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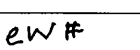
Insert word	Your proof	
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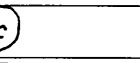
Make capital	your proof	
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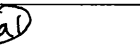
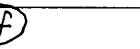
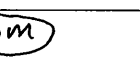
Make bold	Your proof	
Make roman (not italic or bold)	Your proof	
Let it stand; OK as is	Your proof	
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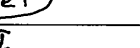
No paragraph	Marked. Your	
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Nasal scraping in diagnosing ciliary dyskinesia

G. Caruso, M.D.,* M. Gelardi, M.D.,§ G.C. Passali, M.D.,* and M. de Santi, Ph.D.# (Italy)

ABSTRACT

Background: *Primary ciliary dyskinesia (PCD) is a congenital, clinically and ultrastructurally heterogeneous disease caused by abnormal structure and/or function of cilia. Kartagener's syndrome is one subgroup of PCD. Acquired ciliary dyskinesia is frequent, generally being associated with or following respiratory tract infections.*

AQ: A **Methods:** *From January 2003 to April 2006, nasal mucociliary transport time was measured in 64 patients and specimens obtained by nasal scraping were examined by transmission electron microscope (TEM).*

Results: *The 64 nasal scrapings led to the diagnosis of 11 (17.2%) cases of PCD and 51 (79.7%) cases of secondary ciliary disorder. In two cases (3.1%) no clear diagnosis was possible.*

Conclusion: *Nasal scraping is an easy, cheap, and efficient tool for detecting ciliary abnormalities by TEM and for distinguishing acquired and congenital modifications.*

(Am J Rhinol 21, 1–00, 2007; doi: 10.2500/ajr.2007.21.3107)

Key words: Acquired ciliary dyskinesia, cilia, Kartagener's syndrome, mucociliary transport time, nasal scraping, primary ciliary dyskinesia, transmission electron microscope

AQ: B Mucociliary clearance and nasal-associated lymphoid tissue are involved in nasal defense. Correct functioning of the aspecific defense system is related directly to the anatomic integrity of mucociliary transport (MCT) structures, *i.e.*, the mechanical integrity of the cilia and the rheological characteristics of mucus (gel and sol components). Physiological clearance depends on the number, length, and density of the beating cilia, moving the overlying mucus toward the oropharynx with a metachronal rhythm, and sufficient mucus with appropriate characteristics.

Primary ciliary dyskinesia (PCD) is a genetically determined disease, usually with autosomal recessive inheritance,[1] characterized by impaired MCT. It occurs as a direct result of heterogeneous morphostructural defects in respiratory cilia,[2,3] manifesting as diverse chronic respiratory disorders of variable severity. The ciliary motility pattern ranges from complete stillness to variable defective beating. The incidence of PCD in the white population is estimated at 1:16,000 births, as extrapolated from radiological findings.[4,5] In children with recurrent respiratory tract infections, PCD is rare although not exceptional (5.6%).[6] The pathogenesis of PCD, of which Kartagener's syndrome is one subgroup, was partially known at the beginning of the 1970s, as a result of electron microscope examination of histological specimens.[7,8]

We now know that ciliary oscillating movements are generated by the interaction of dynein and tubulin molecules. This movement is subordinate to ciliary flexion capacity because of coordinated sliding of microtubule fibers. By convention, the ciliary plane of symmetry is regarded as passing through fiber 1 and between fibers 5 and 6 and is the plane in which beating occurs.[9]

Microscope observation and biochemical tests of ciliary morphological structures show that axoneme subcomponent alterations may cause ciliary impairment. Molecular anomalies, expressed as ultrastructural defects, lead to incorrect microtubule sliding and bending. The randomly orientated cilia move in a chaotic, nonmetachronal manner, with uncoordinated, nonfunctional beating.[10] The clinical entity of PCD is related to the ciliary substructures involved: outer and inner dynein arms, radial spoke defects, microtubule translocation, or random changes in ciliary orientation.[11,12]

All of these alterations lead to modified mucus transport with the following clinical features: chronic rhinitis, nasal polyposis, otitis media, chronic and recurrent rhinosinusitis, chronic bronchitis, bronchiectasias, and obstructive pulmonary disease. Affected male subjects are infertile because of the same axonemal structure displayed by both the sperm tails and the respiratory cilia. Ciliary defects also can be found in patients with chronic respiratory disease not caused by PCD.[13] The term acquired or secondary ciliary dyskinesia is used to indicate ultrastructural abnormalities related to respiratory infections, aging, exposure to pollutants, and smoking. A major diagnostic difficulty is to distinguish primary from acquired alterations: these may resemble congenital defects. It is important to optimize the size and quality of specimens for electron microscope evaluation, obtaining a sample consisting primarily of ciliated epithelial cells. This may be accomplished conveniently and noninvasively by sampling nasal epithelium with a special nasal probe (Rhinoprobe), harvesting cells from the middle third of the inferior turbinate. AQ: C

MATERIALS AND METHODS

From January 2003 to April 2006 we examined 64 patients (34 male patients and 30 female patients) aged 2–76 years (median age, 41 years). Criteria for inclusion were recurrent/chronic inflammation in the upper and lower respiratory tract (nasal polyps, rhinosinusitis, otitis, and/or laryngotracheobronchitis). Five patients manifested situs inversus totalis. We excluded patients with allergic pathology diagnosed by the presence of nasal immunophlogosis (eosinophils and mast cells with or without degranulation). Clinical examination

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and nasal scraping were performed at the Ear, Nose, and Throat Department of the University Hospital of Siena. The ultrastructural studies were performed at the Human Pathology Department, University Hospital of Siena.

All patients underwent ear, nose, and throat examination and MCT time (MCTt) evaluation by the Passali technique,[14] which tests the time a mix of charcoal powder and 3% saccharin takes to reach the oropharynx from the head of the inferior turbinate. We considered only the evidence of oropharyngeal charcoal powder. A specimen of ciliated epithelium was obtained by nasal scraping from the middle third of the inferior turbinate with a spoon-shaped nasal probe (Rhinoprobe). An *in vitro* evaluation of ciliary movement was performed. Ciliary waves and ciliary beat frequency (CBF) were analyzed by phase-contrast microscopy. Cinematography at 100–200 exposures/second was used to determine ciliary frequency by counting the number of exposures for a cilium to return to its original position.

Additional samples of ciliated epithelium were processed for transmission electron microscope (TEM) examination. The specimens were immediately fixed in 2.5% cacodylate-buffered glutaraldehyde, pH 7.3, at 4°C for 3 hours; washed overnight in the same buffer; postfixed in buffered 1% osmium tetroxide for 1 hour; washed; dehydrated through a graded series of ethanol; cleared in propylene-oxide; and embedded in Epoxy resin (Araldite).

Semithin sections (1μ) cut with glass knives on an LKB V Ultratome and stained with toluidine blue, were examined by light microscope for general evaluation of tissue morphology. Ultrathin sections from selected areas were cut with a diamond knife using the same ultramicrotome, retrieved on copper grids, double-stained with uranyl acetate and lead citrate, and examined at 100 kV with a Philips 208 S electron microscope.

At least 50 cross sections of different cilia from different cells were observed in each specimen to study axoneme structure. Only orthogonally cross-sectioned cilia were evaluated, excluding those near the base or tip. Diagnostic electron micrographs were generally taken at a magnification of ×50,000 for a comprehensive view and to determine the orientation of the cilia. A magnification of ×110,000 usually was used to study axoneme pattern.

Dynein arms and microtubules were counted and axoneme organization, presence of radial spokes, spoke heads, and central sheaths were evaluated. The incidence of abnormal cilia was expressed as a percentage.

In each specimen, ciliary orientation was investigated. The ciliary axis was determined drawing a line through the central microtubule pair of each cilium. At least 10 suitable ciliary cross sections per cell were studied: the angle between the ciliary axis and a standard reference line was measured and the SD of the angles was calculated for each cell.

RESULTS

The MCTt was defective in 45 cases (average, 32 minutes), and MCT was completely blocked in 7 cases. The data were confirmed by phase-contrast microscope. Sixty-six nasal scrapings (three in one patient) were performed. TEM examination revealed 11 cases of PCD and 51 cases of acquired ciliary alteration, sometimes associated in the same patient. No clear diagnosis was possible in two cases.

Tables 1 and 2 show correlations between MCTt, evaluating only oropharyngeal charcoal powder and CBF and TEM findings, in PCD and acquired dyskinesia. Patients with PCD showed an absence of outer dynein arms (Fig. 1 *B*) in four cases, lack of outer and inner arms in three cases, lack of only inner dynein arms in two cases, one also showing primary axonemal disorientation, probably because of a radial spoke alteration (Fig. 1 *C*). Central pair defects were present in one case.

The most common electron microscope finding in acquired forms was compound cilia (Fig. 2 *A*), increasing with age and smoking. Randomly orientated cilia (Fig. 2 *B*) as well as abnormalities in the arrangement of peripheral microtubules (Fig. 2 *C*) were common findings. Diagnosis was impossible in two patients because of large areas of squamous metaplasia.

DISCUSSION

Complete study of MCT involves comparison of the results of dynamic evaluation in *in vivo* and *in vitro* examination of ciliary motility and structure. As regards *in vivo* testing, MCT is mostly evaluated as the time necessary for a substance to be carried from the head of the inferior turbinate to the posterior wall of the oropharynx. Because of their low cost and ease of retesting, inert substances are now preferred to radioactive ones and Teflon discs. Many substances have been tested over the years for mucociliary clearance examination. We used a mixture of plant charcoal powder and 3% saccharin.[14] The water-soluble saccharin indicates a relative value for the clearance of soluble substances; the charcoal powder, on the other hand, does not spread into the nasal secretions and therefore is a valid indicator of MCTt.

Our technique consists of measurement of the time taken by the mixture to become detectable on the posterior wall of the oropharynx in the case of charcoal and to be tasted in the case of saccharin, after deposition with a cotton stick on the head of the inferior turbinate. The test is more accurate when

Table 1

Primary Axonemal Alterations	No. of Patients	CBF (range of beat frequency/min)	TMCt (range)
Outer arms lacking	4	0	Not detected at 60 ft
Inner arms lacking	2	35–45	40 ft 32 in.–45 ft 27 in.
Outer and inner arms lacking	3	0	Not detected at 60 in.
Radial spokes	1	65	35 ft 15 in.
Central pair	1	96	32 ft 22 in.

Table 2

Acquired Axonemal Alterations	No. of Patients	CBF (range of beat frequency/min)	TMCt (range)
Compound cilia	46	115–100	12ft12in.–30ft48in.
Demembranated or intracytoplasmic cilia	5	87–75	18ft07in.–44ft57in.
Extra or missing peripheral microtubules	21	103–85	20ft18in.–40ft14in.
Randomly arranged peripheral microtubules	3	105–79	20ft15in.–42ft47in.

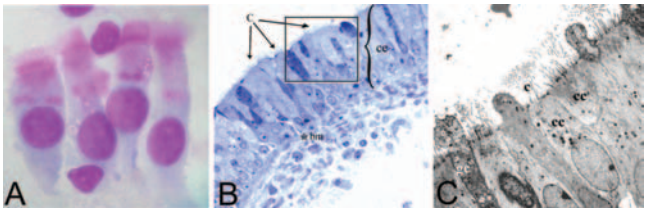

Figure 1. *(A) TEM. Cross section of normal structured cilia showing correct orientation. Inset shows high magnification of normal cilium presenting typical 9 + 2 arrangement (uranyl acetate-lead citrate; original magnification, ×44,000; insert, original magnification, ×89,000). (B) TEM. Cross-sectioned cilium from a patient with PCD, showing total lack of outer dynein arms (arrowhead; uranyl acetate-lead citrate; original magnification, ×180,000). (C) TEM. All cilia from a patient with PCD show loss of inner dynein arm (arrow) and disarrangement of ciliary axis (uranyl acetate-lead citrate; original magnification, ×110,000).*

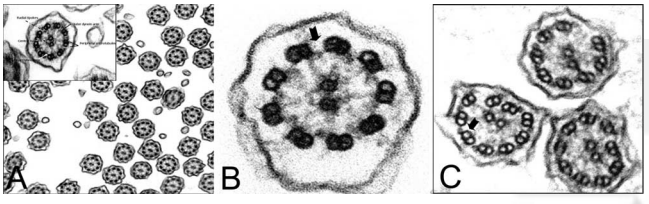

Figure 2. *(A) TEM. Acquired ciliary dyskinesia: a compound cilium with many axonemes within the same membrane (uranyl acetate-lead citrate; original magnification, ×56,000). (B) TEM. Cilia from a patient with recurrent respiratory tract infections, showing random ciliary orientation (uranyl acetate-lead citrate; original magnification, ×89,000). (C) TEM. Extraperipheral microtubules (uranyl acetate-lead citrate; original magnification, ×180,000).*

a number of measurements are made at the same time of day with the patient in the same position and under similar weather conditions, because MCTt may vary with circadian rhythms and ambient temperature and humidity. In this way we established an average MCTt for adults: 12.5 minutes (charcoal) and 17 minutes (saccharin). In children, only charcoal is usually considered and has an average MCTt of ~10 minutes[15] In our study, only the oropharyngeal evidence time of charcoal powder was evaluated because the saccharin test is subjective in children showing low compliance.

Lindberg[16] described a technique for detecting ciliary beat synchrony and CBF with an optic fiber probe connected to a computerized photoelectric transduction system. For *in vitro* analysis, single respiratory cells are sampled by brushing, mucosal lavage, or small biopsies.[17] Ingels *et al.*[18] recommend obtaining a biopsy specimen of intact ciliated epithelium attached to the basal membrane. Our experience shows that nasal biopsy does not provide more information for morphofunctional study of the nasal epithelium than well-performed nasal scraping. We obtained indications regarding various cell sampling methods several years ago while studying cell alterations of nasal mucosa by phase-contrast microscope and May Grunwald-Giemsa staining (Fig. 3 *A*); the scraping method provided excellent cellular material for evaluation of the cytoplasmic component and ciliary system[19,20] (Fig. 3, *B* and *C*). Moreover, nasal scraping does not require sedation or anesthesia and does not cause bleeding; it can be repeated and is appropriate for all ages. F3

A special nasal probe (Rhinoprobe) is used to collect cells from the middle third of the inferior turbinate. One-half the sample is used to evaluate ciliary movement by phase-contrast microscopy, and the rest is used for TEM examination. Because ultrastructural diagnosis centers often receive badly brushed specimens or nasal biopsy specimens in which it is impossible to carefully visualize ciliated epithelial cells, sampling should be done with care by the scraping method.

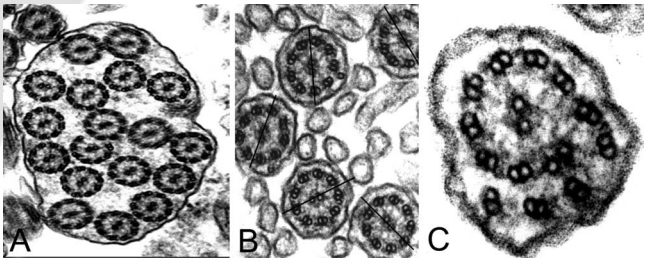

Figure 3. *(A) Well-conserved ciliated cells obtained by nasal scraping (May-Grunwald-Giemsa staining; original magnification, ×1000). (B) LM. Semithin section of nasal mucosa obtained by scraping. Epithelial architecture is well preserved (toluidine blue; original magnification, ×100). (C) TEM. Higher magnification of the same epithelium; ciliated and goblet cells are easily detected and well preserved (uranyl acetate-lead citrate; original magnification, × 3500; bm, basal membrane; c, cilia; cc, ciliated cell; gc, goblet cell; re, respiratory epithelium).* AQ: N

During ultrastructural analysis, ciliary orientation must be studied in the middle section of the cilium to compare axis orientation: in normal subjects this is between 9 and 25°.[10] In our hands, using the scraping method, samples were inadequate in two of patients only, both >65 years of age, because of huge areas of squamous metaplastic tissue.

In our experience bronchial brushing is only necessary when TEM examination of nasal samples provides results incompatible with clinical history. Patients with PCD show heterogeneous morphological defects. The most frequent ultrastructural alteration is total absence or partial defect of dynein arms. Primary lack of central-pair microtubules, with or without transposition, and congenital radial spokes defects leading to axonemal asymmetry are less frequent. A diagnostic problem of PCD is caused by a lack of quantitative data on the incidence of morphological ciliary defects in normal subjects. Acquired ciliary defects usually appear as compound cilia, anomalous peripheral microtubule number and/or position, random axonemal orientation, and changes in ciliary membrane integrity or excessive cytoplasmic matrix in cilia. In our experience and in line with reports in the literature, patients undergoing ultrastructural examination of the upper airway epithelium by nasal brushing or biopsy show at least some pathological cilia. Therefore, care is necessary to differentiate secondary alterations from genetic ones. In this study, we examined three consecutive samples from the same patient. The first and second samples showed different alterations in >20% of the cilia examined, whereas the third, performed after medical therapy, was normal.

The finding of morphologically normal cilia in patients with PCD, including Kartagener's syndrome, means that more sensitive techniques are needed. A major diagnostic difficulty is to distinguish primary defects from acquired alterations, which may resemble congenital defects. Aspecific ciliary alterations may appear in many respiratory diseases other than PCD and may be caused by pathogens, exposure to pollutants, smoking, and other causes. Unlike genetic defects, acquired ones usually are observed in fewer cilia and are heterogeneous. Therefore, it is necessary to estimate the percentage of affected cilia.

CONCLUSIONS

In children with rhinitis, rhinosinusitis, and productive cough due to recurrent respiratory tract infections, systematic investigation of ciliated cells for PCD is mandatory. Kartagener's syndrome is only one subgroup of PCD. Nasal scraping is a far easier, more effective, less expensive, and less invasive method of sampling ciliate cells than nasal brushing, nasal biopsy, bronchial brushing, and tracheal brushing. TEM examination is currently the only available technique to differentiate various morphological abnormalities of cilia and to make a differential diagnosis between primary and secondary forms of PCD.

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