

**RAPID DIAGNOSIS OF PRIMARY CILIARY DYSKINESIA: cell culture and soft computing analysis**

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## **Summary (words = 200)**

**Background** Diagnosis of primary ciliary dyskinesia (PCD) sometimes requires repeated nasal brushing to exclude secondary ciliary alterations.

**Objective** To evaluate whether the use of a new method of nasal epithelial cell culture can speed PCD diagnosis in doubtful cases and to identify which are the most informative parameters by means of a multi-layer Artificial Neural Network (ANN).

**Methods** A cross-sectional study was performed in patients with suspected PCD. All patients underwent nasal brushing for ciliary motion analysis, ultrastructural assessment, and evaluation of ciliary function after ciliogenesis in culture by ANN.

**Results** One hundred fifty-one subjects were studied. A diagnostic suspension cell culture was obtained in 117 nasal brushings. A diagnosis of PCD was made in 36 (29 children) subjects. In 9 out of the 36 patients the diagnosis was made only after a second brushing because of equivocal results of both tests at first examination. In each of these subjects diagnosis of PCD was confirmed by cell culture results.

**Conclusions** Cell culture in suspension evaluated by means of ANN allows the separation of PCD from SCD patients after only 5 days of culture and allows the diagnosis to be reached in doubtful cases, thus avoiding the necessity of a second sample.

## **Keywords**

Ciliary motion analysis, mathematical evaluation, suspension cell culture, transmission electron microscopy

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## **INTRODUCTION**

Primary ciliary dyskinesia (PCD), a rare inherited disorder characterized by chronic infection of the upper and lower airway, (1, 2) is usually diagnosed by the combination of clinical features together with distinctive transmission electron microscopy (TEM) images, and beat frequency and pattern of ciliary motion analysis (CMA) from a nasal brush biopsy (3). In the relatively few patients in whom these tests are equivocal, there are other tests available including ciliary culture, immunofluorescence of ciliary proteins and genetic testing (4). The occasional need for more sophisticated tests is related to the fact that the diagnosis of PCD may be difficult because of respiratory epithelial damage secondary to infection or inflammation. Furthermore, ultrastructural findings may be normal despite alterations in ciliary beat patterns strongly suggestive of a primary disease (5). It is in these patients that ancillary testing may be particularly valuable. If secondary damage is suspected a second sample is usually recommended after a period of antibiotic and anti-inflammatory therapy, but even with this strategy a second brushing may not be diagnostic in at least 5% of cases (1). Alternatively, when CMA and TEM provide equivocal findings, PCD diagnosis can be reached with the culture of ciliated cells since all secondary damage is virtually absent after ciliogenesis in culture. Successful cultures are associated with the ciliogenesis of human respiratory epithelium which takes place after 24–48 h of culture when aggregated cells are floating through the medium in the form of spheroids. These consist of a layer of respiratory epithelial cells which maintain cell polarity and, in subjects without PCD, exhibit normal beating cilia at their outer surface (video clip 1).

If a frankly abnormal pattern of ciliary movement persists after culture the diagnosis is PCD (6). In the past culture was mainly performed on cells obtained by nasal forceps biopsy (6) which, however, is painful. Recently we have demonstrated that cell culture is possible directly from the brushed nasal epithelial cells thus reducing the need for a second and more traumatic sampling (7). With our simplified culture system the evaluation of ciliary activity is only possible on the 21<sup>st</sup> day of suspension culture, when new cilia formation definitely had occurred. However, because of the limited amount of material, we are not able to perform electron microscopy, one of the key diagnostic

tests in PCD, and this is a weakness of the technique. We therefore hypothesised that, using soft computing analysis of functional parameters in our simplified culture system, we could accurately diagnose PCD even without structural analysis which is only available in our unit after a mean time of two months. Soft computing-based models (8) use methodologies such as multi-layer Artificial Neural Network (ANN), that try to mimic the human mind, instructing the computer to deal with imprecision, uncertainty, and partial truth and to include all data in arithmetical algorithms able to counterbalance precision and uncertainty in a way similar to that which is normally done by the human brain (8). They can analyze complex medical data, exploiting meaningful relationships to help physicians in diagnosis, treatment and prediction of clinical outcomes.

Hence the first aim of this study was to determine if a precise evaluation of ciliary activity in suspension culture could by itself lead to a firm diagnosis of PCD in a shorter time, using brushed nasal epithelial cells. The secondary aim of the study was to identify which parameters of ciliary activity assessment were most useful for the definitive diagnosis. We assessed, every 5 days, different ciliary activity parameters in cultures, then with the help of soft computing analysis, we evaluated the whether we could distinguish patients with a final diagnosis of PCD from subjects without PCD.

## **MATERIAL AND METHOD**

### ***Subjects***

All the patients with a clinical history and signs suggestive of PCD (2), such as recurrent upper and lower respiratory tract infections and/or presence of situs inversus, sinusitis and/or bronchiectasis, were consecutively evaluated between January 2009 and January 2011 in the Department of Pediatrics of the University of Pisa. In all subjects, ciliary motion analysis, ultrastructural assessment of cilia, and ciliary function after ciliogenesis in culture of nasal brushing samples were performed, according to standard methodologies, when the subject had been free from respiratory infection for at least 4 weeks (9). Ciliary cultures were performed in all subjects on the first occasion. Thus, the sequence for the study was – brushing and CMA, and samples sent both for TEM and culture (Figure 1). The differential diagnosis between PCD and SCD was made before TEM was available by means of CMA on nasal brushings and ciliogenesis in culture (i.e. not using ANN data). When TEM became available, it was used as a confirmatory test to differentiate between PCD and SCD.

Definite diagnosis of PCD was made on the basis of structural and/or functional ciliary abnormalities both on brushing and culture which take account of both beat frequency and pattern, as previously described (7). Diagnosis of secondary ciliary disease (SCD) was made when PCD was excluded and ciliary abnormalities were considered as acquired. In subjects with inconclusive results from the first evaluation with CMA and TEM the tests were repeated about 4 months later, following appropriate antibiotic and anti-inflammatory treatment. From the outset, only one investigator (FM) who was not involved during the study in any clinical decision making was aware of the cell culture results which would be used for the ANN determinations. At the end of the study and before soft computing evaluation, CMA was also performed by the principal investigator (MP). Informed consent for nasal brushing was obtained from each adult patient, from the children's parents and the local Hospital Ethical Committee approved the study protocol.

### ***Cell culture in suspension***

Cell culture was performed as previously reported (7). Briefly, ciliogenesis of human respiratory epithelium takes place when aggregated cells are floating through the medium in the form of spheroids. Spheroids were identified after 24–48 h of culture using inverse microscopy (Inverted Microscope TE2000-S, Nikon, Tokyo, Japan) at x60 and the evaluation of ciliary activity was performed every 5 days until the 20th day of culture. Diagnosis was based on observation of 5 parameters, considered to be the expression of coordinated ciliary activity *i.e.*: *i*) rotation of the spheroids (yes or no), *ii*) migration of the spheroids (yes or no), *iii*) ability of cilia to remove debris or red blood cells (yes or no), normal motion type (*iv*) or pathologic motion type if it was found in at least 40% of observation fields (*v*). A normal ciliary beat pattern or, by contrast, a pathological pattern, such as a reduced ciliary beat frequency with a markedly restricted beat amplitude and a stiff ciliary motion pattern (10), or an abnormal nonflexible beating pattern with a hyperkinetic beat (11), could be easily identified. These parameters were evaluated every 5 days in relation to the final diagnosis as a binary variable: 0 = absence, 1 = presence and evaluated by ANN.

#### ***Statistical analyses and soft computing evaluation of cell culture results***

Baseline variables were expressed as group mean  $\pm$  standard deviation (SD) or as median and interquartile range (IQR) when the variables were non-normally distributed. The diagnosis obtained, at the end of the usual diagnostic pathway by ciliary motion analysis, TEM evaluation and based on ciliary activity evaluation after ciliogenesis in culture was accepted as the gold standard to evaluate the different parameters of cell culture suspension as previously reported (7).

The sensitivity and specificity in the diagnosis of PCD of parameters used for ciliary activity evaluation of suspension cell culture and their positive and negative predictive values (PPV and NPV) were generated using standard equations. All statistical calculations were performed using SPSS version 18.0 software for Windows (SPSS, Chicago, Illinois, USA) for personal computers.

A soft computing modelling approach was applied to the ciliary activity evaluation during ciliogenesis in culture to make a diagnosis of PCD or SCD, prior to the results of TEM becoming available. In this study, a model was identified by means of a multi-layer Artificial Neural Network

(ANN). They are the digitized model of biological brain and can detect complex nonlinear relationships between dependent as well as independent variables in a data where human brain may fail to detect (12). In order to evaluate the time-dependent diagnostic efficiency of the model to predict the final diagnosis, a cross-validation procedure of the different cell culture features (rotation of the spheroids, migration of the spheroids, ability of cilia to remove debris, normal ciliary beat pattern, pathological ciliary beat pattern) at the 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup> and 20<sup>th</sup> day was applied. Further details on soft computing evaluation of cell culture results are available as supplementary material on-line.

## **RESULTS**

One hundred fifty-one subjects were studied. A CONSORT-style diagram describing what happened to the patients is reported in figure 1.

A diagnostically useful suspension cell culture was obtained from nasal brushing samples in 117 (65 males, 91 children, age range: 1.5 months–57.0 years; median: 9.30; IQR: 12.0) out 151 (77.5%) subjects (all details are shown in Table 1 online). In 23 (15.2%) patients the cultures became infected during the first 48 hours and in 11 (7.3%) subjects the culture was unsuccessful either because of a lack of cells in the brushing sample, or because no cilia could be found in spheroids throughout the period of culture. Moreover, because only a small sample could be obtained, the duration of cell culture in suspension, usually 20 days, was reduced to 10 and 15 days, respectively in 9 (7.7%) and 10 (8.5%) out 117 patients.

Ciliary motion analysis (abnormal motion patterns, including immotile cilia and/or very low ciliary beat frequency) and TEM evaluation of cilia (alterations of the central pair and deficiencies of the dynein arms, associated with a small proportion of swollen cilia and compound cilia) allowed the eventual definitive diagnosis of PCD in 36 (29 children) subjects (30.8%). In 9 out of the 36 patients the diagnosis was obtained only after a second brushing because of equivocal results in 1 subject or the presence of normal ciliary ultrastructure with nonflexible and hyperkinetic ciliary motion pattern

in 8 patients at the first examination (all details shown in Table 2 online). In each of these 9 subjects the diagnosis of PCD was strongly suggested prior to any TEM results being obtained by the results of cell cultures (Table 1) (video clip 2 on-line).

In the remaining 81 (69.2%) patients (68 children), ciliary motion analysis demonstrated abnormal patterns in a small proportion of cilia, prevalence of thick cilia and low ciliary beat frequency, compatible with SCD (secondary changes were found in 1 patient only after the second brushing after intensive and prolonged treatment with antibiotics but SCD was correctly identified from the culture). In these 81 subjects, TEM evaluation clearly showed non-specific abnormalities compatible with chronic inflammation (prevalence of swollen cilia and compound cilia). In all these patients the diagnosis of SCD was robustly suggested by the prior results of cell cultures.

Moreover, as can be seen in table 1, ciliary activity evaluation, over the whole 20 day period of suspension cell culture, demonstrated a rotation of the spheroids in 64 out 81 (79%) subjects with final diagnosis of SCD, and only in 1 out 36 (2.8%) patients with PCD (rotation of 1 spheroid 10 days after the start of the cell cultures). In this patient the diagnosis was confirmed by *DNAH11* compound heterozygous mutations (11). Migration of the spheroids was found in 14 out 81 (17.3%) subjects with SCD, but in no PCD patients, and an ability of cilia to remove debris in 6 out 81 (7.4%) subjects with SCD and in none with PCD. When continuous movements of cilia were detected but migration or rotation of the spheroids with an ability of cilia to remove debris were not observed, a normal ciliary beat pattern was observed at CMA in 52 out 81 (64.2%) subjects with SCD, but in no patient with PCD. By contrast, in 12 out 36 (33.3%) of these PCD patients, but in none with SCD, a pathological ciliary pattern was observed (Table 1). In particular, a reduced ciliary beat frequency with a markedly restricted beat amplitude and a stiff ciliary motion pattern or an abnormal nonflexible beating pattern with a hyperkinetic beat were detected in 4 and 8 subjects, respectively.

The sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of the different parameters for the diagnosis of PCD are reported in table 2.



Taking into consideration together the rotation of the spheroids, migration of the spheroids, ability of cilia to remove debris and type of ciliary motion in the range 5-20 days, the ANN based model was able to identify the final diagnosis in almost all cases (PCD mean: 99, 5% SD: 0,3%; SCD mean: 98,8% SD:1,2%).

Moreover, since the best combination of sensitivity and specificity was demonstrated by rotation of the spheroids, the ability to correctly distinguish the PCD from SCD by the ANN based model, taking into account this parameter together with each of the other three, was analyzed.

As reported in Table. 3, the model was able to correctly identify the PCD or SCD in nearly all cases when the rotation of the spheroids and the type of ciliary motion were considered. This was true from the 5<sup>th</sup> day of cell culture. Other combinations were less good.

## DISCUSSION

The principal finding of the study was that culture in suspension of cells obtained by nasal brushing is possible in around 80% of the samples, and in our hands, using ANNs, this allowed the differentiation of PCD from SCD within one week, and well before TEM results become available.

Strengths of the study include the objective analysis of the data using ANNs *i.e.* by mathematical algorithms generated by computer. With this approach we have been able to identify the two parameters which when combined are most diagnostically accurate (rotation of the spheroids together with type of ciliary motion). These have been used to accurately identify nearly 95% of PCD patients after only 5 days of culture. ANNs learn from standard data and capture the knowledge contained in the data. Further strengths include the relatively atraumatic nature of the brushing as against a forceps biopsy. Originally cells for culture were obtained with more traumatic methods, such as punch biopsy which may cause pain and bleeding. Punch biopsy also has the disadvantage requiring a longer time to establish cell culture compared with cells collected by brushing (7, 13, 14). The technique does however the advantage of a high yield of culture results. Ciliated air-liquid cultures from both punch and brush biopsies (14) result in the culture of sufficient ciliated tissue to allow electron microscopy of cilia which is not possible with our simplified technique, but the success rate of this technique is only 54% (14) not 80% as here. However, with the air-liquid technique the culture success rate decreases to 26% in patients with more severe secondary changes, that is, in the very group in whom the risk of a false-positive diagnosis is more likely, and the cells previously had to be cultured for 21 days, increasing the risk of failure (7). This is exacerbated by the paucity of the starting material which tends further to reduce in subsequent passages. However, this present study clearly demonstrates that prolonged culture is not necessary to make the diagnosis of PCD.

The major weakness of the study is that TEM is not possible with this technique, but the positive predictive values of the different ciliary motion patterns resulted in greater than 98.5% accuracy, thus allowing the diagnosis in almost all patients. Of course, TEM when diagnostic (for example, outer

dynein arm deficiency) is an important test, but it is not the gold-standard, since cases of PCD with normal ultrastructure are well described (5, 11). This was the case in 8 of our patients who were correctly identified from the beginning by the study of ciliogenesis in culture. Even though we cannot perform electron microscopy on cells cultured by our simplified technique, soft computing allowed us to diagnose PCD before the second brushing and without TEM being available in an additional 22% of the patients within less than a week of the first nasal brushing, without recourse to a second brushing. The result is surprising, given that spheroids of epithelial cells in suspension culture lost cilia during the 1st week and developed new cilia only after a further 1–2 weeks (15). We speculate that restoration of normal ciliary activity, in the absence of a congenital defect, does not require the formation of complete new cilia but may occur as a consequence of a normalization of the normal trafficking of protein complexes between the cell basal body and the cilia tip as required for the assembly, maintenance, and function of cilia (16). In this context, it is worth noting that a second ciliary gene mutation in *cis* may correct the abnormal phenotype caused by the first, curiously sometimes without apparently restoring the missing substructure (17, 18, 19, 20). We suggest that the integration in a dedicated system of the optical acquisition and the soft computing algorithm will allow the evaluation of time-dependent cell culture results to be gained rapidly, resulting in a cost saving. TEM will thus become a confirmatory tool, given the long delay in obtaining results.

Ideally we would validate these findings in a second cohort of patients, but it would take a considerable time to generate a sufficiently large number of new patients. However, the network was validated according to a *n*-fold cross-validation method. Cross-validation is one of several approaches for estimating the performance of a model on unseen data. In an *n*-fold cross-validation, the original dataset is randomly partitioned (split) into *n* subsets. For each cross-validation step, a single subset is retained as the validation set (or test set, i.e. validation cohort), and the remaining *n*-1 subsubsets are used as training set. The cross-validation process is then repeated *n* times, with each of the *n* subsets used exactly once as the test set. The *n* results from the folds then can be averaged (or otherwise combined) to produce a single estimation. In our work a 5-fold cross-validation process of

the different cell culture features at the 5th, 10th, 15th and 20th day was applied; each fold consisted of randomly selected data, at least one for each category index was included in each fold. We consider that this cross-validation is very robust; much more so than merely splitting the cohort in half using one half as a discovery cohort and one for validation.

The implication of our study is that PCD is rare and difficult to diagnose, and may require repeated testing in doubtful cases (21, 22). Problems include firstly that TEM changes may be secondary to environmental mucosal injuries, such as inflammation, and bacterial or viral infections, allergies, and smoking (23) or may result from inappropriate preparation of a specimen (24); secondly, in a few patients with immotile cilia, ciliary ultrastructure may be normal or near normal (25, 26, 27, 28). At this regard the complexities of determining the ‘gold standard’ diagnosis have recently been discussed (4). We believe this new technique will make diagnosis indoubtful cases significantly easier.

If there is diagnostic doubt, identification of the causative genetic mutations would be ideal, as in our very atypical PCD patient with rotation of spheroids, but PCD is a genetically heterogeneous disease and more than 250 potential candidate genes have been identified (29). Furthermore, genetic studies have the problem of distinguishing disease producing mutations from harmless polymorphisms. Other investigations such as nasal nitric oxide (nNO) are part of the diagnostic process (30), but may be normal in rare patients with PCD and low in subjects with other diseases such as upper airway infections and cystic fibrosis (31). Under these challenging circumstances culture of ciliated cells is very helpful, since secondary changes are virtually absent after re-growth of the ciliated epithelium in culture while primary defects remain unchanged (32, 33).

In conclusion, the use of a simple culture technique together with the use of ANNs (34) offers new opportunities for the reliable differentiation of PCD from secondary ciliary dyskinesia.

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**Table 1 Ciliary activity parameters evaluated in suspension cell cultures of patients with PCD or SCD**

Study population n. (%)	Suspension cell cultures parameters n. * (%)				
	Rotation of the spheroids	Migration of the spheroids	Ability of cilia to remove debris	Normal ciliary beat pattern	Pathological ciliary beat pattern
PCD 36 (30.8)	1 (2.8)	0	0	0	12** (33.3)
SCD 81 (69.2)	64 (79)	14 (17.3)	6 (7.4)	52 (64.2)	0

\*n. of subjects in which the parameter was observed in at least one occasion.

\*\*a reduced ciliary beat frequency with a markedly restricted beat amplitude and a stiff ciliary motion pattern in 4 subjects; an abnormal nonflexible beating pattern with a hyperkinetic beat in 8 patients.

**Table 2. Sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) of the different parameters used for ciliary activity evaluation in suspension cell culture for the diagnosis of PCD.**

Suspension cell cultures parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Rotation of the spheroids	79.0	97.2	98.5	67.3
Migration of the spheroids	17.3	100	100	34.9
Ability of cilia to remove debris	7.4	100	100	32.4
Normal ciliary beat pattern	64.2	100	100	55.4
Pathological ciliary beat pattern	33.3	100	100	77.1

**Table 3. Capability of the cell cultures parameters to predict the final diagnosis by an ANN based model**

Suspension cell cultures Parameters / days (n. of samples)	Primary Ciliary Dyskinesia correctly identified				Secondary Ciliary Dyskinesia correctly identified			
	5 <sup>th</sup> (117)	10 <sup>th</sup> (117)	15 <sup>th</sup> (108)	20 <sup>th</sup> (98)	5 <sup>th</sup> (117)	10 <sup>th</sup> (117)	15 <sup>th</sup> (108)	20 <sup>th</sup> (98)
Rotation of the spheroids + Type of ciliary motion	94.2* (5.3)**	94.7 (6.1)	95.0 (6.3)	95.9 (5.9)	97.6 (5.4)	96.1 (6.8)	96.6 (1.2)	97.2 (6.5)
Rotation of the spheroids + Migration of the spheroids	80.7 (8.3)	79.4 (9.4)	76.2 (12.1)	76.5 (18.7)	97.0 (6.3)	97.6 (5.5)	90.0 (26.6)	58.3 (39.5)
Rotation of the spheroids + Ability of cilia to remove debris	87.5 (8.1)	81.4 (8.9)	72.2 (9.4)	74.9 (26.6)	97.3 (5.6)	96.8 (6.1)	97.3 (5.9)	63.3 (36.2)

\* % Mean

\*\* (SD)



## FIGURE LEGENDS

Fig. 1. Flow diagram of the studied patients.

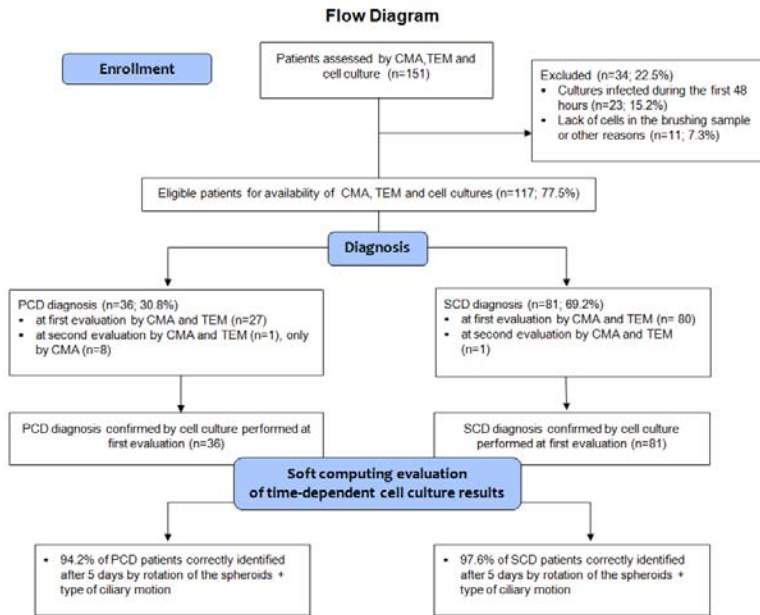


Fig.1

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