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CILIARY CENTRAL MICROTUBULAR ORIENTATION IS OF NO CLINICAL SIGNIFICANCE IN BRONCHIECTASIS

(Running head: Ciliary orientation in bronchiectasis)

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ABSTRACT

It has been suggested that patients with bronchiectasis might have increased central microtubular orientation angle (CMOA), which leads to poor coordination of ciliary beating, and consequently impairment of airway defence. We have employed transmission electron microscopy to assess CMOA of ciliated nasal mucosa in a cohort of 133 (81F, 56.8±16.1 year) stable bronchiectasis and 59 healthy subjects (30F, 49.3±22.1 year). There was no significant difference in CMOA between bronchiectasis (13.2 degree) and control subjects (13.0 degree, p=0.82). There was no significant difference in CMOA among patients according to the etiology of bronchiectasis, presence of nasal symptoms, or sputum status of *Pseudomonas aeruginosa* infection. Patients with more severe bronchiectasis, i.e. those with FEV_1 <60%, FVC <60%, or more than 4 bronchiectatic lung lobes, had significantly lower CMOA than their counterparts ($p<0.05$). There was no correlation between CMOA with age, 24h sputum volume, exacerbation frequency, FEV_1, FVC, or the number of bronchiectatic lung lobes ($p>0.05$). CMOA correlated with ciliary beat frequency (negative), and the percent of cilia showing ultrastructural or microtubular defects ($p<0.05$). Central microtubular orientation angle does not correlate with clinically important parameters, in contrary to the results reported by previously published smaller scale studies.

KEY WORDS

Bronchiectasis, cilia, transmission electronic microscopy, ciliary central microtubular orientation

INTRODUCTION

Cilia are minute hair-like structures present on the surface of mucosal cells in the airways that beat continuously to maintain the sterility of the lower respiratory tract in health. The structure of cilia, which consists of the classical "9+2" microtubular arrangement, is highly conserved at different levels of the airways and across all species (Figure 1). Ciliary dysfunction results in reduced mucosal transport mechanisms leading to chronic retention of secretions and impairment of mucosal function, as manifested by patients with primary ciliary dyskinesia (PCD), a hereditary condition whereby ciliary beat is dyskinetic (1). Patients suffer from chronic upper and lower airway sepsis, and occasionally male sterility and *situs inversus* typical of Kartagener's syndrome (1). Ciliary motility abnormalities, such as immotility and dyskinesia have long been established on PCD patients, and appear to be etiologically related to the underlying ultrastructural ciliary defects.

Many ultrastructural ciliary defects have been described and these include deficiency of dynein arms, nexin links, radial spokes, central sheath, inner microtubules, and even the entire axoneme (2-9). In order for neighboring cilia to beat in a co-ordinated fashion, their central microtubules must orientate in a parallel fashion. Such coordination of ciliary beating could be assessed by measuring the standard deviation of the angles formed between the central microtubules of neighboring cilia with the x-axis (Figure 2). This standard deviation is known as the central microtubular orientation angle (CMOA) (10-12). While ultrastructural defects of cilia, such as absence of dynein arms and microtubular abnormalities, have been reported as etiologically important in bronchiectasis, probably limited by technical requirements, studies on CMOA are scare. It has been proposed that disorientation of ciliary central microtubules alone could lead to the development of bronchiectasis (10), as a few such patients have been reported to develop otherwise idiopathic bronchiectasis, despite normal

ciliary beating and ultrastructure. Indeed, central microtubular disorientation, when the neigboring cilia do not beat in the same directions, has even been proposed to be a new variant of PCD (10).

However, there has not been any systematic evaluation of the clinical significance of central microtubular orientation in bronchiectasis. We have, therefore, performed this prospective study to determine the correlation of CMOA with disease severity (FEV_1, FVC and number of bronchiectatic lung lobes) and activity (24h sputum volume and exacerbation frequency) markers, and sputum infection status on a cohort of 133 stable bronchiectasis patients. We have also determined the relationship between CMOA with ciliary function (as beat frequency) and ultrastructure in the same cohort, which also contained 6 patients with Kartagener's syndrome (dextrocardia, sinusitis and bronchiectasis), a variant of PCD.

METHODS

Non-smoking healthy subjects, who have no respiratory or nasal symptoms for at least 2 weeks and no known systemic diseases, were recruited as controls with written informed consent. Patients with HRCT-proven bronchiectasis, who are stable in spirometry, sputum production and symptoms for at least 3 weeks, were recruited with written informed consent (13). Exclusion criteria included presence of rhinosinusal disease other than rhinosinusitis, previous nasal surgery or radiotherapy, asthma, and other known respiratory diseases. The protocol was approved by the institutional ethics committee.

For each patient, the number of bronchiectatic lung lobes was determined from a thoracic HRCT, performed within 12 months (14). The number of exacerbations occurring in the preceding 12 months, was determined by history taking and review of clinical charts (13). Lung

function indices were measured with a SensorMedics 2200 (SensorMedics, Yorba Linda, USA) package. The etiology of bronchiectasis was determined as described previously (15). The volume of 24h sputum was determined as the mean of a three consecutive day collection (13). Post-physiotherapy sputum was collected for microbiological examination, using enriched and selective agar plates, to identify all the sputum bacteria (16).

Suspensions of nasal epithelium in Medium 199 (Flow Laboratory, New York, USA) was obtained without anesthesia from the inferior turbinate of subjects with a cytology brush (1,11,17), and assessed for ciliary beat movements and frequency with an established photometric technique. Very briefly, the frequency that the beating cilia, examined at 100x and 10 different sites, interrupted a light source was determined as ciliary beat frequency (Hz) (1,11,17). A 70-90nm section through the central portion of each specimen was taken for transmission electron microscopy (TEM) examination at 10,000-22,000x (1,11,17). Microtubular assessment included evaluation of presence of single microtubules, extra central pair of microtubules ("9+4"), extra peripheral microtubule, "8+2", "9+1", "9+0", compound cilium, dynein arm, or microtubular disarrangement was performed on each of the ciliary sections (2-9). The orientation of the ciliary central microtubules was determined as described previously (10,11,17). Briefly, a line was drawn electronically through the central pairs of microtubules with an image analysis package (Improvision, Cambridge, UK). The SD of the angle made by each of these lines to the horizontal axis, drawn to intersect a line linking the centres of both central microtubules, was determined (Figure 2). A mean SD was obtained from all the epithelial cells for each patient, which represented CMOA (10,11,17,18). By convention, only TEM specimens with more than 20 cilia captured in full cross section, obtained from at least 6 different epithelial cells, were analysed (10,11,17).

Unless otherwise stated, data were expressed as mean, standard deviation (SD), and range. Pearson's or Spearman rank correlation analyse was performed to examine the relationship between continuous clinical parameters, CMOA, and ciliary beat frequency. Such correlation analysis was not performed for the Kartagener's patients as there were only 6 cases. Categorical data were compared using t-test or non-parametric tests when appropriate. A p value of < 0.05 was considered to indicate statistical significance. The analysis was performed using the SPSS® 10.0 Version package (SPSS Inc., Chicago, IL, USA).

RESULTS

Subject demography and clinical characteristics

133 bronchiectasis patients (81F) were recruited between June 1997 and May 2001. The mean (± S.D.) age for the subjects was 56.8 ± 16.1 (range 12-82) years. The spirometry data, 24h sputum volume, number of bronchiectatic lobes, exacerbation frequency, etiology of bronchiectasis and sputum bacterial pathogen data, and medications are shown in Table 1. There were 6 patients with Kartagener's syndrome (3F, 28.5 ± 8.9 yrs), who were significantly younger than their control counterparts (p=0.02). There were altogether 59 (30F, 49.3 ± 22.1 yrs) healthy control subjects who donated respiratory cilia for this study.

Light microscopy assessment

Light microscopy assessment of the ciliary specimens was performed on the entire cohort of 133 bronchiectasis and 59 control subjects. The mean ciliary beat frequency for the bronchiectatic patients was significantly lower than that of the control subjects (11.3 ± 2.5, range 0-15.7; and 13.1 ± 1.6, range 9.6-16.2 Hz respectively; p=0.001). Apart from the patients with Kartagener's syndrome, none of the patients or control subjects showed evidence of ciliary

dyskinesia or immotility on light microscopy examination. Among the patients with Kartagener's syndrome, ciliary beat frequency (3.9 ± 6.1 Hz) was significantly lower than that of their bronchiectasis counterparts (11.6 ± 1.6 Hz, $p<0.001$) and control subjects ($p<0.001$).

Transmission electron microscopy assessment (Table 2)

All bronchiectatic and control subjects had adequate TEM examination of microtubules, ciliary matrix and ciliary tail. Significantly more ciliary sections were examined for bronchiectatic (140.6 ± 95.9) than control subjects (106.7 ± 79.4, $p=0.02$). Significantly more patients with bronchiectasis displayed microtubular defects than control subjects ($p=0.001$). Of these, there were significantly more patients displaying microtubular defects, "8+2", or compound cilia, when compared with control subjects ($p<0.05$). However, there were no significant differences between bronchiectasis patients and control subjects in the percent of subjects showing "8½+2", "9+4", extra peripheral microtubules, "9+1", "9+0", absence of outer dynein arm, or disarrangement of microtubules ($p>0.05$). The 6 Kartagener's patients showed significantly more percent of sections showing lack of dynein arm (35.9 ± 24.7%), compared with control (0 ± 0%, $p<0.001$), and their bronchiectasis counterparts (0.04 ± 0.46%, $p<0.001$). Patients with Kartagener's syndrome also showed significantly more percent of sections with microtubular or any ultrastructural defects than their bronchiectasis counterparts ($p<0.001$ for both), and control subjects ($p<0.001$ for both). However, there was no significant difference in their percent of sections showing any of the aforementioned individual defects (data not shown, $p>0.05$).

There was no significant difference in CMOA between bronchiectasis patients (13.2 ± 5.1 degree, range 4.2 – 30.2) and control subjects (13.0 ± 3.6 degree, $p=0.82$). However, there was a significant difference in CMOA between patients from different aetiology subgroups ($p=0.02$).

Patients with Kartagener's syndrome (18.6 ± 7.3 degree) had a significantly higher CMOA than their bronchiectasis (12.9 ± 4.8 degree, p=0.01) and control counterparts (13.0 ± 3.6 degree, p=0.002). Patients with nasal symptoms (n=65, 12.8 ± 5.4 degree), i.e. rhinorrhoea or nasal obstruction for more than one day or any epistaxis within the previous 3 months, had no significant difference in CMOA from control subjects (p=0.79), or their counterparts (13.6 ± 4.8, p=0.36). Patients with *Pseudomonas aeruginosa* infection in their sputum (n=24, 13.31 ± 5.53 degree) did not differ in CMOA from control subjects (p=0.76), or their patient counterparts (13.14 ± 4.99, p=0.88).

Relationship between CMOA and clinical parameters (Table 3)

There was no correlation between CMOA with age of onset, age on presentation, 24h sputum volume, exacerbation frequency, FEV_1 %, FVC %, and the number of bronchiectatic lung lobes (p>0.05). Patients with $FEV_1 \leq 60\%$ predicted (n=47, 12.0 ± 4.7 degree) had significantly lower CMOA than their bronchiectasis counterparts (14.1 ± 5.3 degree, p=0.03), but not different from that of control subjects (p=0.21). Similarly, patients with FVC ≤ 60% predicted (n=30, 11.5 ± 4.3 degree) also had significantly lower CMOA than their counterparts (13.8 ± 5.3, p=0.03), but not different from that of control subjects (p=0.09). Patients with more than 4 or 5 lung lobes affected by bronchiectasis (n=15 and 9, 10.7 ± 4.0 and 8.2 ± 2.1 degree respectively) had significantly lower CMOA than their counterparts (13.7 ± 5.1 and 13.7 ± 5.0 degree, p=0.03 and p=0.001 respectively), as well as control subjects (p=0.04 and <0.001 respectively). There was, otherwise, no significant difference in CMOA between subgroups of patients according to their etiology of bronchiectasis or sputum pathogens (p>0.05, data not shown).

Relationship between CMOA with ciliary and ultrastructural parameters (Table 3)

There was a significant positive correlation between CMOA and the percent of ciliary sections showing microtubular or any ultrastructural defects ($p<0.05$). There was also a significant, albeit weak, negative correlation between ciliary beat frequency and the orientation angle ($p=0.03$). There was no significant correlation between CMOA with ciliary beat frequency, number of ultrastructural defects, or the percent of ciliary section showing ultrastructural or microtubular defects among the control subjects ($p>0.05$), although there was a trend toward a significant correlation between ciliary beat frequency and CMOA ($r=-0.25$, $p=0.06$).

DISCUSSION

Our data, obtained from the largest prospective cohort of bronchiectasis to date, showed no significant difference in CMOA between bronchiectasis patients (n=133) and healthy subjects (n=59). Although etiology of bronchiectasis had no relationship to CMOA, Kartagener's syndrome had significantly higher CMOA than their bronchiectasis counterparts and control subjects. The presence of nasal symptoms was not associated with a difference in CMOA among bronchiectasis patients, despite previous report of increased CMOA among bronchiectasis patients with mucopurulent nasal discharge (19). The presence of *Ps. aeruginosa* infection in sputum was not associated with any significant difference in CMOA although such infection is associated with poor lung function and copious sputum production (16). Patients with more severe bronchiectasis i.e. those with FEV_1 or FVC <60% predicted, or those with more than 4 to 5 lobes affected by bronchiectasis, had significantly lower CMOA than their counterparts ($p<0.05$). It, therefore, appears that central microtubular orientation does not correlate with clinical parameters, except among patients with Kartagener's syndrome or PCD. There were weak, albeit statistically significant, correlations between CMOA and ciliary beat frequency (negative), and the percent of cilia showing

ultrastructural or microtubular defects. The lack of difference in CMOA between patients with and without nasal symptoms strongly suggests that ciliary orientation does not have an important role in the development of rhinosinusitis among bronchiectasis patients. It, therefore, appears that ciliary disorientation is related to abnormalities of ciliary function (beat) and ultrastructure, but has little clinical significance among patients with stable non-PCD bronchiectasis.

There have only been a few published studies designed specifically to examine CMOA on bronchiectasis patients (10-12,18-21). Data from two of these suggest an increase in CMOA in bronchiectasis, particularly in severe cases (12,19), although our data showed no such pattern. It could be argued that we only used nasal cilia for examination, and this did not reflect the changes in the lower airways. However, not only are ciliary function and ultrastructure conserved in all species (22), nasal cilia are also the standard specimens used in ciliary assessment (23). Previous studies on ciliary orientation were not only few, but also of small sample size, ranging from 1-20 bronchiectasis patients with unclear disease stability. These studies, nevertheless, also utilised nasal cilia for examination of CMOA (10,12,18,19). It can also be argued that only the central microtubular orientation of the ciliary shafts was determined, without those of the ciliary tips or basal bodies of the cilia. However, measurements of ciliary deviation at the tip, base, and basal feet have been shown to display very little variation along the ciliary shafts (12). In addition, other published reports on the evaluation of central microtubular orientation also targeted ciliary shafts for such assessment (10,12,18,19).

While current literature suggests that ciliary ultrastructural defects are likely to be primary defects (24-27), ciliary central microtubular disorientation has been suggested to reflect

clinical deterioration, i.e. a secondary defect arisen from noxious insults to the respiratory tract (19). Ciliary central microtubular disorientation, as the only ciliary abnormality, has been proposed to be a variant of PCD and the primary aetiological factor in the development of bronchiectasis among some patients, as it is their only ciliary abnormality and in the presence of otherwise idiopathic bronchiectasis (10,18). Treatment with antibiotics and topical corticosteroids was reported to be associated with normalization of CMOA in one patient (19). Patients with Young's syndrome (obstructive azoospermia with recurrent sinobronchial disease) and PCD were found to have abnormal CMOA (12,21,28). However, non-PCD bronchiectasis and some PCD patients have been shown to display normal CMOA (12). It is, therefore, possible that CMOA is only valuable in helping to make the diagnosis of PCD, rather than of any diagnostic or prognostic value in non-PCD bronchiectasis.

Our data cast doubts on the previously proposed key pathogenic role for ciliary disorientation in the development of non-PCD bronchiectasis among otherwise idiopathic patients (8,29). Bearing in mind that over 80% of bronchiectasis patients have idiopathic disease (30), and in light of our data, it is probable that ciliary disorientation is not of clinical significance on non-PCD bronchiectasis patients. As our data are cross-sectional in nature, further large scale longitudinal follow up studies should help to clarify if CMOA would alter according to clinical status, and whether or not patients with abnormal CMOA could revert to normal, thus on the primary or secondary nature of ciliary disorientation in PCD.

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LEGEND TO FIGURES

Figure 1 Cross sectional view of **A).** a schematic diagram and **B).** a transmission electron micrograph (20,000x) of a cilium showing the classical "9+2" appearance. There are 9 peripheral microtubules and 2 central microtubules, which are arranged in an orderly manner. Central microtubules from neighboring cilia are normally orientated in the same plane and in parallel.

Figure 2 A transmission electron micrograph of the cross section of a cilium showing the way to determine the angle between the horizontal axis and a line drawn between the two central microtubules. Determination of the mean of the standard deviations of such angles of neighbouring cilia will yield an index of ciliary central microtubular orientation for the patient (20,000x).

Figure 3 A transmission electron micrograph of the cilia of **A).** a normal subject showing normal orientation of central microtubules of neighbouring cilia and **B).** patient with severe bronchiectasis who had normal ciliary beat frequency and ciliary ultrastructure but abnormal orientation of the central microtubules (10,000x).

Table 1 Details of patients with bronchiectasis who underwent ciliary central microtubular orientation assessment

Parameter	Bronchiectasis (N=133; 81F)
Mean ± SD age (yr)	56.8 ± 16.12
FEV_1 (% pred)	
Mean ± SD (inter-quartile range)	73.6 ± 30.45, (45.25,100.50)
FVC (% pred)	
Mean ± SD (inter-quartile range)	82.2 ± 24.94, (61.00,99.00)
24h sputum volume (ml)[#]	10.0 (5.0,25.0)
Number of bronchiectatic lung lobes[#]	3 (2,4)
Exacerbation frequency (per year)[#]	2 (1,4)
Aetiology of bronchiectasis	**N**
Idiopathic	104
Post-tuberculous	17
Post-pneumonic	2
Kartagener's syndrome	6
Diffuse panbronchiolitis	3
IgG deficiency	1
Sputum bacterial pathogen	
Commensals	61
Haemophilus influenzae	11
Streptococcus pneumoniae	8
Pseudomonas aeruginosa	24
Medications taken by patients	
Inhaled bronchodilators	41
Inhaled steroids	15
Theophylline	4
Anti-hypertensives	14
Diuretics	4
Allopurinol	7
Aspirin	4
Digoxin	2
Thyroxine	1
Nil	63

FEV_1= forced expiratory volume in one second and FVC = forced vital capacity.
[*]Unless otherwise stated, data shown are medians (inter-quartile ranges).

Table 2 Ciliary ultrastructural abnormalities detected in bronchiectasis and control subjects

Ultrastructural finding	Numbers of patients (%) showing		*p
	Bronchiectasis (N=133)	**Control (N=59)**	
Microtubular defects	105 (80.2%)	32 (54.2%)	**<0.001**
Extra peripheral microtubule	76 (58.0%)	25 (42.4%)	0.05
"9+4"	6 (4.6%)	1 (1.7%)	0.44
"8+2"	36 (27.5%)	8 (13.6%)	**0.04**
"8½+2"	47 (35.9%)	18 (30.5%)	0.47
"9+1"	11 (8.4%)	1 (1.7%)	0.11
"9+0"	8 (6.1%)	1 (1.7%)	0.28
Absence of outer dynein arm	4 (3.1%)	0 (0%)	0.31
Compound cilium	97 (74%)	25 (42.4%)	**<0.001**
Disarrangement of microtubules	51 (38.9%)	21 (35.6%)	0.66

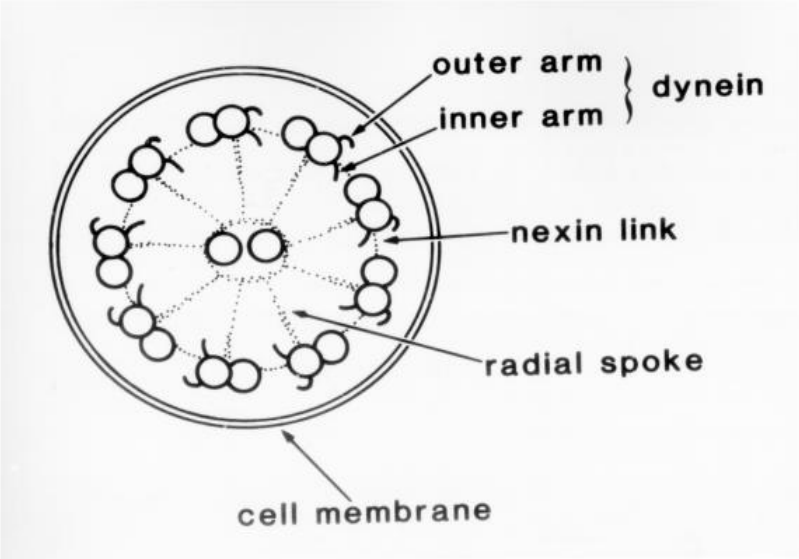
*p value when compared the two subject groups.
Refer to methodology section for description of ciliary ultrastructural defects.

Table 3 Relationship between central ciliary microtubular orientation, and demographic and clinical parameters of disease severity in patients with steady state bronchiectasis

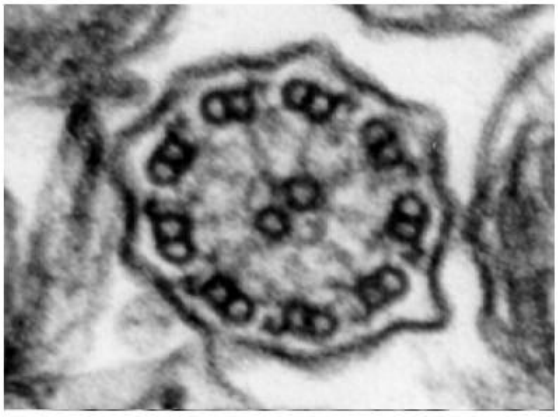
	Central microtubular orientation angle	
	Bronchiectasis patients r (p)	**Control r (p)**
Age of onset of disease (yr)	0.04 (0.75)	
Age at presentation (yr)	-0.09 (0.32)	
24h volume (ml)	-0.08 (0.39)	
Exacerbation frequency (per year)	0.14 (0.13)	
FEV_1 % predicted	0.13 (0.15)	
FVC % predicted	0.15 (0.10)	
No. of bronchiectatic lung lobes	-0.03 (0.72)	
Ciliary beat frequency (Hz)	-0.19 (**0.03**)	-0.25 (0.06)
No. of ultrastructural defects	0.11 (0.21)	0.02 (0.91)
% of cells with ultrastructural defects	0.19 (**0.03**)	0.06 (0.63)
% of cells with microtubular defects	0.26 (**0.003**)	0.13 (0.31)

Data shown are Spearman rank correlation coefficients and p values.
Values with $p < 0.05$ are in bold.

Figure 1


outer arm
inner arm
dynein
nexin link
radial spoke
cell membrane

A



B

Figure 2

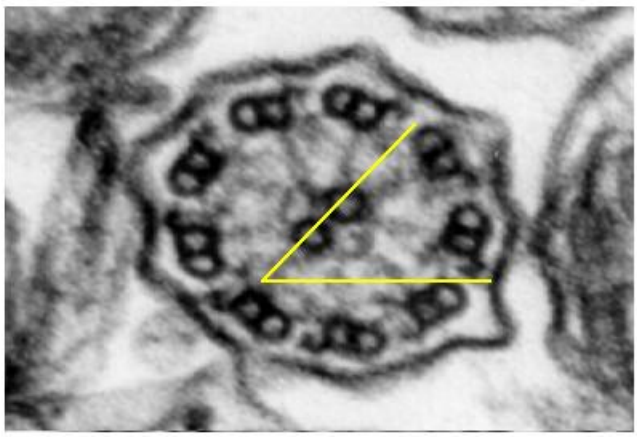
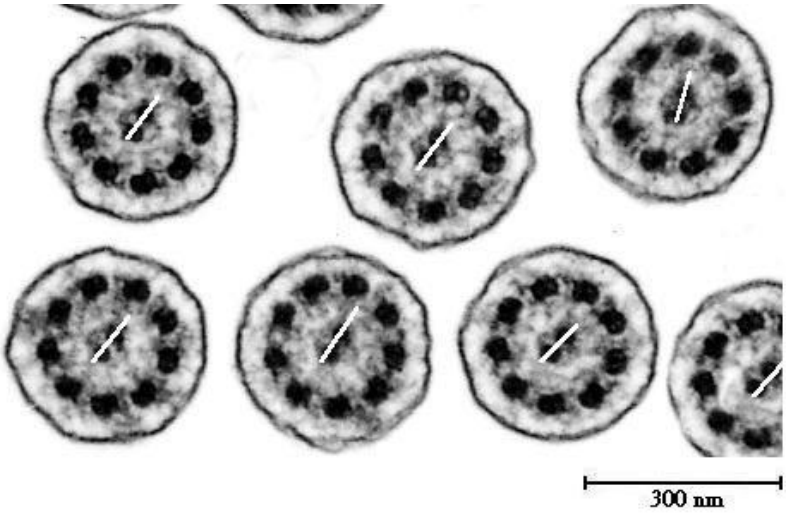
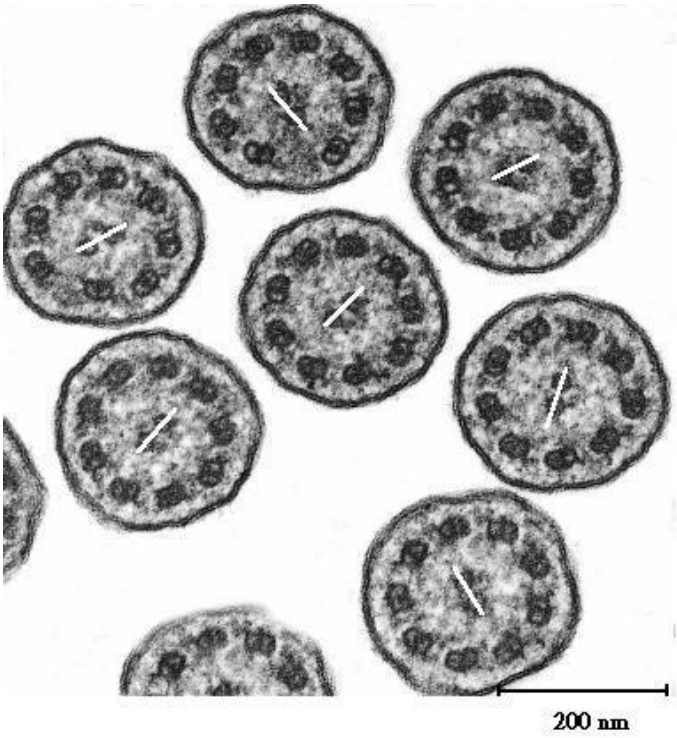


Figure 3



A



B