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### This is the author's manuscript

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/145448> since

*Published version:*

DOI:10.1007/s00414-012-0682-x

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## UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:  
Questa è la versione dell'autore dell'opera:

Int J Legal Med (2012) 126: Issue 3, pp 427–433

DOI 10.1007/s00414-012-0682-x

The definitive version is available at:  
La versione definitiva è disponibile alla URL

[<http://link.springer.com/article/10.1007%2Fs00414-012-0682-x>]

# International Journal of Legal Medicine

## Nasal Ciliary Motility; new tool in estimating the time of death

–Manuscript Draft–

<b>Manuscript Number:</b>	IJLM-D-11-00256R2
<b>Full Title:</b>	Nasal Ciliary Motility; new tool in estimating the time of death
<b>Article Type:</b>	Original Article
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<b>Abstract:</b>	<p>Determination of time since death is one of the most difficult and crucial issue in forensic medicine. Apart from body cooling, which is widespread used in the early post mortem interval (PMI), supravital reactions are the most interesting post-mortem changes for time of death estimation. Nasal ciliary motility has been occasionally observed in post-mortem period although no studies have focused on this phenomenon for forensic purposes. We aimed to evaluate the diagnostic usefulness of ciliary motility as a potential tool in estimating the time of death. Specimens of ciliated epithelium from 100 consecutive cadavers were obtained by scraping the nasal mucosa at three different post-mortem intervals. The samples were then smeared on a slide and an in vitro evaluation of ciliary movement was analyzed by phase-contrast microscopy. A post-mortem nasal ciliary motility was observed and a statistically significant relationship between decreasing ciliary movements and increasing post-mortem interval was detected even in presence of putrefactive changes of nasal ultrastructure integrity. Some peculiar causes of death seem to influence ciliary motility in the early PMI, while no significant correlations with sex or age were observed. According to the results of this study, post-mortem evaluation of nasal ciliary motility may bona fide be a feasible option for estimating the time of death.</p>

## Introduction

The estimation of the time since death (PMI, post mortem interval) is one of the most important points of debate in forensic practice since knowledge of PMI is essential in criminal investigation. Several studies have proposed diverse procedures for PMI determination but the election of the state-of-the-art procedure is still ongoing [1-2].

Henßge developed a nomogram, which is based on measurement of the body core temperature and also considered various parameters of environmental conditions (temperature, humidity, clothing, etc). This method is now well recognized and in widespread use in the early post-mortem period [3-14].

Except for rectal temperature measurement, many studies have reported physiochemical approaches for PMI estimations. Supravital reactions of tissues are considered the practical most efficient post-mortem changes giving information on the time of death [15].

The irreversible circulatory arrest is the starting point for a period of survival of some tissues under a condition of global ischemia. The basis of such phenomenon is the anaerobic glycolytic metabolism, which tends to decrease inconstantly in each individual in the first 24 hours [15]. For practical purposes in forensic medicine most investigations on supravitality have been carried out on different morphological and functional levels: organ systems; isolated organs; tissues; cells.

Nasal mucosa consists of an epithelium resting on a thin basal membrane that separates it from the tunica propria. The pseudostratified columnar epithelium is composed of ciliated and non-ciliated columnar cells. The latter are also called brush border or striate cells, muciparous globet cells and basal cells. All these cells are closely interconnected through desmoidal and hemidesmoidal junction systems. The ciliated cell is the most differentiated and the most numerous cell type, accounting for about 80% of the cells making up the nasal mucosa. The ratio between ciliated and nonciliated cells is 5:1 and increases proceeding distally toward the lower airways where it peaks at 100-200:1. Ciliated cells are elongated polygons, 15-20  $\mu$  high, with nuclei located at various heights above the basal membrane (Figures 1-2).

The top surface is composed of about 100-250 cilia and about 300 microvilli. The cilia grasp a mucous gel layer (an important nonspecific defense system of the upper airways that



decontaminates inhaled air) with their tips toward the nasopharynx where it is eliminated [16-17].

Nasal scraping is the best method for obtaining a representative sample of nasal mucosa cells. It can be performed in clinical practice as a non-invasive procedure that causes the patient minor discomfort and it is used in diagnosing primary and secondary ciliary dyskinesia, rare autosomal syndromes and allergic rhinosinusitis. Usually the cilia propel the gel layer in rhythmic movements at a mean frequency of about 800 beats per minute. Ciliary motility is determined by microtubules scroll and is characterized by two different movement types: a rapid one called "propulsive movement" and a slower one called "return movement". Cilia flex synchronously with a frequency of 600-1000 beats per minute, moving in the same direction, but with a slight phase difference defined "*metachronous movement*" [16].

A previous study conducted by Lee [18] on two cases showed normal ultrastructure integrity of the nasal cells that was maintained for at least 15 hours post-mortem. Moreover, normal motility function was occasionally observed for as long as 18 hours following death, thus pointing to ciliary motility as a clear sign of supravital reaction.

On these bases, in order to improve the reliability of the estimated PMI for forensic purposes, this work aims to study the variation of nasal ciliary motility in the early post-mortem intervals.

## **Material and Methods**

### **Study population and procedures**

We prospectively studied 100 consecutive patients who died at University Hospital of Bari, from March to November 2010. In all cases brain death was legally certified and then cadavers were admitted at the mortuary of local department of Legal Medicine within 2 hours after death, waiting for autopsy or funeral. The bodies laid undressed on mortuary tables; local temperature varied from 17-18° C (March-April, October-November) to 22-26° C (May-September).

The age ranged from 23 to 98 years (average 71,7); the group was made up of 62 males and 38 females.

All pre-mortem information about the cause of death, including the medical treatments performed, were available in the Hospital file (Table 1).

The time elapsed since death was known in all cases.

### **Methods and procedures**

A specimen of ciliated epithelium was obtained by scraping the external portion of nasal mucosa with a spoon-shaped nasal probe (Rhinoprobe). Sampling sites were the inferior turbinate (central portion) or the middle turbinate. The samples were carried out in all cases at three different post-mortem intervals ranging from 4 to 30 hours: T1 (4-6 hr, mean 5), T2 (10-12 hr, mean 11) and T3 (>16 hr, mean average of 25 h, maximum 30 hours). The material picked up was then spread uniformly in the centre of a slide and, after adding 1-2 drops of physiological solution at 36 °C, it was covered by coverglass. After that, an in vitro evaluation of ciliary movement was performed: slides were analyzed by phase-contrast microscopy with 100x objective lens in immersion. About 20 fields were observed for each slide and, once ciliary motility was recognized in some cells, those elements with a more active motility were counted and studied as time passed. Ciliary beat frequency (CBF) was recorded and classified as: present (3-4 beats/sec), hypo-valid (1-2 beats/sec) and absent. We also compared the ciliary beat frequencies with the causes of death, age and sex.

Cases with bleeding nose or with tampons in nasal choanae were excluded from the study because scraping was not possible.

### **Statistical analysis**

The comparison between multiple groups was assessed using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test as post-hoc. All statistical calculations were performed with Excel (Office XP, Microsoft, Seattle, WA, USA) and Graph PadPrism Software (version 5.01, Graph Pad Software, San Diego, CA, USA). P value is considered significant with  $P < 0.05$ .



## **Results**

### **Mucociliary motility and time of death**

Nasal mucosa samples were obtained in all cases examined. Median time between death and scraping was 5 hr at T1 (interval range between 4 and 6 hr), 11 hr at T2 (interval range between 10 and 12 hr) and 25 hr at T3 (interval range between 16 and 30 hr). A comparison of ciliary motility outcome in the three different post-mortem intervals was observed (Figure 3).

In eighty cases in which samples were collected at T1, microscopic observation provided images of ciliary movements: 3-4/sec CBF in sixty-four cases (64%) and 1-2/sec CBF in sixteen (16%). Ciliary activity could not be noted in the remaining twenty cases (20%).

Furthermore, in all these twenty samples in which ciliary motility was absent, microscopic observation and Hospital file analysis provided additional information: six cases (30%) showed bacterial contamination of the sample, four (20%) mycotic contamination, eight (40%) had medical records positive for chemotherapy and two (10%) were negative for all the previous parameters.

Information about ciliary motility was obtained also during T2. Thirty-eight cases (38%) showed activity at 3-4/sec CBF, sixteen (16%) at 1-2/sec CBF, whereas the remaining forty-six samples (46%) didn't show any ciliary movements.

The analysis of the cases with absent motility at T2 showed that at T1 twenty cases (44%) scored negative too, fourteen cases (30%) showed 3-4/sec CBF and twelve cases (26%) 1-2/sec CBF (Figure 4).

When the observation period was longer than 16 hours (T3), ciliary motility at 1-2 CBF was noted in only seventeen (17%) of 100 cases, whereas all of the samples obtained from the remaining eighty-three cases (83%) showed absent activity. Results showed that the seventeen cases with present ciliary movements corresponded to samples obtained when the post-mortem interval was less than 21 hours and that showed 3-4/sec CBF at T1 and T2. The samples with absent motility were detected variably in a range between 16 and 30 hours.

The analysis of ciliary motility outcome at T2 of the cases with absent CBF at T3 revealed that twenty-one cases (25%) showed 3-4/sec CBF at T2, sixteen (20%) 1-2/sec CBF and forty-six (55%) absent movements (Figure 5).

The progressive observation of ciliary motility after death is indicative for a “natural” decrement of ciliary activity with 3-4/sec CBF higher at T1 than at T2 /T3; 0/sec beat frequency rises with growing post-mortem interval. The rate of hypo-valid motility is nearly constant with time. This trend can be explained just observing that with increasing postmortem interval some cases decrease in motility; therefore we can speculate that some of the cases with 3-4/sec CBF in the previous interval show 1-2/sec CBF in the following one as well those with 1-2/sec show absent motility in the subsequent interval.

One-way analysis of variance (Anova) followed by Newman-Keuls showed that ciliary motility rate was significantly lower at T3 than at T1/T2 ( $p < 0,0001$ ), suggesting that the post-mortem interval was relevant in ciliary movements decrement (Figure 6).

#### **Correlation between ciliary motility and age**

Linear regression did not show a significant difference in ciliary activity rate for all the three post-mortem intervals between young and old patients (elderly patients presented 3-4/sec CBF as well as the younger ones).

Figure 7 represents ciliary motility at T1 in different age intervals, but data cannot be considered statistically valid because the number of cases for age group is not comparable (Figure 7).

#### **Correlation between ciliary motility and sex**

We performed student's t-test and we found no significant changes between males and females in terms of mucociliary motility ( $p < 0,0892$ ).

#### **Correlation between ciliary motility and cause of death**

With regard to the cause of death, ciliary motility absence was frequently noted in cases with cancer, septic shock and multiple organ failure (MOF), suggesting that infections and chemotherapy could play a role in ciliary movement impairment (Figure 8).



## Discussion

Hilding [19] has noted ciliary activity in the lower respiratory tract that continues 18 hours postmortem and is prolonged after storage. Earlier publication [18] has shown the evidence of post-mortem ciliary movements that were occasionally observed in only two cases and more than 18 hours after death.

In this study, we firstly confirmed that nasal ciliary motility continues after death and then we observed it tends to decrease with growing post mortem interval. Here for the first time we analysed the progression of such phenomenon in the “early post-mortem period” (usually defined as the time between death and the appearance of generalized putrefaction) and the results obtained raise the intriguing possibility to use the evaluation of such supravital reaction in estimating the time of death for forensic purposes.

The medico-legal evaluation of the time interval since death given at an early stage is of a paramount importance to the criminal investigators.

Several researchers have stressed the importance of supravitality reactions in comparison with body cooling measurement because are not influenced by environmental factors.

Most of these investigations have been carried out on skeletal muscle or smooth muscle (iris, arteries) and evaluate the post-mortem duration of excitability after electrical or pharmacological stimulation [20-22]. The Zsako's phenomenon is a mechanical excitation of muscle that can be seen in the first 2-3 hours post mortem while idiomuscular contraction can be observed in the following hours [23-24].

Postmortem transport of gastric content and spermatozoa viability are claimed to be spontaneous supravital activity even if definitive results regarding the time of death identification are still missing [25-27].

Thus death time estimation using supravital reactions is considered useful and valuable from a theoretical viewpoint although rarely evaluated in everyday forensic practice.

The reason is that most of these techniques, as well as new methods based on biochemical markers or other parameters [28-33], require some type of biopsy or collected biological fluids and laboratory tests. Moreover there is the possibility of altering the crime scene.

Finally, they are also time-consuming and cannot be performed *in situ* (except few of them), thus resulting in a delayed and less efficient investigation.

In our study we investigated 100 cases in three post mortem intervals (T1-T3) within 30 hours after death. Generally speaking 6, 12 and more than 16 hours after death the evaluation of body cooling, that is considered the most scientific sign in this field of evidence, shows an increasing range (of error) in time of death estimation. Our findings indicate that a statistical significant decrease of ciliary motility comparing with time elapsed since death exists. We observed that 3-4/sec CBF rate is higher at T1 than at T2 /T3, moreover 0/sec CBF is correlated with growing post-mortem interval. The rate of hypo-valid motility is nearly constant with time. Regarding this observation we can speculate that some of the cases with higher CBF in the previous interval show a decrease of motility frequency in the following one since showing absent activity if the previous CBF was 1-2/sec. In the present study we then show that after 16 hours in the early postmortem interval there is a great probability to find absence of ciliary movements. Future studies might also include the observation of nasal ultrastructure integrity breakdown as well as the internal temperature measurement to better define the role of absence of ciliary motility in the definition of time death interval.

It is well known that cilia contain a central microtubular core called 'axoneme' that forms a "9+2" arrangement where nine peripheral microtubule doublets (Figure 9).

These cilia beat in a rhythmic manner to propel mucus; the ATPase activity of the dynein arms generates the force required for ciliary beating and bending [34-35]. On these bases we can speculate that spontaneous ciliary activity after death depends on breakdown of ATP that influence the duration of this supravital phenomenon. Our present study indicates that after 16-18 hours most of energetic metabolism of cilia is ending even if the reason why normal ciliary motility in specific cases is maintained till 30 hours is not known and deserves future investigations.

Lee [18] analysed post-mortem respiratory ciliary streaming and ultrastructure after placing epithelial samples of inferior turbinate in Krebs-Henseleit solution and then these specimens stored at 4°C for as long as 60 hours after collection.

We obtained nasal samples from fresh cadavers that were observed three times at least within 16 hours post mortem.



In living people it is well recognized that measurement of Ciliary Beat Frequency may be affected by environmental factors such as PH and temperature [36-37]. Our results seem to show no difference in comparison with the season in which the samples were picked up. These data are obtained, indeed, just by observing the difference existing between the cases analyzed in June rather than in November, where morgue temperatures were evaluated in T1.

No significant correlation was observed between ciliary motility and sex; we can approximately exclude a decreasing activity in older people even if this data has not statistical relevance.

According to international literature, reduction or absence of ciliary function are observed in congenital disease such as primary ciliary dyskinesia that is commonly associated with chronic sinusitis, bronchiectasis, sinus hypoplasia, secretory otitis media [38]. Other causes of ciliary beat frequency modification are polycystic hepatic and renal disease, allergic rhinosinusitis and bacterial or fungal infections [36].

None of these diseases were specifically mentioned in the Hospital file record of the sample studied. Nevertheless, we noted an increasing absence of ciliary motility in people died from cancer, sepsis and multiple organ failure. This data suggested the possible role of immune system in regulating such phenomenon even if further data are requested.

Our data support the idea that ciliary motility analysis may be a useful tool in evaluating the time of death in the early post-mortem period and provide the impetus to study in the future this phenomenon in a large group of patients to better define the relationship between cause of death and time-lag presence of nasociliary motility. Further studies should indeed be undertaken to evaluate the effects of environmental factors (e.g. hypothermia), voluptuary factors (smoking, sniffing) and natural disease on ciliary beat activities.

Overall, in comparison with other supravital phenomena, nasal scraping is a very easy technique by which with a rhinoprobe is possible to keep a large part of ciliated epithelium. This method is non invasive, inexpensive and with a basic microscope is possible to observe the supravital reaction shortly after scraping without delay that is normally required to analyze other markers.



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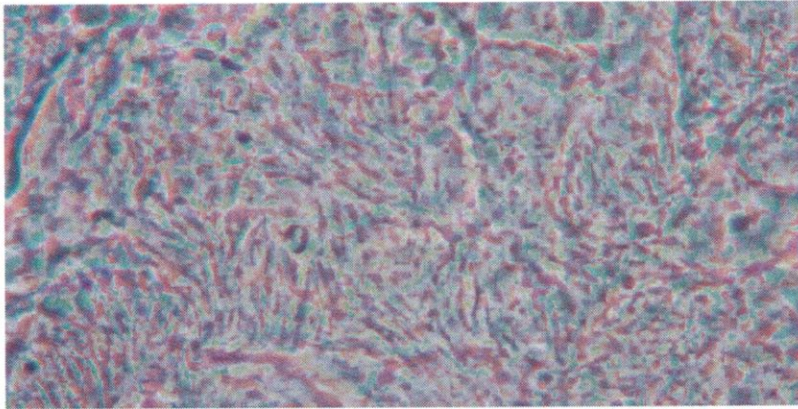


Fig.1 Sample of pseudostratified columnar epithelium of nasal mucosa. It is composed of non-ciliated and ciliated columnar cells. The ciliated cell is the most differentiated and the most numerous cell type, accounting for about 80% of the cells making up the nasal mucosa (5:1 ratio between ciliated and nonciliated cells), (phase-contrast microscopy, 40x).

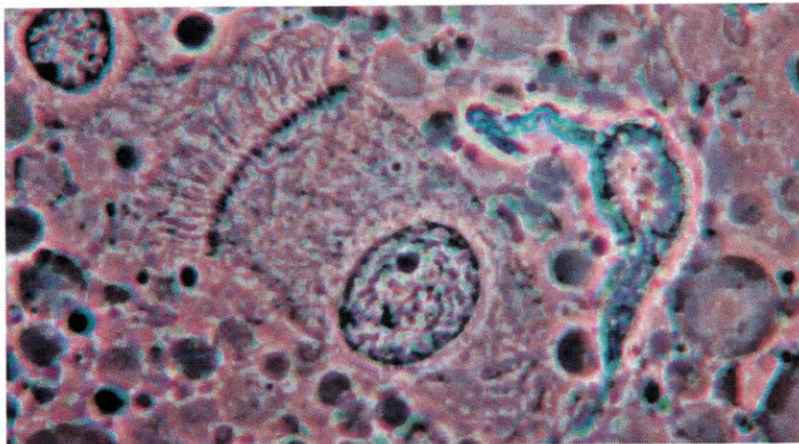


Fig.2 Ciliated cell of nasal mucosa. Ciliated cells are elongated polygons, 15-20  $\mu$  high, with nuclei located at various heights above the basal membrane. The ciliated cell is divided in three regions: apex with ciliary apparatus, body and tail, (phase-contrast microscopy, 100x in immersion).

	N (%)	Male/ Female	Age (years) range
<i>Natural deaths</i>			
Cancer	23 (23)	15/8	25-84
Heart failure	14 (14)	7/7	38-98
Heart attack	15 (15)	11/4	28-97
Hepatic disease	3 (3)	1/2	52-70
Cerebral ictus	4 (4)	2/2	76-81
Septic shock	15 (15)	8/7	43-91
Ruptured aortic aneurysm	4 (4)	4/0	38-84
MOF	10 (10)	7/3	23-83
Respiratory failure	9 (9)	5/4	59-90
<i>Traumatic deaths</i>			
Burns	3 (3)	2/1	30-81

**Table 1.** The causes of death.



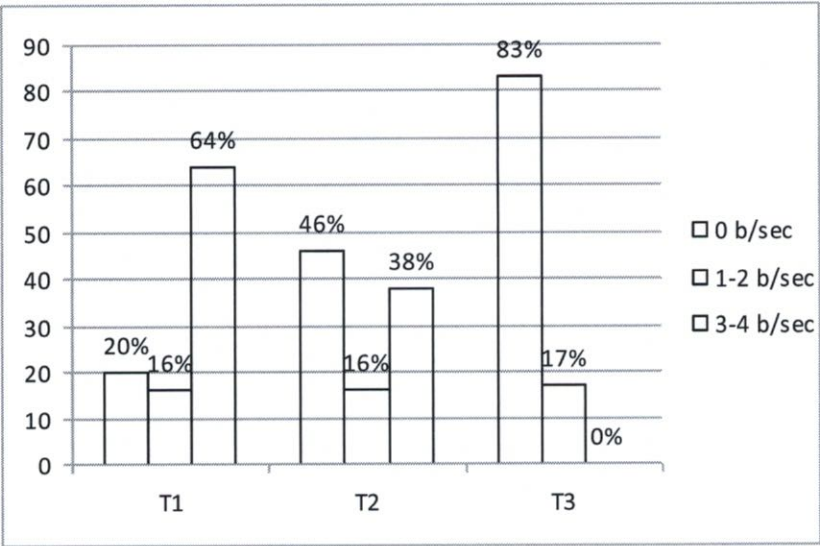


Fig.3 Ciliary Motility observed at T1, T2, and T3.

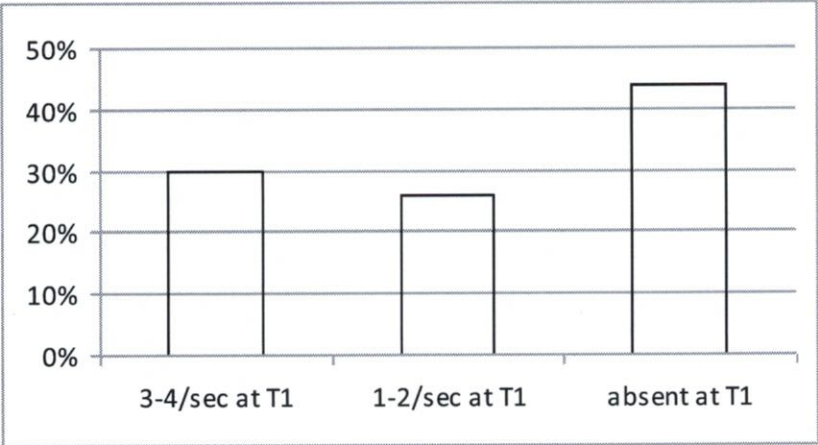


Fig.4 Ciliary motility at T1 of the cases with absent CBF at T2.

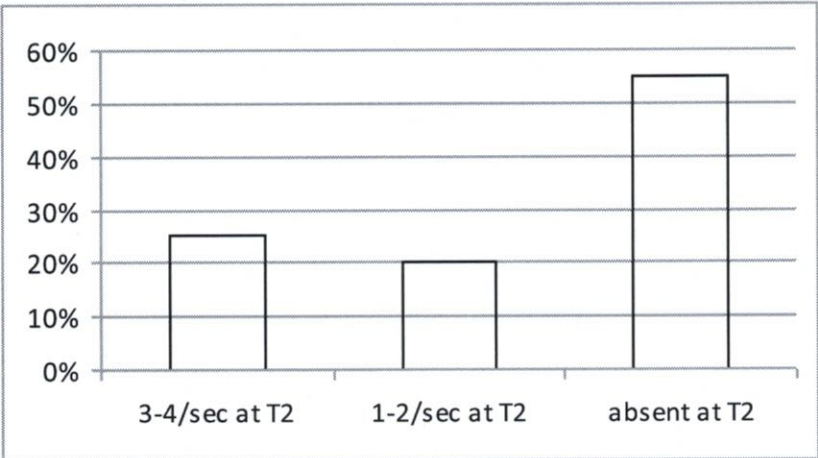


Fig.5 Ciliary motility at T2 of the cases with absent CBF at T3.



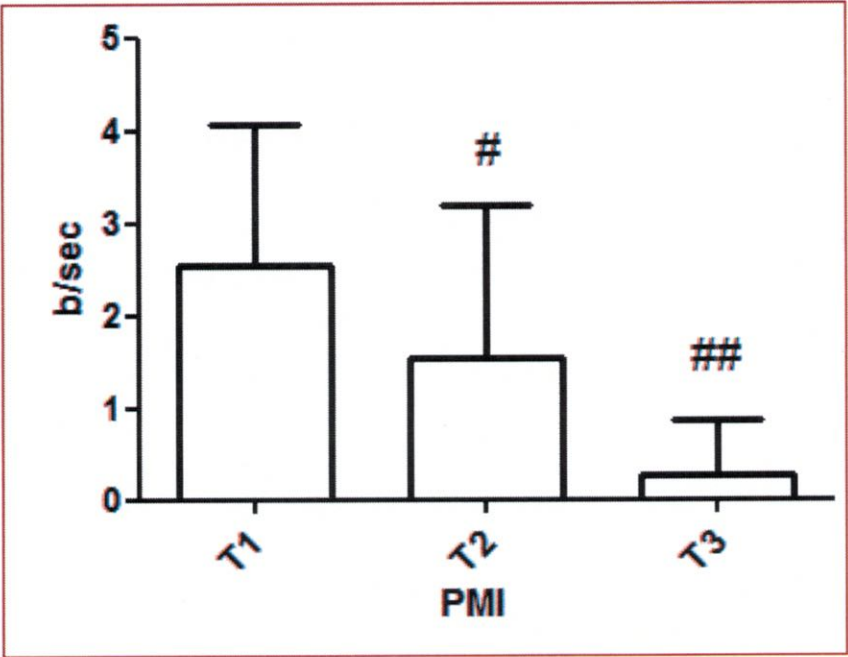


Fig.6 Ciliary beat frequency at T1, T2 and T3; at T1 the majority of cases shows 3-4 b/sec CBF more than in the other intervals with a constant decrement of motility rate ( $P < 0.0001$ ). # indicates significant difference vs T1; ## indicates significant difference vs T1 and T2 (ANOVA followed by Newman-Keuls).

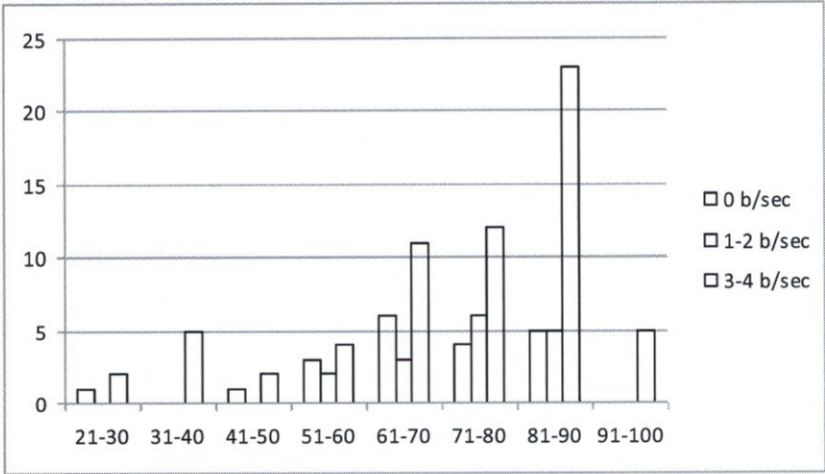


Fig.7 Ciliary motility distribution at different age groups.

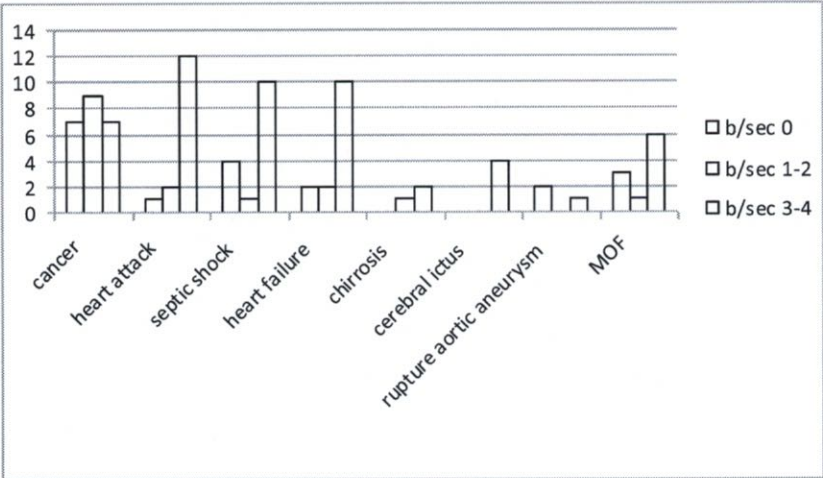


Fig.8 Ciliary motility distribution compared with different cause of death groups.



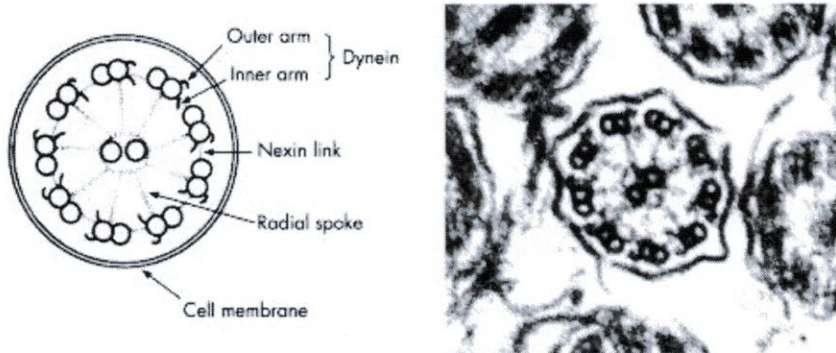


Fig.9 Ciliary ultrastructure: cilia are composed of an array of nine double microtubules arranged in a peripheral ring surrounding a central pair (9+2), (schematic diagram and electron microscopy, 50000x).