

## **Accuracy of immunofluorescence in the diagnosis of Primary Ciliary Dyskinesia**

Amelia Shoemark<sup>1,2</sup>, Emily Frost<sup>1</sup>, Mellisa Dixon<sup>1</sup>, Sarah Ollosson<sup>1</sup>, Kate Kilpin<sup>1</sup>, Andrew V Rogers<sup>1</sup>, Hannah M Mitchison<sup>3</sup>, Andrew Bush<sup>1,2</sup>, Claire Hogg<sup>1</sup>

<sup>1</sup> Department of Paediatrics, Royal Brompton & Harefield NHS Trust, London, UK

<sup>2</sup> National Heart and Lung Institute, Imperial College London, UK

<sup>3</sup> Genetics and Genomic Medicine Programme, Institute of Child Health, University College London, UK

Correspondence to:

Amelia Shoemark

Primary Ciliary Dyskinesia Service

Electron microscopy unit

Department of Paediatrics

Royal Brompton Hospital

London SW3 6NP

Statement of contribution:

AS, CH and AB designed the study. EF, KK, SO and AS consented patients, conducted light microscopy, collected nasal brushings and prepared slides. EF and AS conducted IF staining and analysis. MD conducted light and electron microscopy. HM provided genotyping. AS and EF analysed the data. AS, CH and AB drafted the manuscript. All authors contributed to manuscript drafts and preparation. AS is custodian of the data and takes responsibility for its accuracy.

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**At a Glance Commentary:** Scientific Knowledge on the Subject

Primary Ciliary Dyskinesia is a genetically heterogeneous chronic condition. Early diagnosis is key to attenuating disease progression by implementation of appropriate medical management. Currently diagnosis requires expensive and complex equipment

What This Study Adds to the Field

This study validates the clinical use of a panel of commercially available antibodies to diagnose Primary Ciliary Dyskinesia by immunofluorescence, a simpler, more widely available, cost effective alternative to current confirmatory diagnostic tests.

Immunofluorescence is a useful diagnostic test for PCD, reduces the need for repeat biopsies, and improves turnaround time without compromising diagnostic accuracy.

"This article has an online data supplement, which is accessible from this issue's table of content online at [www.atsjournals.org](http://www.atsjournals.org)"



## **ABSTRACT**

**Rationale** Diagnosis of primary ciliary dyskinesia is by measuring ciliary beat frequency and pattern by video microscopy and ciliary ultrastructure by electron microscopy; equipment and expertise is not widely available. Fluorescent antibodies to ciliary proteins are widely used to validate genetic studies in many settings, but diagnostic utility in this disease has not been systematically evaluated.

**Objectives** Determine the sensitivity and specificity of a panel of fluorescent labelled antibodies as a diagnostic tool for primary ciliary dyskinesia.

**Methods** Immunofluorescent labelling of nasal brushings from 35 patients with primary ciliary dyskinesia, and 386 patients referred with symptoms suggestive of the disease, using six commercially available antibodies. Each was assessed on a smear slide in a double label with acetylated tubulin to demonstrate the presence of cilia. The results were compared to diagnostic outcome by conventional pathways.

**Measurements and Main Results** Immunofluorescence correctly identified primary ciliary dyskinesia in the discovery cohort. In the diagnostic accuracy study immunofluorescence successfully identified 22 of 25 patients diagnosed with primary ciliary dyskinesia and all 252 in whom the diagnosis was excluded, positive predictive value 1.0 and negative predictive value 0.01. Sensitivity was 88%, specificity was 100%. Immunofluorescence additionally correctly diagnosed 55% (39) of cases which were inconclusive using the standard diagnostic protocol. Immunofluorescence results were available within 14 days, costing \$187 per sample compared to electron microscopy which took 27 days and cost \$1452.

**Conclusions** Immunofluorescence is a sensitive and highly specific diagnostic test for primary ciliary dyskinesia, and can improve the speed and availability of diagnostic testing.

**Abstract word count: 252**

**Key words: Cilia, Electron microscopy, Antibody**

**INTRODUCTION**

Primary Ciliary Dyskinesia (PCD) affects approximately 1 in 15,000 of the population. Manifestations are caused by defective ciliary beating and reduced mucociliary clearance. Diagnosis is frequently delayed, and delay is associated with significant impairment of lung function. Diagnostic delay is related to two factors: the non-specificity of symptoms (cough, rhinitis) and the lack of an easy and widely available diagnostic test for the condition. [1]

The diagnosis pathway for PCD includes measurement of nasal nitric oxide, followed by a nasal brush biopsy for light and electron microscopy. Light microscopic assessment of ciliary function is by high speed video analysis of the frequency and pattern (waveform) of cilia movement on live cells. Electron microscopy allows visualisation of the ultrastructure of cilia and can often provide a definitive diagnosis [1]. All tests require sophisticated equipment and considerable expertise, and in consequence are only available in very few centres. Genetic testing for PCD is also increasingly used, but there are at least 200 potential genes which are widely scattered through the karyotype. To date, more than 30 disease-associated mutations have been identified, which are estimated to account for 60-65% of known cases [2,3].

Immunofluorescence allows specific antibodies with fluorescent tags indirectly to image target proteins by fluorescent or confocal microscopy. The use of double labelling allows the co-localisation of proteins to be determined. Immunofluorescence for the diagnosis of PCD was first described in 2005 and was subsequently recommended in the ERS expert consensus statement for diagnosis and treatment of PCD [4,5]. Despite this recommendation limited commercial availability of validated antibodies and lack of evidence for the diagnostic accuracy of the technique has limited its use. Immunofluorescence has been used extensively in PCD research in confirming protein absence due to genetic mutations. A number of

antibodies to proteins defective in PCD have been developed and validated. These include DNAH5 (an outer dynein arm heavy chain) [4,6], DNALI1 (an inner dynein arm light chain)[6,7], GAS8 (a component of the nexin dynein regulatory link) [7] and RSPH4A, RSPH9 and RSPH1 (components of the radial spoke)[8]. These antibodies represent the four key ultrastructural abnormalities detected by electron microscopy which are the end products of multiple gene defects (table 1 and figure 1). It can be seen that absence or mislocation of a single protein allows the effects of mutations in multiple genes to be detected. We hypothesised that immunofluorescence using a panel of antibodies would be a sensitive and specific diagnostic test for PCD, and aimed to assess this in a large cohort of patients with possible PCD referred for diagnostic testing.

## **METHODS**

### *Subjects*

Discovery cohort- Nasal brushings were analysed from a cohort of 35 patients with a known PCD ultrastructural defect.

Diagnostic accuracy cohort- Sequential nasal brushings were analysed from 386 patients referred to the Royal Brompton Hospital for PCD diagnosis. Referrals were due to symptoms suggestive of PCD such as situs inversus, neonatal respiratory distress, bronchiectasis, recurrent chest infections, rhinosinusitis and otitis media.

### *Diagnosis of PCD*

All patients underwent a standardised diagnostic protocol regularly audited across the 3 UK PCD centres [5].

1. Assessment for symptoms suggestive of PCD (n= 378/386; 8 external samples had a limited clinical history).
2. Nasal Nitric Oxide measurement – In children >4 years. Two readings from each nostril were taken during a breath hold manoeuvre using a chemiluminescent analyser LR2000 (Logan Research, Rochester, UK) [9] (n= 129/386)
3. High speed video microscopy for cilia beat frequency and ciliary beat pattern measured at 37°C using a 100x objective [10] (n=386/386)
4. Quantitative transmission electron microscopy of ciliary ultrastructure [11] (n=208/386)
5. Air liquid interface culture of difficult samples and repeat light and electron microscopy [12] (n=115/386)
6. Genotyping from blood sample in patients with likely PCD based on other investigations (n=16/386)

In the absence of an established gold standard, a diagnosis of PCD was made following a review of clinical and laboratory findings in a monthly multidisciplinary meeting (MDT) lead by a consultant clinician with expertise in PCD

#### *Experimental methods*

Methodological details are provided in the online data supplement [4]. All slides were double labelled with acetylated alpha tubulin (T7451, Sigma Aldrich) in order to visualise cilia.

Antibodies of interest were used in a two step protocol. All nasal brushings were assessed for Panel 1: DNAH5 (HPA037470), DNALI1 (HPA053129), RSPH4A (HPA031196). A second round of antibodies were used in selected cases. Panel 2: RSPH9 (HPA031703), RSPH1 (HPA017382) or GAS8 (HPA041311)

Slides were scanned under a fluorescent microscope for ciliated cells at x40 magnification to identify acetylated tubulin. In each ciliated cell, the co-localised ciliary protein of interest was assessed in a second channel. If there was visual co-localisation of the antibody label with acetylated tubulin the target protein was considered present. If more than seven of ten ciliated cells were clearly labelled the sample was considered normal for that protein. PCD was defined as at least ten of ten ciliated cells with absent or partial staining of the target protein and was repeated on a duplicate slide for confirmation. Insufficient and inconclusive slides were repeated. If the second assessment was normal the second assessment was included in results (n=71).

#### *Statistical methods*

The subject number was based on a 10% prevalence of PCD in the population tested, and a sensitivity of 85%, obtained from pilot data during antibody testing. Power calculations predicted 271 patients with a confirmed diagnosis of 'PCD' or 'Non-PCD' would be required to predict sensitivity and specificity with 95% confidence. Results were analysed in graph pad prism 5 and a p value <0.05 considered significant. Diagnostic sensitivity and specificity was calculated for immunofluorescence and compared to the MDT diagnosis of PCD. This calculation included only samples in which a positive or negative conclusion was reached. Exclusions are shown in Figure 2.

#### **ETHICS**

This study was conducted according to the recommendations of the Declaration of Helsinki. The protocol was approved by the Hounslow and Hillingdon ethical review committee (08/H0709/73). Written consent was obtained from subjects or their parent/guardian.

## **RESULTS**

### **Immunofluorescence can be used to confirm a diagnosis of PCD**

Antibodies were tested on nasal brushings from a discovery cohort of 35 patients with PCD with a confirmed ultrastructural ciliary defect. Results shown in table 2 demonstrated complete agreement between absence of structure by electron microscopy and absence of associated protein by immunofluorescence.

### **Diagnostic accuracy of immunofluorescence in the validation cohort**

The immunofluorescence technique successfully demonstrated an absence of target proteins from the axoneme in 22 of 25 patients with a MDT diagnosis of PCD. Normal results were obtained in all 252 patients who did not have PCD.

Figure 2 shows an example of immunofluorescent staining of ciliated epithelial cells from two patients who were ‘PCD-positive’ and a patient who was ‘PCD-negative’. The IF protocol had a positive predictive value (PPV) of 1.0 and negative predictive value (NPV) of 0.01 in this population. Sensitivity was 88% and specificity 100%.

Results of the diagnostic tests and immunofluorescence tests are shown in tables 3 and 4.

Immunofluorescence identified all patients who had an identifiable ultrastructural defect.

Sensitivity and specificity of the test was the same as that of electron microscopy.

Three patients who were ‘PCD- positive’ were not identified using the immunofluorescence protocol and were examined in closer detail. Genetic tests in two patients showed mutations in *DNAH11*. Both patients had a beat pattern consistent with this defect (hyperfrequent, stiff

and static) on more than one biopsy and nasal NO <77nl/min. One patient had consanguineous parents and was homozygous for a nonsense mutation (c.3380G>A , p.Trp1127\*) the other had heterozygous nonsense changes (c.5506C>T , p.Arg1836\* and c.5636T>A p.Leu1879\*) . The third individual was also from consanguineous parentage and was homozygous for a frame shift mutation in the *HYDIN* gene (c.2196dupT, p.Y372fs). The ultrastructure from these three patients was considered normal by standard electron microscopy. Electron tomography from the patient with the *HYDIN* mutation showed absence of the c2b central pair projection [16].

### **Insufficient and inconclusive samples**

*Immunofluorescence as part of the diagnostic pathway could reduce the requirement for repeat nasal brushing.*

In 71 patients a conclusive MDT diagnosis was not made on first sampling due to insufficient tissue for light and/or electron microscopy. The immunofluorescence protocol was able to produce a definitive result for 55% (39) of these cases. Two of these cases showed an absence of DNAH5 by immunofluorescence and were confirmed subsequently on a repeat biopsy as an outer dynein arm defect by electron microscopy. The remaining 37 samples, in which immunofluorescence testing was normal, were confirmed 'PCD-negative' on second testing either by repeat nasal brushing (n=31) or by culturing the original sample at air liquid interface (n=6).

*Insufficient and inconclusive results by immunofluorescence*

In 42 samples in which a MDT diagnosis was made there were not enough cells for immunofluorescence. In a further 32 samples in which a MDT diagnosis was made the

immunofluorescence result for one or more antibody was inconclusive on more than one occasion. Sixty nine of these patients (represented by 71 samples) did not have PCD, however, two were diagnosed with PCD at the MDT meeting. One of these two patients yielded a sample which was insufficient for immunofluorescence, light microscopy and electron microscopy but found to have a mutation in the *CCNO* gene (c.258\_262dupGGCCC, p.Gln88Argfs\*8). This patient was related to a previously genetically characterised family with reduced generation of multiple motile cilia (14). The second patient with PCD had DNAH5, DNALI1 and GAS8 present, however, inconclusive results were obtained by immunofluorescence for RSPH4A on two separate slides and the other radial spoke head proteins RSPH1 and RSPH9 were absent. This patient had a circular beat pattern on light microscopy and a transposition /central pair complex defect detected by electron microscopy. These insufficient and inconclusive patients were excluded from the sensitivity and specificity analysis, inclusion of these inconclusive results as 'missed cases' modifies the sensitivity to 81% but does not affect the specificity. In thirty five patients a diagnosis of 'PCD positive' or 'PCD negative' could not be reached during the study period.

We investigated factors relating to insufficient and inconclusive immunofluorescent results. Blood and mucus in the sample both appeared to be confounding factors. In 63% of samples with inconclusive results by immunofluorescence viscous mucus was surrounding the cilia on the high speed video light microscopy assessment compared to 46% of conclusive samples ( $p < 0.05$ ). In 25% of samples insufficient for immunofluorescence blood was seen in the sample compared to 8% in sufficient samples ( $p < 0.01$ ). The cause of this relationship is unknown, we hypothesise that increased blood could prevent cells from attaching to the slides or increased blood in a sample is due to a damaged mucosa denuded of cilia as a result of a recent infective or inflammatory process

Samples were deemed insufficient or inconclusive if a result could not be obtained for one or more antibodies. 30% were inconclusive or insufficient in just one antibody, 16% in two antibodies and 54% in three or more antibodies. Couriers and cultured samples showed similar results compared to nasal brushings taken on site.

### **Time for results to be available and cost of investigation**

Immunofluorescence was compared to electron microscopy. The turnaround time, defined as time from the sample being taken to the results being reported was median 14 days (range 1 - 40) for immunofluorescence compared to 27 days (range 9 - 61) for electron microscopy  $p < 0.05$ . Additionally a cost assessment exercise which included staff hours, equipment running costs and consumables showed that the cost per sample was \$187 for immunofluorescence and \$1452 for electron microscopy. The assessment did not include the purchase and set up of equipment required.

## **DISCUSSION**

All defects were correctly identified by immunofluorescence in a cohort of patients with a known ultrastructural diagnosis of PCD. In a subsequent cohort of consecutively referred patients immunofluorescence successfully identified 22 of 25 patients diagnosed with PCD and 252 of 252 in whom the diagnosis was excluded. The sensitivity and specificity of immunofluorescence was the same as that of electron microscopy. Immunofluorescence failed to identify 12% PCD cases in the present study and is therefore not suitable for use as a stand-alone test. This report provides strong evidence for introducing this test into clinical practice

The main strength of the immunofluorescence technique is the cost reduction and improved turnaround time relative to electron microscopy to confirm a diagnosis of PCD. The cost of the test and the basic equipment and the simplicity of the test may allow improved accessibility to a wider population of patients. The immunofluorescence technique also works on small samples where there are too few cells to process for electron microscopy analysis. In the diagnostic cohort an additional 34 cases could have been diagnosed with the inclusion of an immunofluorescence in the diagnostic pathway.

The strengths of this study include that it has been conducted in clinical setting which follows a nationally audited and standardised algorithm for PCD diagnosis giving a realistic indication of how this test performs. . Other strengths include the use of discovery and validation cohorts, with large numbers in the latter in particular.

A weakness of this study, and the field of PCD diagnostics, is the lack of gold standard for the diagnosis of PCD. Despite use of a multidisciplinary diagnosis to maximize diagnostic

capability fifteen patients had an indeterminate diagnosis (a situation also seen in cystic fibrosis, for example). It is likely that some of these patients have PCD and that our current diagnostic protocol has not allowed us to make a firm diagnosis. These cases could have PCD, which would impact the sensitivity and specificity of the test; however, this is conjectural currently.

The major limitation of the immunofluorescence technique is that the antibodies used are directed to specific proteins of interest and therefore defects in unrelated proteins will be missed. This antibody panel represents the end products of multiple gene defects, however three cases were still missed. The three cases missed in this study had biallelic mutations in *DNAH11* and *HYDIN*. This is expected since previous publications have demonstrated the outer and inner dynein arms, nexin links and radial spokes are present in patients with these defects [15,16]. Antibodies to both *HYDIN* and *DNAH11* proteins are commercially available however we have not been able to validate the use of either antibody sufficiently for use in PCD diagnosis, nor is there any report in the literature of the successful use of these antibodies to identify PCD. New reliable antibodies to the *DNAH11* and *HYDIN* proteins would benefit diagnosis and research in this area. Furthermore, the cilium consists of over 200 proteins and it is therefore likely that other cases of PCD missed by this panel will emerge.

Given the similar sensitivity and specificity we envisage that immunofluorescence could be useful where transmission electron microscopy equipment or expertise is not available. In specialist diagnostic centers where these facilities are available the technique could be added to the diagnostic protocol to improve diagnostic success and reduce the number of electron microscopy tests required. Application of the immunofluorescence technique in this study

was incorporated into our PCD diagnostic pathway following light microscopy assessment. Firstly, a core panel of three antibodies is applied and then a second panel based on the primary panel and the light microscopy findings. This two-step protocol allows cost, time and tissue savings but introduces a selection bias. In this study three patients were diagnosed using the second panel by the GAS8 antibody. As absence of DNALI1 always co-existed with absence of GAS8 or DNAH5 we suggest DNALI1 might be substituted for GAS8 in the first panel. Recent data show that use of a RSPH9 antibody may detect a broader range of central pair complex defects than RSPH4A [17]. Therefore GAS8, DNAH5 and RSPH9 might be a more appropriate selection for the first panel.

In conclusion immunofluorescence is a useful diagnostic test for PCD, reduces the need for repeat biopsies, and improves turnaround time without compromising diagnostic accuracy. We suggest it should be included in the routine PCD diagnostic pathway.

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## FIGURE LEGENDS

Figure 1: Diagram of the ultrastructure of a motile cilium in transverse section. Labels indicate ultrastructural features targeted by immunofluorescence with corresponding antibodies (**bold text**).

Figure 2; Workflow diagram indicating the pathway of patients from referral to diagnosis during the study period. Numbers in bold indicate the number of patients at each stage of the study (not the number of samples). Excluded groups are indicated in red. Groups included in the sensitivity and specificity analysis are indicated in green.

Figure 3: Example results in PCD and non PCD samples for three antibodies used in the immunofluorescence panel. The left column shows the cell nucleus in blue by DAPI, the next column shows presence of cilia on the cell in green by acetylated tubulin. The third column shows the protein of interest in red and the final column a merged image of the three channels. In the merged image yellow suggests co-localisation and presence of the protein of interest, green suggests absence of the protein of interest. The top images show DNALI1 (an inner dynein arm component), the middle images DNAH5 (an outer dynein arm component), and the bottom RSPH4A (a radial spoke head component)

## TABLES

Ultrastructural Defect	Gene	Immunofluorescence
Outer dynein arm defect	<i>DNAH5</i> <i>DNAI1</i> <i>ARMC4</i> <i>CCDC114</i> <i>TXNDC3</i> <i>(NME8)</i> <i>DNAI2</i> <i>DNALI1</i> <i>CCDC151</i>	DNAH5 Absent
Inner and outer dynein arm defect	<i>C21orf59</i> <i>ZYMND10</i> <i>CCDC103</i> <i>DNAFF2</i> <i>(KTU)</i> <i>DNAFF1</i> <i>(LRRC50)</i> <i>LRRC6</i> <i>DNAFF3</i> <i>(C19orf31)</i> <i>HEATR2</i> <i>DYX1C1</i> <i>SPAG1</i>	DNAH5 Absent DNALI1 Absent
Central complex / transposition defect	<i>RSPH4A</i>  <i>RSPH9</i> <i>RSPH1</i> <i>RSPH3</i>	RSPH9, RSPH4A,RSPH1 absent RSPH9 absent RSPH9, RSPH1 absent All present
Microtubular disorganisation with loss of inner dynein arm	<i>CCDC39</i> <i>CCDC40</i>	DNALI1 Absent GAS 8 Absent
Microtubular disorganisation with present inner dynein arms	<i>CCDC65</i> <i>CCDC164</i> <i>GAS8</i>	GAS 8 Absent
Normal ciliary ultrastructure	<i>HYDIN</i> <i>DNAH11</i> <i>OFD1</i> <i>RPGR</i>	All present
Ciliary 'aplasia'	<i>CCNO</i> <i>MCIDAS</i>	All present

Table 1: The major classes of ultrastructural defects seen in PCD and the gene associated with each defect (former gene name is shown in brackets). Column three shows the assumed coverage of the six antibodies investigated in this study over the known ultrastructural and gene defects.

<b>Electron microscopy defect</b>	<b>Absent or mislocalised antibody</b>	<b>Number of patients with PCD tested (total n=35)</b>
Outer dynein arm defect	DNAH5	14
Outer and inner dynein arm defect	DNAH5 DNALI1	10
Inner dynein arm and microtubular disorganisation defect	DNALI1 GAS8	7
Transposition defect / central pair absence	RSPH4A RSPH9 RSPH1	4

Table 2: Immunofluorescence antibody results from 35 patients with primary ciliary dyskinesia due to a known ultrastructural defect

		<b>Standard diagnosis</b>	
		PCD positive	PCD negative
<b>Immunofluorescence diagnosis</b>	PCD positive	22	0
	PCD negative	3	252

Table 3: Diagnostic outcome table comparing immunofluorescence technique to the standard diagnostic approach in 271 patients referred to a national referral centre for investigation into symptoms suggestive of primary ciliary dyskinesia (PCD)

MDT diagnosis	Light microscopy			Electron microscopy	Immunofluorescence						
		Ciliary Beat Pattern	CBF Hz mean (stdev)		DNAH5	DNALI1	RSPH4A	GAS8	RSPH9	RSPH1	
PCD negative n= 252	148	stiff areas (116), slow beat (87), possible area of circling/ semi circling (11), un-coordinated (41), dyskinetic (28), fast beat (4),static areas (9)*	9.8 (2.1)	Normal ultrastructure -97% <i>both dynein arms present (+/- 7%) 89% normal microtubular arrangement (+/-6%)</i>	present (148)	present (148)	present (148)	present (37)	present (11)	present (11)	
	104	normal beat pattern	11.1 (1.4)		ND	present (104)	present (104)	present (104)	ND	ND	ND
			<b>10.2 (2.0)</b>								
PCD positive n=25	8	completely static (7), mostly static some moving cilia (1)	0Hz-8.4Hz	IDA and ODA defect	<b>absent (8)</b>	<b>absent (7)</b> <b>partial absent (1)</b>	present (8)	present (8)	ND	ND	
	8	completely static (1), static with occasional cilia with residual 'twitching or slow beat (7)	0Hz	ODA defect	<b>absent (8)</b>	present (8)	present (8)	ND	ND	ND	
	2	stiff (1), stiff incomplete beat with static patches (1)	4.77Hz*	IDA and MTD defect	present (2)	<b>absent (2)</b>	present (2)	<b>absent (2)</b>	ND	ND	
	1	dyskinesia, circling pattern viewed from above	8.4Hz	Transposition defect	present (1)	<b>present (1)</b>	<b>absent (1)</b>	present (1)	<b>absent (1)</b>	<b>absent (1)</b>	
	3	stiff incomplete beat (3), pause in beat (2)	12.5-13.3Hz	MTD defect	present (3)	<b>present (3)</b>	present (3)	<b>absent (3)</b>	present (3)	present (3)	
	3	mostly static (1), stiff (3), jittering (3), hyperfrequent (1), movement at tips static at base (2), incomplete beat(3)	0-16.4Hz	Normal ultrastructure	present (3)	present (3)	present (3)	present (3)	present (3)	present (3)	
		<b>4.5 (5.1)</b>									

Table 4: Comparison of results from light microscopy, electron microscopy and immunofluorescence testing of nasal brushings from patients referred due to clinical symptoms suggestive of primary ciliary dyskinesia. CBF = ciliary beat frequency \*one patient in this group did not have CBF measured due to dyskinesia