outer doublets with 2 central pairs; 8 outer doublets with 4 central single microtubules; 8 outer doublets without a central pair and 1 doublet outside of the outer ring.

Figure 1. Electron Micrographs Findings

A total of 871 ciliary cross sections were scored from 7 PCD families (8 subjects) with biallelic mutations in *RSPH4A*, and representative images are shown. **(A)** Normal axonemal structure including central apparatus [9+2] (50% of cilia images); **(B)** Complete absence of the central apparatus [9+0] (9% of cilia images) or **(C)** electron dense material in central area [9+0] (9% of cilia images); **(D)** Translocation of outer doublet to replace the central pair [8+1] (11% of cilia images); **(E)** Translocation of outer doublet into central region [8+4] (1% of cilia images); **(F)** A single microtubule in the center instead of a central pair [9+1] (5% of cilia images); **(G)** Eccentric 9 outer doublets with 9+2 arrangement (3% of cilia images); **(H)** Central pair off-center with 9+2 arrangement (3% cilia images); **(I, J)** Extra singlet or multiple microtubules in center of the cilia (6% of cilia images); **(K)** Multiple single microtubules outside the 9 outer doublet ring (1% of cilia images); **(L)** Other abnormalities includes 8 outer doublets with or without a normal central apparatus; 7 outer doublets with a central pair and 2 single microtubules in the center; 8 outer doublets with 2 central pairs; 8 outer doublets with 4 central single microtubules; 8 outer doublets without a central pair and 1 doublet outside of the outer ring (2% of cilia images).

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Table 1

Demographic, Clinical and *RSPH4A* mutations in 11 PCD patients from 9 unrelated families

Family - patient #	Sex	Age in yrs	Situs Solitus	Ciliary Ultra-structural Defect	Ethnicity	nNO nl/min†	Neo RDS	Bxsis	Sinus-itis	Otitis Media	Allele 1				Allele 2			
											Exon / intron	Base Change	Amino-Acid Change	Segre-gation‡	Exon / intron	Base Change	Amino-Acid Change	Segre-gation‡
Homozygous Mutations																		
#135–853††	F	6	yes	CA defects	Hispanic	22	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Paternal	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#135–854††	F	10	yes	CA defects	Hispanic	10.5	yes	no	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Paternal	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#214–1116	F	25	yes	CA defects	Hispanic	17.8	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Paternal	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#603–1842	F	17	yes	CA defects	Hispanic	22.6	yes	yes	na	no	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#650–1989	F	9	yes	CA defects	Hispanic	10	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Paternal	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#839–2331	F	34	yes	CA defects	Hispanic	24	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal
#856–2359‡‡	F	37	yes	na	Hispanic	na	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na
#856–2388‡‡	F	37	yes	na	Hispanic	na	na	na	na	na	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	na
Compound Heterozygous Mutations																		
#501–1623	F	10	yes	CA defects	Hispanic	15.2	yes	yes	yes	yes	Int 2	c.921+3_6del§	p.Tyr230Glnfs*8	Maternal	Ex 4	c.1732_33delGA	p.Asp578Argfs*3	Paternal
#668–2033	M	3	yes	CA defects	non-Hispanic (white)	5.9	yes	yes	yes	yes	Ex 1	c.116C>A	p.Ser39*	Paternal	Int 3	c.1662+2_5del§§	Splice-site	Maternal
Heterozygous Mutations																		
#235–1147	F	14	yes	CA defects	non-Hispanic (white)	42.7	no	yes	yes	yes	Ex 1	c.116C>A	p.Ser39*	na	na	unknown//	unknown	na

Mutations in *RSPH4A* are numbered according to the cDNA sequence NM_001010892.2, where +1 corresponds to the 'A' of 'ATG' translation start codon. Mutations are described according to Human Genome Variation Society (HGVS) guidelines (http://www.hgvs.org/) and analyses by *in silico* program known as Mutalyzer (https://mutalyzer.nl).

† Normal nNO levels are 376+124 nl/min (mean+SD), calculated from 27 healthy subjects (Noone et al 2004).

‡ Mutant allele shown to segregate with either from the paternal or the maternal side of the family (see Supplemental Figure 2 for full pedigree).

†† Both affected siblings from a family carried identical biallelic mutations.

‡‡ Both affected maternal first cousins from a family carried identical biallelic mutations.

§ c.921+3_6delAAGT (g.IVS2+3_6delAAGT) splice site mutation interrogated for transcript analysis (see details in Supplemental Figure 3).

§§ c.1662+2_5delTAGG) (g.IVS3+2_5delTAGG) is a known splice-site mutation (Zietkiewicz et al 2011) and transcript analysis has not been interrogated.

// Second mutation not identified despite sequencing all coding exons and splice junctions.

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Abbreviations: M = Male; F = Female; CA = central apparatus; na = Not Available; Neo RDS = neonatal respiratory distress in full term birth; Bxsis = bronchiectasis; nNO = nasal nitric oxide