

Primary ciliary dyskinesia: Diagnosis in children with inconclusive ultrastructural evaluation

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The purpose of this study was to distinguish between acquired and genetically determined ciliary abnormalities in children with severe chronic respiratory diseases. Samples of nasal ciliated epithelium from 50 subjects (25 male, 25 female; age-range 2–19 years) with severe chronic respiratory diseases were examined using transmission electron microscopy (TEM). Based on TEM findings, patients were divided into two groups: A and B. Group A comprised 39 children with ciliary alterations compatible with a condition probably occurring secondary to chronic inflammation (alterations of peripheral pairs, swollen cilia, and compound cilia). The other 11 patients, Group B, exhibited a greater number of alterations of the central pair and dynein arms ($p < 0.001$), which were qualitatively similar to, but less numerous than, those observed in primary ciliary dyskinesia (PCD). In both groups, analysis of ciliary beat frequency and waveform was performed by phase contrast microscopy (PCM). All the children with a ciliary beat frequency of < 7 Hz were treated with daily physiotherapy and with antibiotics, as recommended for PCD, for a 6-month period. After this treatment, the children were reexamined by PCM. Almost 50% of the children from Group B (i.e. those with a small proportion of specific ultrastructural defects) showed permanence of low ciliary beat frequency. This was also observed in two children of Group A. These children were considered to be affected by PCD. Our study describes a method for the diagnosis of PCD in the absence of specific ultrastructural defects or when these defects are present in only a small proportion of the cilia.

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Wheezing is one of the most common symptoms of respiratory disease in childhood. Epidemiologic studies from various countries have shown that 25–30% of infants in the first year of life and up to 40% of children under 3 years of age present to their physician with wheezing respiratory illness (1). The majority of children older than 5 years of age with recurrent wheezy, lower respiratory tract symptoms have a high probability of having asthma, regardless of age of onset, evidence of atopic disease, precipitating causes, or frequency of wheezing (2). However, asthma in infancy can be confused with various other conditions which produce similar signs and symptoms, and physiologic changes. For this reason,

the initial evaluation of a wheezing infant should aim to exclude alternative diagnoses. One of these could be primary ciliary dyskinesia (PCD), which may well be misdiagnosed as asthma with a consequent delay in the appropriate treatment.

PCD is a highly heterogeneous disease, caused by one of a number of different ciliary ultrastructural defects, each of which may result in ineffective mucociliary transport in the airways (3,4). Transmission electron microscopy (TEM) is an essential part of diagnostic testing (5–8), but the benefit of ultrastructural findings as criteria for the diagnosis of PCD is sometimes limited (9). Normal ciliary ultrastructure has been reported in few patients with immotile cilia, suggesting

that abnormal ciliary motion could be related to cellular defects not detectable by ultrastructural study (10–14). Furthermore, ciliary functional alterations are not always characterized by immotile cilia, as uncoordinated and dyskinetic ciliary motions can also impair the mucociliary clearance (15–23).

On the other hand, respiratory infections, physico-chemical injury of the mucosa, locally applied drugs, and exacerbations of asthma can cause focal and reversible ultrastructural ciliary abnormalities (16,24–30). This condition is termed 'secondary ciliary dyskinesia' (SCD) to distinguish it from the autosomal-recessive inherited and persistent disorder called PCD. Moreover, abnormalities in up to 10% of cilia may be seen in normal subjects (9,31–33). For these reasons TEM may not be conclusive for the diagnostic definition of PCD and the specific diagnosis has to rely on the demonstration of persistent inefficient ciliary function by using microscopic and photometric techniques (3,4,33).

In the present study we verified the possibility of distinguishing between genetically and acquired ciliary abnormalities, assessing the persistence of ciliary motion abnormalities in children with chronic respiratory disease, who were reexamined after 6 months of regular daily physiotherapy and prompt prolonged treatment of intercurrent lung infections, as usually recommended for PCD (34).

Patients and methods

Biopsies of nasal ciliated epithelium were obtained from 50 patients (25 male, 25 female), 2–19 years of age (mean $\pm$ SD: 8.5 ± 4.2), with severe chronic respiratory diseases, including four patients with situs invs. (SI). No patient had recently had respiratory infections or exacerbations of the chronic pathology, and no patient had taken medications in the 48 h preceding the study. Samples, obtained from two or more sites of the inferior turbinate using a cytology brush (Microvasive, Milford, MA, USA), as described by Rutland & Cole (35), were suspended in 2 ml of Medium 199 fluid cell culture or in 2 ml of physiological solution for immediate light-microscopic or TEM evaluation, respectively.

The samples for ciliary motion analysis were kept at 37°C and transferred to a variable-thickness culture chamber, as described by Braga (36). The cilia were observed using an optical Diaplan Leitz (Leica, Wetzlar, Germany) microscope equipped with Nomarsky differential interference contrast set at $\times 100$. The microscope was connected to a high-resolution digital color video camera (TK-C1380; JVC, Yokohama, Japan).

The video camera was connected to an image-processing unit (Magic DaVE 3D Digital Effects System; Snell & Wilcox, Saffron Walden, UK) and that to a video recorder (BR-D80E Digital S; JVC) and to a monitor (TM-1500PS; JVC). The length of normal cilia on the screen was 3–5 cm. Ciliary morphology, motion pattern, and beat frequency were evaluated, by an experienced observer, on magnified slow-and-stop motion images at 25 frames per second. For each sample at least 50 cells, single or in clusters, were examined. We looked for the presence of a rotational, multiplanar motion pattern at the apex resembling the movement of an eggbeater (18,37); a vibrational, metronome-like pattern on different planes (18,37); a quivering motion pattern (17,37); a biphasic rotation about the vertical axis of the cilium, i.e. a corkscrew motion (16,37); a grabbing-type motion pattern with the base rigid (16,37); and, finally, absent motion (immotile cilia). The motion type was attributed, whenever it was found, in at least 40% of observation fields (15). Beat frequency was determined in no less than 25 randomly chosen sites in each sample. The presence of ciliated cells was always confirmed by cytologic analysis after cytocentrifugation and staining with May–Grunwald–Giemsa stain.

For the ultrastructural studies, samples of ciliated epithelium from the nasal mucosa were centrifuged and fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35, for 3 h at 4°C, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded alcohol series and embedded in an epon-araldite mixture. From each sample, semi-thin sections were cut for orientation purposes and stained with toluidine blue for light microscopy. Thin sections were obtained with an Ultratome III LKB and stained with lead citrate for TEM observation (Philips 301, Philips CM10, FEI Co, Hillsboro, OR, USA; Zeiss 902, C. Zeiss, Okberkochen, Germany) (38). Cilia were studied at an initial magnification of $\times 45,000$ – $91,000$. For each specimen an average of 14 ciliated cells and 120 transversely sectioned cilia were examined. Because, in controls, some 3–5% of cilia can exhibit ultrastructural defects (16), a percentage of abnormal cilia of $>10\%$ was considered to be clearly pathologic (16,39). Ciliary motion analysis and ultrastructural studies were performed by different experienced observers who were each unaware of the results obtained by the other.

According to the ciliary motion analysis the patients were divided into those with a synchronous, coordinated ciliary motion pattern and normal or near-normal frequency beat, i.e. a

Table 1. Ciliary motion patterns in the patients

Ciliary motion pattern	Patients	Group A	Group B
Synchronous, coordinated, metachronal	31 (62%)	31 (62%)	0 (0%)
Eggbeater	3 (6%)	2 (4%)	1 (2%)
Metronome	10 (20%)	4 (8%)	6 (12%)
Quivering	1 (2%)	1 (2%)	0 (0%)
Immotile cilia	2 (4%)	1 (2%)	1 (2%)
Eggbeater + immotile cilia	2 (4%)	0 (0%)	2 (4%)
Metronome + immotile cilia	1 (2%)	0 (0%)	1 (2%)

frequency beat of >7 Hz (25,40), and those with a clearly abnormal frequency beat of <7 Hz.

According to the ultrastructural evaluation, the patients were then divided into two groups: A and B. Group A included patients with an ultrastructure pattern consistent essentially with chronic inflammation, such as swollen cilia, compound cilia, missing or supernumerary peripheral microtubules or pairs, translocation of one or more peripheral pairs and probably secondary lesions. Group B consisted of patients with more severe ultrastructure alterations such as missing or supernumerary central microtubules or pairs, central translocation of a peripheral pair as well as missing or shortened dynein arms, probably related to PCD (4,16,33).

Patients with a ciliary frequency beat of >7 Hz and a normal beat pattern were followed-up in the out-patient clinic every 2 months, where a clinical examination was performed together with spirometry, but no other specific investigation for PCD was carried out. In patients with lower-frequency ciliary motion (<7 Hz), considered clearly abnormal (25,40,41) and associated or not associated with an abnormal motion pattern (including immotile cilia), the ciliary beat frequency and pattern were studied again after 6 months of treatment with aggressive use of physiotherapy and antibiotics, as suggested for patients with PCD (34). Samples for ciliary motion analysis were obtained using the protocol described above. Again, these patients were studied when they had experienced no recent respiratory infections or exacerbations of the chronic pathology and had not taken any medications in the 48 h preceding the study.

Informed consent was obtained from the parents before enrolling children in this study,

and the study protocol was approved by the local hospital Ethical Committee.

Statistical analysis

The results were analyzed using the Student's *t*-test for quantitative unpaired data. For non-quantitative data, comparison of proportions was made using the chi-square test. When small numbers invalidated the chi-square approximation, Fisher's exact test was used. Significance was established at $p=0.05$.

Results

The results of the functional study performed by phase-contrast microscopy showed the motion patterns reported in Table 1. Sixty-two per cent of our patients had a synchronous, coordinated, metachronal motion pattern. The remaining subjects showed abnormal patterns with immotile cilia, isolated or associated with other defects, being present in 10% of our study population. The measurements of ciliary beat frequency are reported in Table 2. Twenty-one (42%) of our patients presented a clearly pathologic beat frequency (<7 Hz); of these, 10 (20%) had minor ultrastructural defects (Group A) and 11 (22%) had major ultrastructural defects (Group B). None of the patients with a beat frequency of >7 Hz presented severe ultrastructural defects. The morphological study by phase-contrast microscopy showed the presence of thick cilia in 23 patients (46%) (22 of Group A, one of Group B).

The ultrastructural analysis (Fig. 1), facilitated the identification of two groups of patients characterized by a similar proportion of defective cilia but with significantly different patterns of

Table 2. Measurement of ciliary beat frequency in the patients

Ciliary beat frequency	Patients	Group A	Group B
<7 Hz	21 (42%)	10 (20%)	11 (22%)
≥ 7 Hz	29 (58%)	29 (58%)	—

*Normal range in our laboratory: 12.1 ± 1.8 Hz (mean $\pm$ SD).

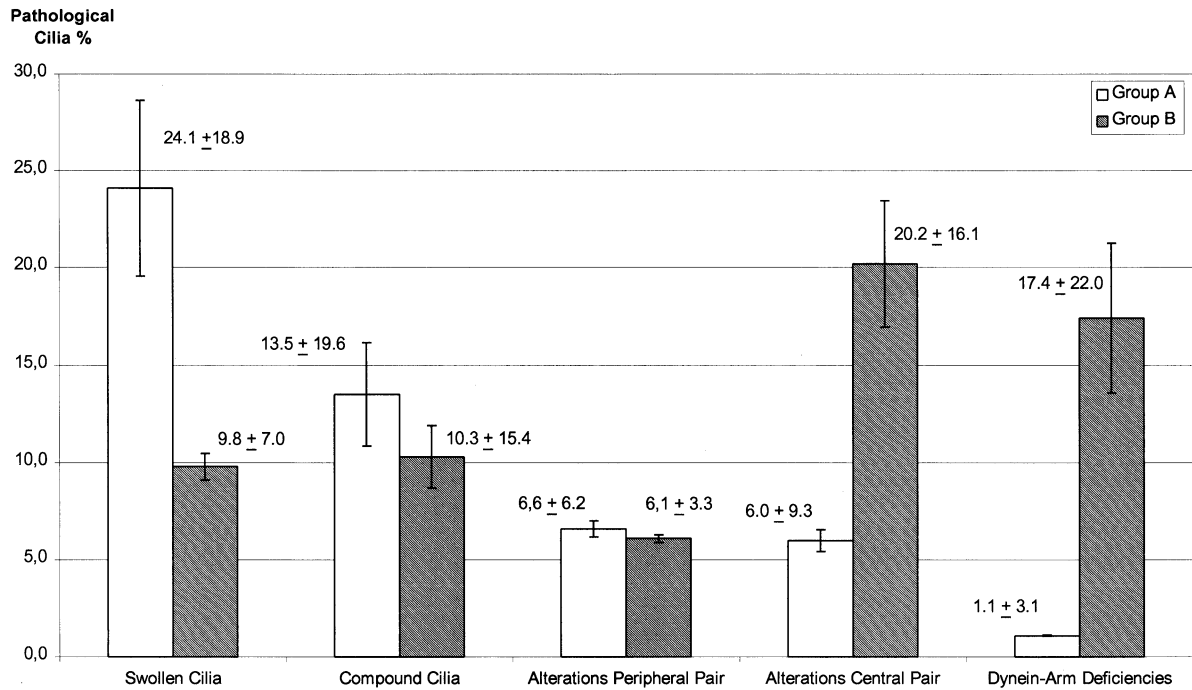


Fig. 1. Ultrastructural alterations in our series.

axonemal alterations, both in terms of quality and quantity, especially as regards lesions potentially

indicative of PCD (alterations of the central pair, dynein arms deficiencies).

In the first group (Group A), which accounted for 78% of our patients (39 subjects, including one with SI), the ultrastructural pattern was consistent with chronic inflammation and presented the following qualitative alterations: compound cilia

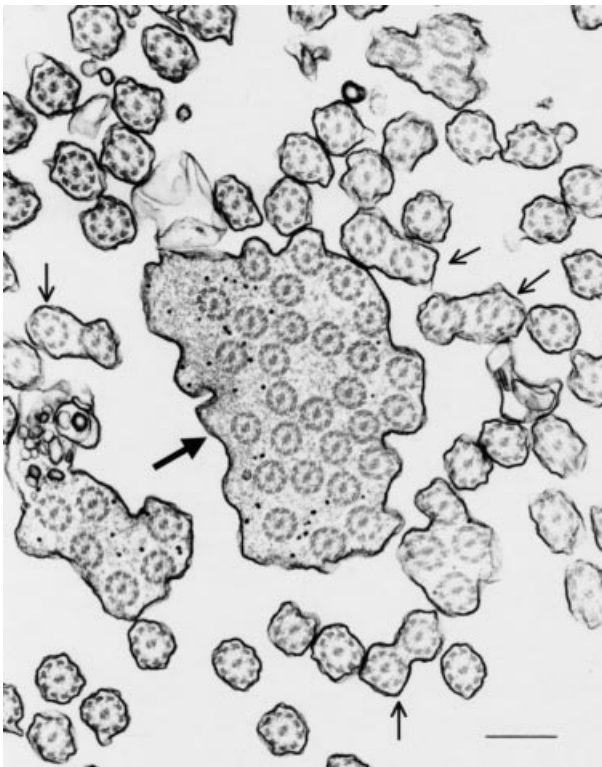


Fig. 2. Transmission electron micrograph of transverse sections of compound cilia from the nasal mucosa of a patient with chronic respiratory infections. Compound cilia are multiple axonemes within a common cell membrane: in this field compound cilia contain two (thin arrows) to 28 axonemes (thick arrows) within a membrane. Magnification $\times 62,000$. Bar: 300 nm.

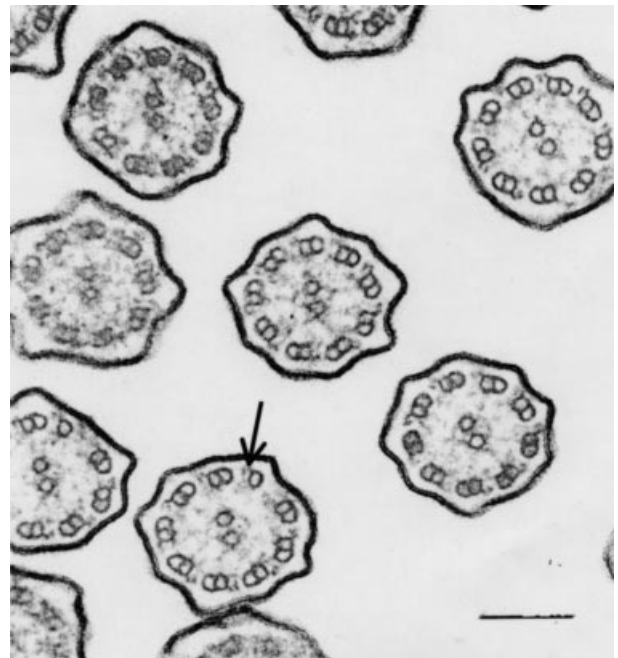


Fig. 3. Transmission electron micrograph of cross-sectional profiles of cilia from the nasal brushing of a patient with chronic respiratory infections, showing both normal ultrastructure and one missing peripheral microtubule (arrow). Magnification $\times 150,000$. Bar: 100 nm.

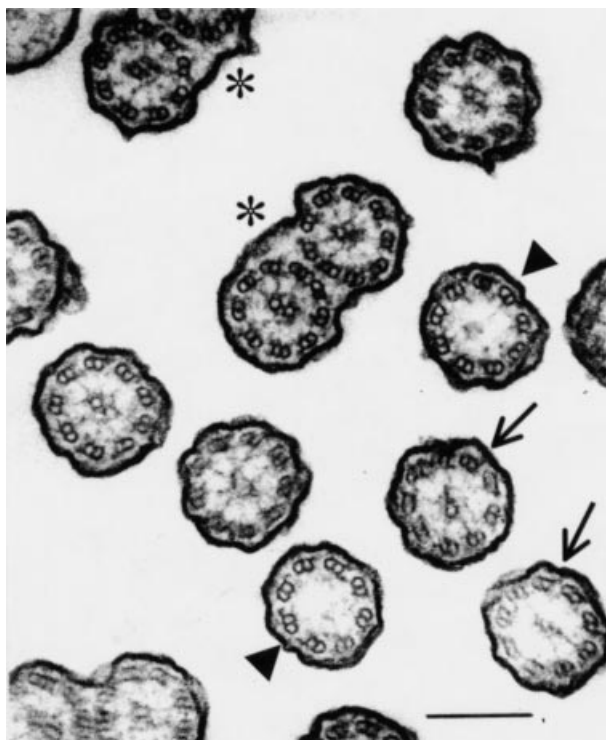


Fig. 4. Transmission electron micrograph of cross-sectioned nasal cilia showing missing central singlets (arrows) or pairs (arrow heads). Note the presence of two compound cilia (*). Magnification $\times 97,000$. Bar: 150 nm.

(Fig. 2), swollen cilia, one or more missing or supernumerary peripheral microtubules or peripheral pairs (Fig. 3), and translocation of one or more peripheral pairs. In this group, the mean proportion ($\pm$ SD) of defective cilia was $52.2 \pm 22.2\%$. The mean data ($\pm$ SD), referring to both pathological and normal cilia evaluated in this group, were: alterations of the central pair, $6.0 \pm 9.3\%$; alterations of dynein arms, $1.1 \pm 3.1\%$; compound cilia, $13.5 \pm 19.6\%$; swollen cilia, $24.1 \pm 18.9\%$; and alterations of peripheral pairs, $6.6 \pm 6.2\%$. However, the most frequent anomaly was swollen cilia, that in 18 out of 39 patients (46.1%) reached percentages ranging from 51.2 to 96.4% of pathological cilia.

In the second group (Group B), accounting for 22% of our patients (11 subjects, including three with SI), there were significantly more ($p < 0.001$) ultrastructural alterations of the central pair compared with Group A (missing or supranumerary central microtubules or pairs, central translocation of a peripheral pair; see Fig. 4). The association of central pair alterations with missing or shortened dynein arms (Fig. 5) was statistically significant ($p < 0.001$). In all 11 patients of this group, central-pair and/or dynein-arm alterations were observed in at least 50% of pathological cilia. The mean proportion ($\pm$ SD) of pathological cilia in Group B was $58.5 \pm 23.1\%$ and was slightly

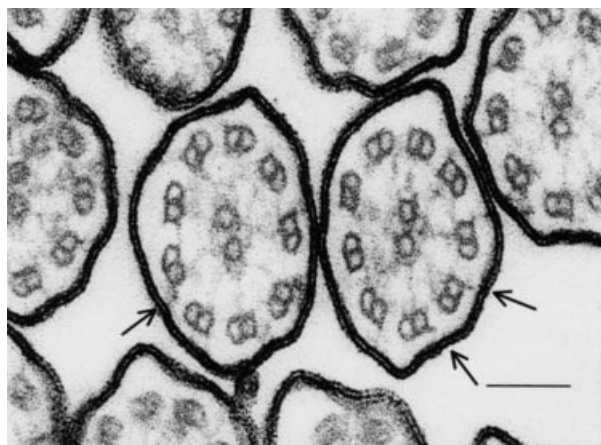


Fig. 5. Transmission electron micrograph of a cross-sectional profile of cilia from the nasal mucosa showing absence of inner dynein arms. The outer dynein arms, when visualized, appear short and stubby (arrows). Magnification $\times 277,000$. Bar: 70 nm.

higher than in Group A. Their mean distributions ($\pm$ SD) with respect to the total number of cilia examined were: alterations of the central pair, $20.2 \pm 16.1\%$; dynein-arm deficiencies, $17.4 \pm 22.0\%$; compound cilia, $10.3 \pm 15.4\%$; swollen cilia, $9.8 \pm 7\%$; and alterations of peripheral pairs, $6.1 \pm 3.3\%$ (Fig. 1). All the patients in Group B presented a clearly abnormal low ciliary beat frequency (< 7 Hz).

A highly significant correlation ($p < 0.001$) between immotile cilia and the prevalence of dynein-arms deficiencies was found. In Group B, immotile cilia were found in four patients, the remaining patient was from Group A, thus confirming that immotile cilia without major ultrastructural defects can be identified (10–14). In contrast, no significant correlation between pathological motion patterns and the prevalence of dynein-arms deficiencies was found.

The comparative analysis of phase-contrast microscopy and TEM data showed a significant correlation ($p < 0.001$) between thickened and swollen cilia.

When the children with dynein-arms deficiencies (Group B) were compared with all the other patients, no correlation was found for ciliary beat frequency and the various types of ultrastructural alterations. Nevertheless, in Group A, ciliary beat frequency (mean $\pm$ SD: 7.9 ± 1.7 Hz) was significantly ($p < 0.001$) lower than that considered normal by our laboratory. In view of the ultrastructural data alone, we could speculate the presence of PCD in Group B patients, but the definitive diagnosis could not be performed mainly in relation to the relatively low prevalence of abnormal cilia.

The 10 patients of Group A and all the 11 subjects of Group B who presented low frequency ciliary motion (<7 Hz), were studied again after 6 months of daily physiotherapy and antibiotic treatment of recurrent infections, thus allowing the repair of secondary abnormalities. When reexamined, ciliary motion abnormalities were observed in seven patients only: in two patients from Group A (5.1%) and in five patients from group B (45.4%). In these children the abnormal motion pattern and ciliary beat frequency were not significantly different with respect to those observed at the first examination. These patients were considered to be affected by PCD. The remaining 14 children showed a normal ciliary motion pattern and a normal ciliary beat frequency at the second examination and were considered to have had SCD. The correlation between persistent ciliary motion abnormalities and small proportion ($<70\%$ with respect to the total number of cilia examined) of specific inherited ultrastructural defects was statistically significant ($p < 0.005$).

Discussion

Pediatric allergists should know about PCD and how to diagnose it. There are two arguments for this reasoning. The first is that PCD commonly presents with recurrent coughing and wheezing and may well be misdiagnosed as asthma. Furthermore, as there are common nasal and middle ear problems, this can further confuse the issue. Second, there has been suggestion that atopy might be more common in patients with PCD because the repeated inflammation of mucosa will facilitate allergen sensitization (16,42). Thus, again, there is a need to understand when this condition might exist and how to diagnose it in order to prescribe the most appropriate treatment.

Concerning the diagnosis, the use of samples of nasal ciliated epithelium in our series appears to be justified on the basis of previously published data which demonstrate that ultrastructural nasal ciliary changes reflect changes of ciliated epithelium in other areas of the respiratory tract (43). In our study we observed that ciliary motion patterns, similar to the one initially described for PCD, can be found also in children with acquired respiratory tract pathologies. This is in accordance with previously published studies (16,18,25,44). Moreover, the reduction of ciliary beat frequency is not an exclusive feature of congenital diseases (16,18,44). In our study population, abnormal ciliary motion was initially observed in 42% of the patients with chronic

respiratory tract infections. After appropriate treatment, ciliary motion anomalies persisted and remained unchanged in 14% of our subjects, who were therefore considered to be affected by PCD. In another study, in children suffering from recurrent respiratory tract infection, abnormal ciliary motion was found in 14% of the study population, and PCD was detected in 5.6% (41). Thus, PCD is rare but not exceptional in children with recurrent respiratory infection, once immunologic and other congenital diseases have been excluded.

The components necessary for a detailed evaluation of a patient suspected to have PCD include: clinical evaluation, mucociliary transport studies, ciliary motion analysis, and electron microscopic examination of the nasal cilia. The clinical suspicion relies on a combination of recurrent lower airways infections together with chronic upper airway secretions from the nose and the ear. Assessment of mucociliary clearance, using saccharin or technetium-labeled albumin placed on the inferior turbinate are important diagnostic procedures (45) but may miss cilia moving in a dyskinetic manner. Furthermore, the saccharin test requires complete cooperation of the patient and therefore is difficult to perform in children (46). Electron microscopy analysis is indicated, at once, in patients with SI who suffer from chronic, recurrent infections of the respiratory tract. In all other cases, to avoid unnecessary expenditure, it has been suggested that ultrastructure investigation of the cilia is indicated only after exclusion of all other possible causes of signs and symptoms of the patients (47), and after demonstration of ineffective ciliary function by microscopic and photometric techniques (4,34).

The presence of immotile cilia in at least 40% of phase-contrast microscopy observation fields, especially when it is accompanied with cilia exhibiting pathological motility patterns, is particularly helpful for the diagnosis (15). When immotile cilia or a low ciliary beat frequency is associated with a high percentage of abnormal cilia, this suggests the potential role of defective cilia in the respiratory disease of the patients. The next step is to determine whether these ciliary abnormalities have a congenital origin or are secondary to environmental noxious agents.

A number of insults, including environmental pollutants, and viral and bacterial infections, may cause disruption of mucociliary clearance (24,48). In these circumstances the alterations are transient and improve after appropriate treatment or removal of the insulting factors (49). Mucociliary impairment caused by physical trauma of the airways may occur as a result of airway manip-

ulation with tracheal tubes, suction catheters, and bronchoscopes, as well as a consequence of cough (50). Long-term exposure to low-to-intermediate concentrations, or short-term exposure to high concentrations, of cigarette smoke, atmospheric pollutants, and oxygen may also cause transient impaired mucociliary clearance (51). Moreover, some viral infections can cause a transient, but severe, impairment of mucociliary clearance for up to 4 months (52). Mucosal biopsies taken from the airways of patients with bacterial infections have revealed impaired ciliary beating with a return to normal after antibiotic therapy (53). Infections, especially those caused by *Pseudomonas* species, *Hemophilus*, *Pneumococci*, and *Mycoplasma*, are ciliotoxic and cause reversible ciliostasis, which may disturb mucociliary clearance for a number of months (54).

In our study, when the children were reexamined after 6 months of treatment with physiotherapy and prompt antibiotic use for intercurrent infections, it was clearly evident that almost half of the subjects from the group with specific and severe ultrastructural defects, even if present in a relatively small proportion (Group B), showed permanence of low ciliary beat frequency. The persistence of abnormal beat frequency was also identified in two children from the group with no specific ultrastructural defects (Group A). These patients were considered to be affected by PCD, the others by SCD. Therefore, the type and severity of ultrastructural defect may help to differentiate between PCD and SCD only partially. Secondary ciliary abnormalities consist predominantly of microtubular defects in up to 10% of cilia in normal individuals (55), and compound cilia (4). Moreover, the finding of thickened cilia by phase-contrast microscopy, which at TEM appear as swollen cilia, probably indicates an inflammatory condition. They are frequently found in patients with different respiratory-tract pathologies (such as cystic fibrosis, bronchial asthma, and bronchitis) and are considered to be nonspecific alterations (55). Their meaning is thus similar to that of compound cilia with anomalous microtubular patterns, of membrane-deficient cilia, and of giant cilia (19,55). Cilia not normally oriented with respect to each other can occur both as a primary defect or secondary to infection (56). In our study these alterations were mainly found in Group A children. The ultrastructural alterations of the 11 patients of the second group (Group B) were different mainly for the qualitative patterns of lesions. Although these children shared with those of the first group similar overall proportions of defective cilia, alterations of peripheral pairs,

swollen cilia, and compound cilia, they exhibited a greater number of alterations of the central pair and dynein arms (Fig. 1). These alterations were qualitatively similar to, although less numerous than, those usually observed in congenital pathologies. We therefore believe that a small proportion of ultrastructural defects specific for PCD allow identification of patients who are more prone to maintain the ciliary beat frequency defect over time. However, PCD has also been observed in subjects with normal ultrastructure of the cilia (10–14) and for this reason ultrastructure morphology cannot be considered as an absolute discriminatory diagnostic criterion. In our study population it was possible to correctly diagnose PCD in two patients presenting with normal ciliary ultrastructure. In these patients the motion defect can be probably ascribed to different alterations in the complex construction of the axonema, which contains at least 200 species of polypeptides (4), that might not be apparent at the ultrastructural analysis.

Furthermore, to reduce possible misinterpretation, as a result of secondary ultrastructural defects potentially confounding the final diagnosis (57), biopsy should be obtained at least 4–6 weeks after complete resolution of the respiratory infection. Alternatively, it has been recently demonstrated that it is possible to culture the epithelial cells *in vitro*, in an environment free of noxious agents, thus allowing the regeneration of the cilia and reducing potential confounding factors (9). In addition, to avoid false diagnosis of PCD, it is essential to obtain the mucosal biopsy in at least two different sites (58). Effectively, in the two children with minimal and nonspecific ultrastructural anomalies, but with a persistent low beat frequency, samples taken from tracheal and bronchial biopsies confirmed the ultrastructural alteration previously observed in the cilia obtained from the nose. Therefore, it was possible to confirm the diagnosis of PCD, both demonstrating the presence of specific abnormalities in other areas of the respiratory tract (universality criterion) and their persistence at different times (permanence criterion), unlike acquired abnormalities that are focal and transient (33). Finally, the value of an early diagnosis of PCD should be emphasized, as this condition, if untreated or undertreated, is accompanied with a progressive deterioration in lung function. Conversely, lung function can be maintained if appropriate antibiotic treatment and regular physiotherapy are promptly carried out (59).

For this reason it is important to investigate potentially appropriate screening tools such as the

concentration of nasal nitric oxide (NO), which is low in patients with primary dyskinesia (34,60), and membrane polarity of polymorphonuclear leucocytes, which has been found to be increased in children with PCD (61). The genetic loci responsible for these defects are under investigation and one gene involved was found to be located at 9p13-p21 (62). However, it seems unlikely that a genetic test will become available in the near future (63). At present, the specific investigation for PCD includes measurement of ciliary beat frequency and identification of the ultrastructural configuration of the cilia. To avoid overdiagnosis and overexpenditure, this should be performed only after exclusion of all other possible causes of recurrent respiratory infections and after appropriate treatment for all the conditions responsible for secondary abnormalities of the cilia. This approach will also avoid misdiagnosis of PCD when specific ultrastructural defects are not present or are present in only a small proportion of the cilia.

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