AIRWAY BIOLOGY

Functional analysis of cilia and ciliated epithelial ultrastructure in healthy children and young adults

M A Chilvers, A Rutman, C O’Callaghan

Background: There are very few data on normal ciliary beat frequency, beat pattern, and ultrastructure in healthy children and adults. A study was undertaken to define ciliary structure, beat frequency and beat pattern in a healthy paediatric and young adult population.

Methods: Ciliated epithelial samples were obtained from 76 children and adult volunteers aged 6 months to 43 years by brushing the inferior nasal turbinate. Beating cilia were recorded using a digital high speed video camera which allowed analysis of ciliary beat pattern and beat frequency. Tissue was fixed for transmission electron microscopy.

Results: The mean ciliary beat frequency for the paediatric population (12.8 Hz (95% CI 12.3 to 13.3)) was higher than for the adult group (11.5 Hz (95% CI 10.3 to 12.7 Hz), p<0.01, t test); 10% (range 6–24%) of ciliated edges were found to have areas of dyskinetically beating cilia. All samples had evidence of mild epithelial damage. This reflected changes found in all measurements used for assessment of epithelial damage. Ciliary ultrastructural defects were found in less than 5% of cilia.

Conclusion: Normal age related reference ranges have been established for ciliary structure and beat frequency. In a healthy population localised epithelial damage may be present causing areas of ciliary dyskinesia.

Respiratory cilia beat in a coordinated manner with a specific frequency and pattern, clearing mucus and debris from the airways. Acquired or congenital ciliary ultrastructural defects result in cilia which are either stationary or beat in a slow or dyskinetic fashion. Ineffective movement impairs mucociliary clearance. In primary ciliary dyskinesia this causes sinusitis and recurrent chest infections which may lead to bronchiectasis.1 2

An early diagnosis of primary ciliary dyskinesia is important as institution of appropriate respiratory care has been shown to halt the progressive decline in lung function.3 A diagnosis is made on the basis of a supportive clinical history and an abnormal ciliary beat frequency accompanied, in most cases, by specific abnormalities of the ciliary axoneme on electron microscopy.4 Studies by Rossman and colleagues suggest that evaluation of beat pattern, in addition to beat frequency, may be helpful in the diagnosis of patients with primary ciliary dyskinesia.5

Making a confident diagnosis of primary ciliary dyskinesia can at times be very difficult as abnormalities of the epithelium and cilia may also be found purely as a result of acquired ciliary defects.6 7 It is therefore important to differentiate between primary and secondary ciliary structural and functional abnormalities.

Secondary ciliary ultrastructural defects are common.8 Defects may persist for up to 12 weeks following resolution of an upper respiratory tract infection9 10 and ultrastructural interpretation may be difficult.11 Quantitative ultrastructural analysis in healthy adult subjects is limited.11 12 Paediatric studies of the ciliary ultrastructure have been small and have consisted of patients rather than healthy controls. Data suggest that 5% of cilia have abnormalities,12 13 with reports only analysing microtubular defects.14 15 The analysis of the dynein arms has been limited to patients with respiratory infections; up to 30% of cilia have been found to be affected.16 17 Reference ranges for healthy children are not available for either ciliary microtubules or the presence of dynein arms, and there are no data on the damage of nasal ciliated epithelium in a healthy control population.

While normal ranges of ciliary beat frequency in adults have been published,18 19 there are few data for children. Cilia from neonatal patients10 and adolescents18 were found to beat at a higher frequency than cilia from adults. Other studies have suggested that ciliary beat frequency may either fall with age19 or remain constant.20 21 Evaluation of cilia in children involves the sampling of nasal epithelium and analysis of ciliary beat frequency, beat pattern, and ultrastructure.22 Few data from normal children are available to allow comparison with findings in patients suspected of having primary ciliary dyskinesia.

We have adopted a digital high speed imaging method that allows the exact movement of a cilium to be rapidly evaluated throughout the beat cycle and measurement of the ciliary beat frequency. The direct observation of ciliary movement in slow motion and the ability to archive such material for audit and research is a major advantage over existing methods. It is likely that high speed video analysis will become the preferred method for evaluation of ciliary beat pattern and beat frequency for the diagnosis of primary ciliary dyskinesia. Using digital high speed imaging we have been able to describe precisely the normal ciliary beat cycle and found it to differ from previously published data.7

The high speed video method has also been evaluated against other existing indirect techniques such as the photodiode and photomultiplier methods for the measurement of ciliary beat frequency and significant differences were found.1 This again emphasises the need for a normal range to be established for each method. Reference ranges exist for both the photomultiplier1 and photodiode16 methods, but no normal reference range exists for digital high speed imaging.

The aim of this study was to measure ciliary beat frequency and to determine the ciliary beat pattern and ultrastructure in healthy children and adults. The second aim of the study was to determine the ultrastructure of the respiratory epithelium from healthy children and adults.
METHODS
Fifty three healthy children (31 male, age range 6 months to 17 years) were recruited from subjects undergoing elective surgery and 23 adult volunteers (16 male, age range 18–43 years) were also recruited. Subjects were excluded if they had a history of chronic respiratory or nasal disease or a symptomatic upper respiratory tract infection during the previous 6 weeks, were taking regular medication, or were known smokers.

Paediatric samples were obtained immediately after induction of anaesthesia with propofol. This agent has been shown to have no effect on ciliary beat frequency. No premedication had been given to any subject before surgery. In all subjects ciliated samples were obtained by brushing the inferior nasal turbinate with a 2 mm cytology brush. Nasal brushings were placed in medium 199 (pH 7.3) which contained antibiotic solution (streptomycin 50 µg/ml, penicillin 50 µg/ml; Gibco, UK).

The study was approved by the Leicestershire ethical review committee and written consent was obtained before sampling.

Figure 1  Transmission electron micrographs illustrating the parameters assessed to examine epithelial damage in comparison with normal epithelium shown in fig 2A. (A) Severe loss of cilia, grade 3 [bar 10 µm]. (B) Projection of a cell from the epithelial edge, grade 3 [bar 10 µm]. (C) Cytoplasmic blebbing, grade 2 [bar 10 µm]. (D), (E) Mitochondrial damage: a cell with a normal healthy mitochondrion (arrowhead, E) is shown against a cell with a damaged mitochondrion (arrow, D), grade 1 [bar 2 µm].
Evaluation of ciliary structure and function

Tissue obtained by nasal brushing was fixed in 2.5% glutaraldehyde and processed through to resin by standard techniques as previously described. Ultrathin sections were cut at 70 nm. These were collected on 200 mesh thin bar copper grids, stained in 1% uranyl acetate, counterstained in Reynolds’s lead phosphate, and examined by transmission electron microscopy.

The ciliated epithelium was assessed blindly for both epithelial and ciliary ultrastructural changes. Epithelial integrity was assessed by firstly examining cell type. The number of ciliated cells, mucous cells, and dead cells were totalled and expressed as a percentage of all cells examined. Disruption and damage to the tissue was quantified using the scoring system previously described. Briefly, the tissue was scored for the following parameters: loss of cilia from ciliated cells: 0 (fully ciliated), 1, 2, 3 (a few cilia visible); projection of cells from the epithelial edge: 0 (normal alignment), 1, 2, 3 (cell projected from edge but some contact with other epithelial cells); cytoplasmic blebbing: 0 (absent), 1 (minor), 2 (major); mitochondrial damage: 0 (absent), 1 (present) (fig 1).

To give an overall evaluation of epithelial damage an epithelial integrity score was given to the epithelium which incorporated ciliary loss, cellular projection, cytoplasmic blebbing and mitochondrial damage. A healthy intact epithelial edge was scored 0 and a severely disrupted edge was scored 5 (0=no damage, 1=minor, 2=mild, 3=moderate, 4=major, 5=severe damage; fig 2). The scoring system was evaluated by comparing it against all measurements used to measure epithelial damage.

Damage to individual cilia was evaluated by examining the ciliary ultrastructure for microtubular and dynein arm defects. Alignment of individual cilia within a cell was assessed by measuring ciliary orientation as previously described.

The percentage of cells with loss of cilia, cellular projections, cytoplasmic blebbing, mitochondrial damage and with microtubular or dynein arm defects was calculated.

Ciliary beat frequency and beat pattern

Ciliary beat frequency and beat pattern were evaluated as previously described. Briefly, ciliated strips of epithelium were suspended in a chamber created by the separation of a cover slip and glass slide by two adjacent cover slips. The slide was placed on a heated stage (37°C) of a Leitz Diaplan microscope mounted on an anti-vibration table (Wentworth Laboratories Ltd, UK). Specimens were examined using ×100 interference contrast lens. Only undisturbed ciliated strips longer than 50 μm devoid of mucus were studied. Beating ciliated edges were recorded using a digital high speed video camera (Kodak Motioncorder Analyser, Model 1000) at a rate of 400 frames per second. The camera allows video sequences to be recorded and played back at reduced frame rates or frame by frame. The ciliated edge, projected onto a high resolution monitor, was divided into five adjacent areas measuring 10 μm. Two measurements of ciliary beat frequency were made in each area, resulting in a total of 10 measurements of beat frequency along each ciliated strip. A maximum of 10 edges were analysed per subject. Ciliary beat frequency was determined directly. Groups of beating cilia were identified and the number of frames required to complete 10 cycles recorded. This was converted to ciliary beat frequency (CBF) using the calculation (CBF = 400/(number frames for 10 beats) × 10).

As the digital high speed video system was to be used to establish reference ranges for the measurement of ciliary beat frequency, the reproducibility of the method was evaluated. A single point on the ciliated edge was identified on a grid placed on the monitor. Ciliary beat frequency at that point was measured independently by two observers (O1, O2), and this was repeated for each of the five areas displayed on the monitor. A total of five readings were obtained for each edge and the analysis was performed in 10 subjects. One observer repeated the series of measurements two days later (M1, M2). From this the inter-observer and intra-observer coefficient of variation (CV) could be calculated.

To assess the ciliary beat pattern each edge was analysed. Coordinated ciliary beating in a forward backward motion along the whole epithelial edge was defined as normal. Edges which appeared to have dyskinetically beating cilia were noted and the percentage of edges exhibiting areas of dyskinetically beating cilia was then calculated.

Analysis of data

As ciliary beat frequency may change with age, we wanted to see if other parameters showed such variation. As suggested by Roth et al., a cut off was made at 18 years of age. To allow sufficient subjects in each age group, three age ranges were used: 0–6, 7–12, and 13–18 years of age. Adults were classified as ≥19 years.

To form reference ranges the mean ciliary beat frequency, standard deviation, 5th and 95th percentiles were calculated for individual age groups. A one way analysis of variance was performed between groups. Individual groups were compared using a Student’s t test. Similarly, the mean percentage, 5th and 95th percentiles of edges exhibiting areas of dyskinetically beating cilia were calculated. For all ultrastructural

Figure 2. Transmission electron micrographs showing assessment of epithelial integrity. (A) Normal tissue with an intact well ciliated surface and minimal disruption; epithelial integrity score=0 (bar 10 μm). (B) Abnormal tissue with severely disrupted cell surface and marked loss of cilia; epithelial integrity score=5 (bar 10 μm).
parameters the mean and the 5th and 95th percentiles were calculated. A one way analysis of variance was performed between groups.

RESULTS
Ciliary beat frequency and beat pattern were measured in all subjects; 56 had sufficient tissue for epithelial integrity measurements and 60 for ciliary ultrastructure. Ciliary beat frequency and beat pattern were the initial measurements to be made after which samples were then processed for electron microscopy. During this procedure tissue may be lost. Consequently, some subjects had an inadequate sample for full ultrastructural analysis.

Table 1 shows the percentage of different cell types seen in the epithelial strips obtained. Analysis showed no difference between the percentage of different cell types identified and the age of the subject. Ciliated cells formed 65% of the cell population.

Analysis of the factors involved in epithelial integrity are summarised in table 2. Even within the healthy population there is evidence of loss of cilia, cellular extrusion, cytoplasmic blebbing, and mitochondrial damage. Analysis of variation found no difference between groups for all measurements analysed. The epithelial integrity score, which reflects a combination of all the measurements used to assess epithelial damage, also showed no significant difference between the age groups.

A summary of the results of the ultrastructural analysis of individual cilia is shown in table 3. Dynein arm defects were found in less than 3% of cilia observed. It was possible to visualise, on average, seven of the expected nine dynein arms when counting both inner and outer dynein arms. Again no differences were found in the ultrastructural analysis of individual cilia between the various age groups. Microtubular abnormalities were uncommon in all age groups; ciliary orientation did not vary with age (table 3).

Ciliary beat frequency and the percentage of cells with ciliary dyskinesia are shown in table 4. No significant difference in mean ciliary beat frequency between individual age groups was found (ANOVA, p=0.10). However, there was a significant difference in ciliary beat frequency between patients under the age of 18 (12.8 Hz [95% CI 12.3 to 13.3]) and those over the age of 18 (11.4 Hz [95% CI 10.2 to 12.6 Hz], p<0.01, t test). Approximately 10% of all edges analysed exhibited areas of

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Table 1  Analysis of cell type by transmission electron microscopy

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>n</th>
<th>Ciliated cells (%)</th>
<th>Unciliated cells (%)</th>
<th>Mucous cells (%)</th>
<th>Dead cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>16</td>
<td>69.1 [45.9, 81.0]</td>
<td>21.0 [13.1, 42.3]</td>
<td>9.9 [5.2, 17.5]</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>7–12</td>
<td>13</td>
<td>62.6 [33.7, 76.0]</td>
<td>26.8 [16.2, 52.9]</td>
<td>10.6 [4.9, 16.4]</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>13–18</td>
<td>10</td>
<td>71.3 [59.6, 79.1]</td>
<td>20.6 [13.6, 32.2]</td>
<td>8.1 [6.7, 10.4]</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
<tr>
<td>&gt;19</td>
<td>15</td>
<td>67.5 [43.0, 78.6]</td>
<td>22.1 [12.4, 43.1]</td>
<td>10.4 [6.4, 19.7]</td>
<td>0.0 [0.0, 0.0]</td>
</tr>
</tbody>
</table>

Results are expressed as the mean percentage (5th and 95th percentiles) for individual age groups.

Table 2  Transmission electron microscopy assessment of epithelial integrity

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>n</th>
<th>Cells with loss of cilia (%)</th>
<th>Cells extruding from surface (%)</th>
<th>Cells with cytoplasmic blebbing (%)</th>
<th>Cells with mitochondrial damage (%)</th>
<th>Epithelial integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7–12</td>
<td>13</td>
<td>24.7 [6.0, 58.2]</td>
<td>21.5 [10.5, 35.8]</td>
<td>11.8 [5.5, 24.2]</td>
<td>8.9 [0.0, 21.9]</td>
<td>1.3 [0.8, 1.9]</td>
</tr>
</tbody>
</table>

Results are for individual age groups and expressed as the mean (5th and 95th percentiles).

Table 3  Analysis of ciliary ultrastructure by transmission electron microscopy

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>n</th>
<th>Dynein arm counts</th>
<th>Dynein arm defects (%)</th>
<th>Microtubule defects (%)</th>
<th>Central microtubule defects (%)</th>
<th>Ciliary orientation °</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inner</td>
<td>Outer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–6</td>
<td>19</td>
<td>7.4 [7.2, 7.7]</td>
<td>7.9 [7.6, 8.2]</td>
<td>1.8 [0.0, 7.1]</td>
<td>2.1 [0.6, 5.6]</td>
<td>0.3 [0.0, 1.2]</td>
</tr>
<tr>
<td>7–12</td>
<td>15</td>
<td>7.2 [7.0, 7.4]</td>
<td>7.7 [6.4, 8.3]</td>
<td>1.3 [0.0, 4.4]</td>
<td>1.9 [0.0, 4.4]</td>
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<tr>
<td>13–18</td>
<td>10</td>
<td>7.1 [6.7, 7.3]</td>
<td>7.5 [5.6, 8.1]</td>
<td>1.0 [0.0, 1.9]</td>
<td>2.3 [1.2, 3.6]</td>
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</tr>
<tr>
<td>&gt;19</td>
<td>16</td>
<td>7.2 [6.5, 7.9]</td>
<td>6.8 [5.7, 8.1]</td>
<td>1.0 [0.0, 2.8]</td>
<td>1.9 [0.8, 3.8]</td>
<td>0.3 [0.0, 1.0]</td>
</tr>
</tbody>
</table>

Results for individual age groups are expressed as the mean (5th and 95th percentiles).

Table 4  Summary of analysis of ciliary beat frequency measurements

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>n</th>
<th>Ciliary beat frequency (Hz)*</th>
<th>Dyskinetically beating edges (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–6</td>
<td>24</td>
<td>12.9</td>
<td>10.0, 18.1</td>
</tr>
<tr>
<td>7–12</td>
<td>19</td>
<td>12.9</td>
<td>10.9, 15.0</td>
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<td>11.5</td>
<td>7.7, 15.3</td>
</tr>
</tbody>
</table>

*Mean ciliary beat frequency, standard deviation (SD), and 5th and 95th percentiles. †Mean (5th, 95th percentiles) percentage of edges exhibiting areas of ciliary dyskinesia.

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Dyskinetically beating cilia. This was found to be higher in the 13–18 year group.

To establish a reference range the mean ciliary beat frequency was plotted against age (fig 3A). A weak negative correlation was found between mean ciliary beat frequency and increasing age ($r=-0.30$, $p=0.008$). This was modelled and a linear relationship was found. Quadratic and other relationships were modelled and found not to be significant.

Within each sample cilia were found to beat at different frequencies. To evaluate sample variation in ciliary beat frequency the edges with the highest and lowest ciliary beat frequencies from several edges and from different sites may be because of the healthy nature of the tissue.

The CV for measurement of ciliary beat frequency along an edge was found to have slower beating cilia$^{[6–9]}$ with frequencies as low as 6–9 Hz.$^{[24]}$ Two healthy children were also reported to have cilia beating as slowly as 6 Hz.$^{[21]}$ The ciliary beat frequency was found to vary between edges within a sample, with a mean beat frequency of $<11$ Hz in some subjects. This is in keeping with other reports which have found cilia in healthy adults to beat maximally at $<11$ Hz in some subjects. This is in keeping with other reports which have found cilia in healthy adults to beat maximally at $<11$ Hz in some subjects.

The quantification of inner and outer dynein arms is important in the diagnosis of primary ciliary dyskinesia as dynein arm defects are the most common abnormality found in these patients.$^{[7]}$ The majority of inner and outer dynein arms were visualised in all subjects. Our results are consistent with other published data on the number of outer dynein arms visible, but we were able to identify a greater proportion of inner dynein arms than has previously been reported.$^{[8]}$ This may be because of the healthy nature of the tissue.

As suggested by Veale and colleagues, we examined ciliary beat frequencies from several edges and from different sites along an edge.$^{[14]}$ The ciliary beat frequency was found to vary between edges within a sample, with a mean beat frequency of $<11$ Hz in some subjects. This is in keeping with other reports which have found cilia in healthy adults to beat maximally at a frequency of $>10$ Hz (range 10.2–14.6) and minimally at a frequency of $>7$ Hz (range 7.5–11.2).$^{[31]}$ This was limited to 20 volunteers and no children were included. Adults were found to have slower beating cilia$^{[6–9]}$ with frequencies as low as 6–9 Hz.$^{[24]}$ Two healthy children were also reported to have cilia beating as slowly as 6 Hz.$^{[21]}$

The CV for measurement of ciliary beat frequency along an epithelial edge has been shown to vary from 9% to 58% with other published data.$^{[9]}$ However, these data were from organ culture models and it is possible that, in the process of brushing and tissue preparation, minor damage may have occurred. Although two previous studies have evaluated the use of nasal brushing to sample cilia for ultrastructural measurement, they did not assess epithelial damage.$^{[7, 8]}$

A scoring system for evaluation of epithelial integrity has been developed. This has been validated against the measurements used to assess epithelial ultrastructural damage and found to be representative of the minor epithelial damage observed in healthy subjects.

The percentage of dynein arm and microtubular abnormalities were both found to be less than 5%, which agrees with other published data.$^{[10]}$ The mean orientation of cilia in the paediatric population has only been described in eight children under the age of 2 years and was reported to be 14.9°.$^{[31]}$ This is higher than the values we obtained (10.7–10.9°) in 60 subjects of differing ages.

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The CV for measurement of ciliary beat frequency along an epithelial edge has been shown to vary from 9% to 58% with other published data.$^{[9]}$ Compared with 10% in our study. We found no significant difference in inter-observer or intra-observer CV using the digital high speed video technique.

The ciliary beat frequency of the children was found to be significantly greater than the adult population. This is supported by studies which have shown ciliary beat frequency...
of neonates and teenagers to be greater than adult subjects. Our data suggest a slight fall in ciliary beat frequency with increasing age, which is in agreement with other studies, although Jorissen et al. found ciliary beat frequency to be independent of age. However, their readings were conducted at 22°C rather than at body temperature which makes the comparison difficult. At this temperature cilia beat at a much slower frequency and the association may therefore have been lost.

Digital high speed video imaging allowed us to visualise precisely the normal ciliary beat pattern in healthy subjects; 10% of edges had evidence of dyskinetically beating cilia. The remainder of the cilia were found to beat forward and backwards within the same plane without a classical sideways recovery sweep. This is consistent with our earlier description. Analysis of ciliary beat pattern may improve our understanding of the actions of various respiratory pathogens—for example, cilia following infection have been found to have a dyskinetic beat pattern despite beating at a normal ciliary beat frequency.

In summary, we have established an extensive age related normal reference range for both ciliary structure and function. We have also examined the epithelial integrity in a healthy population. Such data will help with our evaluation of patients suspected of having primary ciliary dyskinesia and in research studies looking at the effects of various pathogens on nasal ciliary ultrastructure, function, and epithelial integrity.

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