Primary Cilia Dyskinesia: an update with molecular correlation

Joint Long Course in EM and Paediatric Pathology: EM in the multidisciplinary management of paediatric diseases
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Primary Ciliary Dyskinesia (PCD)

- Inherited as an autosomal recessive, genetically heterogeneous, disorder
- Incidence 1:15,000 live births
- Causes dysfunction of motile cilia and sperm flagella
- Abnormalities affect ciliary ultrastructure and are usually detected by electron microscopy
- About 50% of patients have situs inversus
- Majority of patients are symptomatic from birth and characterized by persistent or recurrent oto-sino-pulmonary infections
- However diagnosis is often delayed and only about half the patients are diagnosed by age 5
Importance of early PCD diagnosis

Late diagnosis is associated with worst disease severity and poorer lung function

<table>
<thead>
<tr>
<th>Variable</th>
<th>Whole cohort (n=30)</th>
<th>Presentation &lt; 18 years (n=18)</th>
<th>Presentation ≥ 18 years (n=12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT confirmed bronchiectasis</td>
<td>17/19 (89.5%)</td>
<td>6/8 (75%)</td>
<td>11/11 (100.0%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Sputum culture</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Any Pathogen</td>
<td>14/30 (46.7%)</td>
<td>7/18 (38.9%)</td>
<td>7/12 (58.3%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>9/30 (30.0%)</td>
<td>3/18 (16.7%)</td>
<td>6/12 (50.0%)</td>
<td>0.10</td>
</tr>
<tr>
<td>FEV1 Z score</td>
<td>-1.78 (-5.05, -0.16)</td>
<td>-1.35 (-1.93, -0.16)</td>
<td>-3.66 (-5.05, -0.38)</td>
<td>0.076</td>
</tr>
<tr>
<td>% with low FEV1</td>
<td>7/20 (35%)</td>
<td>0/8 (0%)</td>
<td>7/12 (58.3%)</td>
<td>0.015</td>
</tr>
<tr>
<td>FVC Z score</td>
<td>-1.74 (-5.38, 0.93)</td>
<td>-1.33 (-2.04, -0.32)</td>
<td>-3.41 (-5.38, 0.93)</td>
<td>0.076</td>
</tr>
<tr>
<td>% with low FVC</td>
<td>9/20 (45%)</td>
<td>2/8 (25%)</td>
<td>7/12 (58.3%)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Patients diagnosed in adulthood present more frequently with Pseudomonas infection

Patients diagnosed in adulthood have worse lung function from patients diagnosed in childhood

Yiallouros PK 2015
CILIA: Primary and motile

**Motile monocilium**: with 9+0 arrangement present in the embryonic node. Rotational movement. Produces nodal flow – Left right asymmetry.

**Motile Cilia**: 9+2 arrangement present at various sites on the human body. Characterised by planar beating, produce flow. Airways, brain, reproductive tract

**Non-motile (Primary cilia)**: with 9+0 arrangement, sensory function. Kidney tubule, bile duct, eye, ear, bone, fibroblasts

Praveen K 2015
Norris DP 2012
Primary Ciliary Dyskinesia

Signs and Symptoms:

- Recurrent respiratory infections
- Chronic cough & rhinorrhea
- Bronchiectasis
- Laterality Defects (Situs Inversus, 50%)
- Otitis
- Nasal polyps
- Male infertility (Female subfertility?)

Other complications:

- Congenital heart defects -30%
- Hydrocephalus (rare)
- Retinitis pigmentosa (rare)

Knowles MR 2013  Boon M 2013
Ciliary ultrastructure

- Motile cilia characterized by a core axoneme structure of cilia and flagella which consists of a central pair (CP) of single microtubules, surrounded by nine doublet microtubules giving the classic “9+2” arrangement when viewed in cross-section.
- Motile monocilia that initiate L-R body axis patterning in the embryo, lack the CP apparatus in the “9+0” arrangement. Have a circular beat giving rise to a leftward fluid flow.
- A number of microtubule associate protein complexes attach, along the axoneme length in a regular 96nm repeat formation to provide stability.
- Pairs of ODA and IDA motor complexes attach to peripheral doublets, responsible for ATP generated ciliary beating.
- Nexin-dynein arm complexes (N-DRC), link the peripheral doublets and radial spokes.
Diagnosis of PCD – state of the art

- Diagnostic investigations of PCD are highly specialized, laborious, require expensive equipment and experienced teams of clinicians and scientists
- Currently there is no “single gold standard investigation”
- Historically defects were first identified using EM and this was the “gold standard” for several years
- Samples of ciliary epithelia are examined in EM; up to 100 cross sections of cilia may be evaluated in order to detect abnormalities
PCD diagnosis – State of the art routinely available tests

Transmission Electron Microscopy (TEM)
- Ultrastructural assessment: sensitivity 70%
- Up to 20% disorientation = normal
- Normal Ultrastructure

High Speed Video Microscopy (HSVM)
- Estimation of Ciliary Beat Frequency & Ciliary Beat Pattern
- Much lower in PCD patients vs controls 10-20% normal

Nasal Nitric Oxide (nNO)
- Normal values up to 77nl/min or up to 300ppb
- Mostly used as a screening test
PCD diagnosis – State of the art specialised tests

In many cases EM and HSVM are inconclusive so need to use additional tests

**Molecular Genetics**
- NGS panels (30 or more genes (ciliome), 150 genes, expanded ciliome WES, WGS)

**Immunofluorescence (IF)**
- Localization of cilia proteins

**Culture of airway ciliated cells**
- Ciliogenesis before performing HSVM and TEM
- Reduction of secondary defects, caused by damage, infection

- Secondary defects
  - Compound cilia
  - Swollen cilia
  - Additional, single microtubules

[Link to cilia pathology]

[Image of molecular genetics methods]
[Image of immunofluorescence methods]
[Image of culture of airway ciliated cells methods]
PCD diagnosis - TEM

Transmission Electron Microscopy

IN THE PAST: Considered as the Gold Standard

Typical ultrastructural defects
- Could be used as a guide for genetic testing
- Dynein Arm Defects
- Mircrotubular defects
- Central Pair defects
- Nexin links defects
- Orientation defects

Normal Ultrastructure

Outer Dynein Arm Defect

Central pair defect
Ciliary Beat Frequency (CBF)/ pattern using HSVM

- Cilia beat in synchrony with a coordinated whiplash motion

- CBF is normally between 11-18Hz, but in patients with PCD, beat frequency is zero (static), slow or fast

- In some cases CBF normal but beat pattern is abnormal
  
  - e.g. in ODA defects cilia are static but in central pair defects cilia make a rotating motion
  
  - Cilia with ODA defects are either static or show a low amplitude motion
  
  - Central Pair defect: cilia show a circular beat pattern

- Normal beat pattern: Cilia sweep forward bend and flick backwards

- IDA: Cilia show a stiff backward forward motion
PCD use of HSVM

Respiratory cilia: mechanical defense mechanism

- Mucociliary clearance
- Cilia produce a whiplash, wave like motion removing pathogens, cell debris or inhaled particles by expelling mucus
PCD Diagnosis - TEM

TEM is no longer the Gold Standard

- PCD with normal ultrastructure in TEM
- Mutations in DNAH11

Knowles MR 2012
CP Defect

HSVM results: rotational pattern
PCD Genetics >30 genes identified

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein localisation/function</th>
<th>Ultrastructural defect in PCD</th>
<th>Typical functional defect in PCD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA1</td>
<td>ODA</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>DNAH5</td>
<td>ODA</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>DNA1I2</td>
<td>ODA</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>TXNDC3 (NME8)</td>
<td>ODA</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>DNAL1</td>
<td>ODA</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>CCDC114</td>
<td>ODA docking complex</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>ARMC4</td>
<td>ODA docking complex</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>CCDC151</td>
<td>ODA targeting and docking</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>CCDC103</td>
<td>Cytoplasmic, ODA assembling</td>
<td>ODA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>KTU (DNAAF2)</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>LRRC50 (DNAAF1)</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
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<tr>
<td>DNAAF3</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>DYX1C1</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
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<tr>
<td>HEATR2</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>LRRC6</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
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<tr>
<td>ZMYND10</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
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<tr>
<td>SPAG1</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
</tr>
<tr>
<td>C21orf59</td>
<td>Cytoplasmic, DA assembling</td>
<td>ODA+IDA defect</td>
<td>Immotile cilia</td>
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<tr>
<td>CCDC39</td>
<td>N-DRC</td>
<td>MT disorganisation and IDA defect</td>
<td>Hyperkinetic, stiff cilia</td>
</tr>
<tr>
<td>CCDC40</td>
<td>N-DRC</td>
<td>MT disorganisation and IDA defect</td>
<td>Hyperkinetic, stiff cilia</td>
</tr>
<tr>
<td>CCDC164</td>
<td>N-DRC</td>
<td>N-DRC defect; not visible</td>
<td>Reduced amplitude</td>
</tr>
<tr>
<td>CCDC65</td>
<td>N-DRC</td>
<td>N-DRC defect</td>
<td>Impaired motility</td>
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<tr>
<td>RSFH4A</td>
<td>RS</td>
<td>MT disorganisation (CP-RS defect); not visible</td>
<td>Circular beat or stiff cilia</td>
</tr>
<tr>
<td>RSFH9</td>
<td>RS</td>
<td>MT disorganisation (CP-RS defect)</td>
<td>Circular beat</td>
</tr>
<tr>
<td>RSFH1</td>
<td>RS</td>
<td>MT disorganisation (CP-RS defect)</td>
<td>Different beating patterns</td>
</tr>
<tr>
<td>HYDIN</td>
<td>CP</td>
<td>CP projection defect; not visible</td>
<td>Reduced amplitude, discoordination</td>
</tr>
<tr>
<td>DNAH11</td>
<td>ODA</td>
<td>Normal ultrastructure</td>
<td>Hyperkinetic cilia, reduced amplitude</td>
</tr>
<tr>
<td>RPGR</td>
<td>Cytoplasmic</td>
<td>Syndromic PCD with retinitis pigmentosa</td>
<td></td>
</tr>
<tr>
<td>OFD1</td>
<td>Cytoplasmic</td>
<td>Syndromic PCD with orofacialdigital syndrome</td>
<td></td>
</tr>
<tr>
<td>CCNO</td>
<td>Apical cytoplasm</td>
<td>Reduction of multiple motile cilia</td>
<td></td>
</tr>
</tbody>
</table>
Assessment of dynein arms, microtubular organisation, central apparatus, cilia orientation, nexin links?

Horani et al. 2016
A 30 gene PCD diagnostic panel is now available. It is estimated that this panel will pick up approximately 70% of all PCD genetic defects. Primary panel - ciliome

- ARMC4 C21orf59 CCDC103 CCDC114 CCDC151 CCDC39 CCDC40 CCDC65 CCNO DNAAF1 DNAAF2 DNAAF3 DNAAF5 DNAH11 DNAH5 DNAH8 DNAI1 DNAI2 DNAL1 DRC1 DYX1C1 MCIDAS NME8 OFD1 RPGR RSPH1 RSPH4A RSPH9 SPAG1 ZMYND10

Nearly half of all PCD patients have some form of a congenital heart defect (CHD), so PCD panels also include genes for CHD or - 150 PCD candidate genes (Onoufriadis et al, 2014) - expanded ciliome

- WGS or WES will lead to the discovery of novel PCD genes.
Immunofluorescent studies

Use antibodies directed at different protein components of the cilium; act as structural markers

<table>
<thead>
<tr>
<th>Cilium components</th>
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<tbody>
<tr>
<td><strong>Microtubules</strong></td>
</tr>
<tr>
<td>Acet. Tubulin</td>
</tr>
<tr>
<td>Alpha/beta Tubulin</td>
</tr>
<tr>
<td><strong>ODA</strong></td>
</tr>
<tr>
<td>DNAH5, DNAH9, DNAH11</td>
</tr>
<tr>
<td>DNAI1, DNAI2</td>
</tr>
<tr>
<td><strong>IDA</strong></td>
</tr>
<tr>
<td>DNALI1</td>
</tr>
<tr>
<td><strong>RS</strong></td>
</tr>
<tr>
<td>RSPH1, RSPH4A, RSPH9</td>
</tr>
<tr>
<td><strong>N-DRC</strong></td>
</tr>
<tr>
<td>GAS8, LRRC48, CCDC39</td>
</tr>
</tbody>
</table>

Mutations in *DNAH5*

Available only in the University of Munster, Germany

*Am J Respir Crit Care Med. 2005;171(12):1343-9*
FP7 BESTCILIA project

- To address the many limitations and gaps of knowledge that are characterizing PCD diagnosis and management.
- Need for a multi-centre/multi disciplinary collaboration
- BESTCILIA consortium (clinicians and researchers from 9 countries)
  - Led by Prof Heymut Omran (Germany)
  - Participants from
    - UK
    - Switzerland
    - Cyprus
    - Netherlands
    - Greece
    - Poland
    - Denmark
    - USA
  - Supported by international scientific organizations (European Respiratory Society) and patient advocate groups.

www.pcdregistry.eu

Better Experimental Screening and Treatment for Primary Ciliary Dyskinesia
Summary

• PCD is not a single disorder, but embraces a range of genetically inherited, heterogeneous disorders. Associated with oto-sinopulmonary disease and infertility in males

• Accurate diagnosis of PCD requires a multidisciplinary approach and expertise which currently, is not widely available. Modalities such as TEM, nNO and molecular genetics have a sensitivity of up to 70%

• No PCD diagnostic test is perfect so currently information from a combination of different tests, is required; however no diagnostic algorithm is available.

• Genetics is rapidly emerging and leading to the discovery of novel PCD genes, involved in cilia assembly, structure and function. Most patients have homozygous bi-allelic mutations

• PCD is best diagnosed in the neonatal period so that patients benefit from appropriate treatments and better quality of life
Acknowledgments

- Collaborators
  - Cyprus Institute of Neurology and Genetics
    - Kyriacos Kyriacou, PhD
    - Andreas Hadjisavvas, PhD
    - Marianna Nearchou, MSc
    - Maria Loizidou, PhD
    - Panayiota Pirpa, MSc
    - Kyriaki Michailidou, PhD
  - Cyprus University of Technology
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  - University of Cyprus
    - Panayiotis Yiallouros
- Funding
  - FP7 BESTCILIA
- Cyprus Research Promotion Foundation
Genetics of PCD

To date more than 30 genes found to be associated with PCD

Estimated that several hundred genes encode proteins responsible for normal ciliary function; many more genes to be discovered

Historically the first gene associated with PCD was DNAH5 discovered in 2000.

Approximately 50-60% of PCD patients have bi-allelic mutations in genes associated with PCD

Genetically heterogeneous

Most of these mutations correspond to a specific ultrastructural defect e.g

- mutations in DNAH5 cause defects of dynein arms
- mutations in ZMUND10, DYX1C1 associated with IDA and ODA defects
- mutations in CCDC39 and CCDC40 lead to axonemal disorganization and absent IDA