Comparison of ciliary beat frequencies at different temperatures in young adults

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ABSTRACT

Rationale: Direct visualisation of ciliary beat pattern (CBP) and ciliary beat frequency (CBF) has been recommended as the first-line diagnostic test in patients suspected of having primary ciliary dyskinesia (PCD). However, the test procedure is not yet completely standardised, and centres measure the CBF at different temperatures.

Objectives: It was the aim of the study to compare CBF at different temperatures, to establish normative values, to check for age dependency and to measure the temperature on the nasal mucosa of the participants.

Methods: High-speed video-microscopy analysis with a Sisson-Ammons Video Analysis (SAVA) system was used to determine CBP and CBF in the participants.

Measurements: Nasal brushings were taken and CBF was measured in randomised order at three temperatures: 25°C, 32°C and 37°C.

Main results: In total, 100 healthy young adults (74 female, 26 male), aged 20.2–31.9 years, were included in the study. We found a highly significant difference among the groups: the median CBF was 7.0 Hz at 25°C, 7.6 Hz at 32°C and 8.0 Hz at 37°C. The maximum time period ex vivo was 65 min and did not differ significantly. However, CBF was significantly higher when the cilia were kept at a higher temperature before the measurements were made. We found no correlation between CBF and the age of the participants. The median nasal mucosal temperature in our study participants was 30.2°C (range 24.7–35.8°C) comparable to the 30.2–34.4°C described in the literature.

Conclusions: The most appropriate temperature at which to measure CBF is 32°C. In our study, with 95% confidence for this temperature the CBF was between 6.3 and 9.0 Hz.

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Equivalent to the nasal mucosa, the most appropriate temperature to measure ciliary beat frequency is 32°C https://bit.ly/2GCr2fP

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Introduction

Primary ciliary dyskinesia (PCD) is a heterogeneous group of morphological and functional abnormalities in cilia that can lead to neonatal respiratory distress, recurrent upper or lower respiratory tract disease and male infertility [1]. In almost 50% of cases dextrocardia is present, and this together with chronic sinusitis and bronchiectasis was originally described as Kartagener’s syndrome [2]. PCD is usually considered to be an autosomal recessive genetic disorder. Homozygous or compound heterozygous mutations in >44 genes are known to be causative for PCD [3] and correlate with the ciliary beat pattern (CBP) variations [4]. It has been shown that the ultrastructural phenotype has an influence on lung function and body mass index of the patients [5]. The reported prevalence varies widely from 1:2000 to 1:40,000 [6]. It is generally accepted that PCD is underdiagnosed and that the diagnosis is made too late [7]. In a longitudinal study over 7 years adult patients with late diagnosis showed impaired forced expiratory volume in 1 s (FEV1) and increased Pseudomonas aeruginosa colonisation [8–10]. There is no single gold standard test available to diagnose PCD [11, 12]. Appropriate tests include nasal NO measurement, high-speed video-microscopy analysis (HVMA), transmission electron microscopy or immunofluorescence microscopy. Nowadays genotyping of the most common mutations can additionally be used as a diagnostic tool [4, 13]. Direct visualisation of CBP and ciliary beat frequency (CBF) by HVMA has been recommended as the first-line diagnostic test in patients suspected of having PCD [12]. The accuracy of HVMA has recently been proven [14]. However, ciliary function varies under differing conditions such as temperature and pH [12]. The physiological nasal mucosal temperature has been shown to range from 30.2°C to 34.4°C [15]. Some centres measure CBF at 37°C [16–19] and others at lower temperatures, usually at 25°C [4, 20–22]. Therefore, centres need to define their own normative data until a consensus is reached to allow standardisation of methods and reporting between centres [12]. So far, no study has compared CBF at different temperatures. A correlation between CBF and age of the individuals has been discussed controversially. It was the aim of the study to compare CBF at the three temperatures 25°C, 32°C and 37°C to establish normative values for these temperatures and check for age dependency. Due to restrictions of the ethics committee, the study could only be performed in adults and not in healthy children. In the same individuals we also intended to measure the temperature on the nasal mucosa noninvasely to determine the optimal temperature for analysis of CBF.

Study subjects

For ethical reasons it was not possible to include healthy children in the study. We asked healthy adult students without lung disease, allergy or acute infection to take part. All students who showed interest filled in a questionnaire and were interviewed on their medical history. Exclusion criteria were asthma or asthma symptoms, hay fever, eczema, exercise limitation, smoking or any medication that might have an influence on lung function or mucociliary clearance. Students who met no exclusion criteria were considered “lung healthy”. In dubious cases lung function testing was offered. The healthy volunteers eligible for the study were informed verbally and in writing about the trial. Informed consent was obtained from all participants. The participants were free to withdraw from the study at any time. Approval from the ethics committee was obtained before the start of the study. The declaration of Helsinki was always followed.

Samples were only taken in individuals who were not receiving nasal steroids or decongestants, and who did not show signs of acute respiratory infection for at least 3 weeks in order to minimise secondary ciliary dyskinesia.

Study design

CBP was assessed and CBF was measured in every participant at three temperatures in randomised order: in participants with an uneven birth date in ascending order first at 25°C, then at 32°C and finally at 37°C; in participants with an even birth date in descending order first at 37°C, then at 32°C and finally at 25°C. The time interval from nasal brushing to measuring CBF was recorded for the different temperatures.

Methods

CBP and frequency

Ciliated respiratory epithelial cells were obtained from the participants by nasal brushing as previously described [3]. The material was suspended in RPMI 1640 medium and stored in a water bath preheated to the required temperatures. Microscopy was carried out using a Nikon microscope solution system with an inverted phase-contrast microscope ECLIPSE Ts2R-FL, CFI Super Plan Fluor ELWD ADM 40x C/0.60/3.6 objective and TPX-TS2R temperature control plate (NIKON GMBH Microscope Solutions, Düsseldorf, Germany).

CBP and CBF were evaluated according to the published recommendations [4, 23] using a Sisson-Ammons Video Analysis (SAVA) system [24]. Videos of beating cilia were recorded using a Basler
acA1300–200 µm USB3 video camera with 640×480-pixel resolution and a frame rate of 120 frames per second (Basler AG, Ahrensburg, Germany). CBP was evaluated in real time and in slow motion and considered normal if cilia showed regular forward and recovery strokes and were not disrupted, static, almost static with minimal movements, stiff or beating with reduced amplitude or abnormal circular pattern [4]. Videos with ciliated cell bundles were accepted for analysis while videos of disrupted cells or single ciliated cells were considered to be of insufficient quality and excluded from the analysis [23].

CBF was measured at five different ciliated cell bundles and two different regions of interest for every ciliated cell bundle. To ensure reliable results the measurements were only accepted if the difference between the two regions of interest did not exceed 10% and if the difference among the different ciliated cell bundles did not exceed 20%. The mean of the two regions of interest was taken first before the mean from both measurements for every video was calculated as CBF. Whole field analysis was not performed to exclude the influence of outlying measurements. In this way five videos respectively ten measurements were taken into analysis of each individual.

Nasal mucosal temperature
Participants were enrolled in the study throughout the year. They had time to acclimatise to the room temperature of 20 to 23°C for ∼30 min while filling in the questionnaire. The nasal mucosal temperature was measured before nasal brushing using a contactless infrared thermometer Geratherm Contact GT-101 (Geratherm Medical AG, Geschwenda, Germany). The object temperature measurement mode was selected, and the temperature of the nasal mucosa was measured twice on both sides from a distance of 4–5 cm. The mean temperature from each side and the mean temperature of both sides were calculated.

Analysis
Data were analysed with GraphPad Prism version 6.0.7 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). To test for deviations from the “normal” Gaussian distribution the Kolmogorov–Smirnov test was used. In not normally distributed data the nonparametric Wilcoxon test was used to compare matched pairs. The Mann–Whitney test was used to compare unpaired data. Correlation coefficients were determined using the nonparametric Spearman regression analysis. Percentiles were calculated according to the method described by HYNDMAN and YANAN FAN [25]. Differences associated with probabilities p<0.05 were considered significant.

Results
Participant characteristics
In total, 100 healthy young adults (74 females and 26 males) were included in the study. The age was not normally distributed (Kolmogorov–Smirnov normality test: p=0.005). The median age was 23.5 years (range 20.2–31.9), and the mean age was 23.9 years (sd 2.7 years).

Participant subgroups
The participants were randomised depending on their birth date to measure CBF at the three temperatures in ascending order or in descending disorder. In 48 participants with uneven birth dates (35 females and 13 males) CBF was measured in ascending temperature order from 25°C, 32°C to 37°C. In 52 participants with even birth dates (39 females and 13 males) CBF was measured in descending temperature order from 37°C, 32°C to 25°C.

The age was not normally distributed in the two groups (Kolmogorov–Smirnov normality test: p=0.001 in the group with uneven birth dates, p=0.039 in the subgroup with even birth date). The age did not differ significantly between the two groups. The median age was 23.4 years (range 20.3–31.0), the mean age was 23.7 years (sd 2.5) in the subgroup with uneven birth dates compared with the median of 23.6 years (range 20.2–31.9), mean 24.1 years (sd 2.8), in the subgroup with even birth dates (Mann–Whitney test p=0.454).

Ciliary beat pattern
CBP showed regular forward and recovery strokes in all participants. No obvious change in beat pattern was observed at the different temperatures.

CBF at different temperatures
CBF measured at the different temperatures was not normally distributed (Kolmogorov–Smirnov normality test for 25°C: p<0.001; 32°C: p=0.036; 37°C: p<0.01). The median CBF at 25°C was 7.0 Hz (range 6.2–9.6), mean 7.2 Hz (sd 0.6). The median CBF at 32°C was 7.6 Hz (range 5.8–9.1), mean 7.6 Hz (sd 0.6). The median CBF at 37°C was 8.0 Hz (range 6.5–9.8), mean 8.1 Hz (sd 0.7). There was a highly significant difference among the three groups (figure 1).
Normal range of CBF

Since CBF was not normally distributed, the "normal range" defined as 95% confidence interval was calculated as the interval between the 2.5th percentile and the 97.5th percentile according to HYNDMAN and YANAN FAN [25]. The results are shown in table 1.

CBF in the subgroups

CBF measured at the three temperatures 25°C, 32°C and 37°C in ascending order (↑) were not normally distributed (Kolmogorov–Smirnov normality test 25°C: p=0.001, 32°C: p=0.049, 37°C: p=0.031), while the subgroups in descending order (↓) passed the normality test (Kolmogorov–Smirnov normality test 25°C: p=0.084, 32°C: p=0.10, 37°C: p=0.10). The median CBF at 25°C↑ was 6.9 Hz (range 6.2–9.6), mean 7.0 Hz (SD 0.6) versus median CBF at 25°C↓ of 7.2 Hz (range 6.4–9.1), mean 7.4 Hz (SD 0.6). The median CBF at 32°C↑ was 7.5 Hz (range 5.8–8.9), mean 7.5 Hz (SD 0.5) versus median CBF at 32°C↓ of 7.7 Hz (range 6.2–9.1), mean 7.8 Hz (SD 0.7). The median CBF at 37°C↑ was 7.8 Hz (range 6.5–8.9), mean 7.8 Hz (SD 0.5) versus median CBF at 37°C↓ of 8.3 Hz (range 6.9–9.8), mean 8.4 Hz (SD 0.7). The differences between the corresponding subgroups were significant (figure 2).

Time periods ex vivo

Due to the study design the time intervals from nasal brushing to the start of measuring CBF at the three different temperatures, i.e. the time periods ex vivo, were not normally distributed (Kolmogorov–Smirnov normality test for 25°C: p<0.0001, 32°C: p<0.001, 37°C: p<0.0001). The median time period ex vivo at 25°C was 38.5 min (range 1.0–63.0), mean 25.5 min (SD 22.6). The median time period ex vivo at 32°C was 24.0 min (range 15.0–38.0), mean 24.5 min (SD 4.2 min). The median time period ex vivo at 37°C was 4.0 min (range 1.0–65.0), mean 23.6 min (SD 22.8). The time periods ex vivo did not differ significantly among the three temperatures (Wilcoxon matched pairs test 25°C versus 32°C: p=0.916; 25°C versus 37°C: p=0.763, 32°C versus 37°C: p=0.500).

Time periods ex vivo in the subgroups

The time periods ex vivo to measure CBF at the three temperatures 25°C, 32°C and 37°C in ascending order (↑) or descending order (↓) were not normally distributed except for 37°C↑ (Kolmogorov–Smirnov normality test 25°C↑: p<0.0001, 25°C↓: p=0.049, 32°C↑: p=0.032, 32°C↓: p=0.023, 37°C↑: p=0.10, 37°C↓: p<0.0001).

For measuring CBF at 25°C↑ the median time ex vivo was 2.0 min (range 1.0–11.0), mean 2.6 min (SD 2.2), for 25°C↓ the median time was 46.6 min (range 35.0–63.0), mean 46.6 min (SD 6.1). For measuring CBF at 32°C↑ the median time ex vivo was 26.0 min (range 15.0–38.0), mean 26.3 min (1 SD 24.3), for

### Table 1 Normal range of ciliary beat frequencies at the different temperatures

| Temperature °C | 2.5th percentile | 97.5th percentile |
|----------------|-----------------|------------------|
| 25             | 6.3             | 8.8              |
| 32             | 6.3             | 9.0              |
| 37             | 6.7             | 9.8              |

Figure 1: Median and individual results for ciliary beat frequency (CBF) shown at the three temperatures. CBF was significantly higher when measured at higher temperatures (Wilcoxon matched pairs test for 25°C versus 32°C: p<0.0001; 25°C versus 37°C: p<0.0001; 32°C versus 37°C: p=0.0001).
32°C ↓ the median time *ex vivo* was 22.0 min (range 17.0–33.0), mean 22.9 min (SD 3.4). For measuring CBF at 37°C ↑ the median time *ex vivo* was 47.5 min (range 34.0–65.0), mean 46.8 min (SD 6.3), for 37°C ↓ the median time was 2.0 min (range 1.0–5.0), mean 2.2 min (SD 1.3).

Due to the randomisation design, the subgroup analysis for 25°C respectively 37°C revealed significantly shorter time intervals *ex vivo* when the measurement for the corresponding temperature started immediately after nasal brushing and was not performed at the end (figure 3). The time period *ex vivo* to measure CBF at 32°C was significantly shorter in the subgroup with descending order (figure 3). The time periods *ex vivo* were not significantly different between the subgroups for 25°C and 37°C when the measurements started with the respective temperature first (p=0.584) or last (p=0.715).

**Association between CBF and time period *ex vivo***

Due to the study design, the time intervals from nasal brushing to measurement of CBF at the different temperatures 25°C, 32°C and 37°C varied. The association between the time periods *ex vivo* and CBF measured at the three temperatures 25°C, 32°C and 37°C is shown in figure 4.

**Association between CBF and age**

The association between the age of the participants and CBF measured at the different temperatures 25°C, 32°C and 37°C was calculated. There was no correlation for any of the temperatures. Detailed results are shown in figure 5.

**Nasal mucosal temperature**

The nasal mucosal temperatures measured at the right and the left side were not normally distributed on both sides (Kolmogorov–Smirnov normality test right mucosa p=0.001, left mucosa p=0.015). The median temperature on the right nasal mucosa was 30.6°C (range 24.9–35.8), mean 30.0°C (SD 2.3) and the median temperature on the left side was 30.1°C (range 24.5–35.8), mean 29.8°C (SD 2.3). The difference between the right and the left side was significant (figure 6). Assessing both sides, the median temperature on the nasal mucosa was 30.2°C (range 24.7–35.8), mean 29.9°C (SD 2.3°C).
Discussion

The CBF in healthy individuals is dependent on several parameters such as pH, surrounding temperature and possibly age. In neonatal respiratory cilia the mean CBF measured at body temperature was 14 Hz and decreased by 9.1% on cooling to 32°C, respectively increased by 8.5% on warming to 40°C [26]. In an early study a linear increase in CBF between 19°C and 32°C and a plateau between 32°C and 40°C has been described [27]. In another study no significant difference in CBF was observed between nasal and tracheal cilia when comparing extreme temperatures from 5°C to 50°C [28].

Pulmonary centres usually measure ciliary function at 37°C [16–19] or at 25°C [4, 20–22], while the physiological nasal mucosal temperature on the nasal mucosa is ~30°C to 34°C [15]. In our study we compared the CBF at the three temperatures 25°C, 32°C and 37°C and established normative values for these temperatures. We were able to show a highly significant difference among the groups: the median CBF was 7.0 Hz at 25°C, 7.6 Hz at 32°C and 8.0 Hz at 37°C. Such difference might have been expected but has never been proven before. Due to a sufficient number of healthy participants, we were able to calculate 95% confidence intervals for the three temperatures. Previously published data showed higher CBF of ~13 Hz (range 11.5–14.6 Hz) at 37°C [18, 29] suggesting that CBF is not only affected by temperature. Other factors such as pH of the media, storage time and conditions, observer and the technique used to measure CBF might be involved. Even if normative ranges may still need to be centre dependent in the near future, our data not only establish normative values in our own institution but can also contribute to establish a consensus among different centres, because the influence of temperature on CBF was clearly shown in a setting in which other factors were kept constant. In such context, it might also be useful to investigate the influence of other potential factors such as pH that were not measured in this study.

It has been shown that the ex vivo lifetime of nasal ciliary cells is limited and that these cells have to be transferred to a culture medium for analysis immediately [30]. Under optimal conditions the CBF can
even increase initially during the first 3 h, followed by a plateau for up to 9 h and reduces thereafter. Therefore, measurements of CBF can be performed most reliably for up to 9 h ex vivo [30]. In our study the maximum time period from nasal brushing to measurement of the CBF was 65 min. The mean time period for the cilia to be ex vivo ranged from 23.6 to 25.5 min for the measurements at 25°C, 32°C and 37°C and was not significantly different among the three groups. However, due to the randomisation measuring in ascending or descending order, the time period ex vivo was significantly different between the corresponding subgroups. This could be expected for the corresponding subgroups at 25°C and 37°C when the measurement was started immediately after nasal brushing or was postponed to the end.

FIGURE 5 No significant correlation between age of the participants and ciliary beat frequency (CBF) measured at a) 25°C, b) 32°C and c) 37°C: 25°C, p=0.051, Spearman’s rank r=0.196, slope of the regression line +0.022; 32°C, p=0.521, Spearman’s rank r=0.065, slope of the regression line 0.000; 37°C, p=0.336, Spearman’s rank r=−0.097, slope of the regression line −0.019.

FIGURE 6 Nasal mucosal temperatures are shown as median and individual results. The temperature was significantly higher on the right mucosa compared to the left mucosa (Wilcoxon test, p=0.046).
However, for 32°C the median time interval ex vivo was only 22 min and significantly shorter in descending order compared to 26 min in ascending order. This might be explained by the fact that the heating plate had to be cooled down only by 5°C when the measurement was made in descending order and had to be heated up by 7°C in ascending order.

We also investigated the association between CBF and the time period ex vivo. For that measurements at 37°C and 32°C CBF decreased over time but surprisingly CBF increased over time at 25°C. This observation cannot be explained by the time period ex vivo. CBF was always significantly higher when the cilia were kept at a higher temperature before the measurements were made. This might indicate that CBF can be obtained ex vivo for a certain time period if cilia are kept at the required temperature but that CBF cannot recover anymore to previous levels if the cilia slowed down ex vivo due to a lower storage temperature. Such a theory would also explain the predisposition to common colds in the winter season.

In our study we found no correlation between the CBF and the age of the participants. This is in accordance with a study investigating 203 individuals between the ages of 3 months and 74 years [31]. In contrast a higher CBF of 13.6 Hz was reported in adolescents aged 10 to 19 years compared with 12.2 Hz in adults older than this [32]. Similar results were obtained in another study in 76 children and adult volunteers aged 6 months to 43 years. The mean CBF was 12.8 Hz for the paediatric population and significantly higher than 11.5 Hz for the adult group [19]. From these results the authors even established normal age-related reference ranges. However, the correlation was only weak (r = -0.30, p = 0.008). Due to the restrictions given by the ethics committee, we were only able to investigate young adults aged 21 to 32 years. As there are contradicting results published in the literature, it would be of clinical and scientific interest to investigate children in our hospital in the future too.

It was our intention to measure the nasal mucosal temperature with a simple noninvasive method, and therefore we chose a contactless infrared thermometer. Using this device, it was hardly possible to standardise the exact site of measurement on the mucosa. The participants had to keep still for a short time period and hold their breath. The mean mucosal temperature in our patients was 30.2°C and the range was 24.7 to 35.8°C. We found a significant difference between each side of the mucosa: the median temperature was 0.5°C higher at the right nasal mucosa. Differences of 2.0°C between inspiration and expiration have already been shown [33]. In a recent publication the nasal mucosal temperature was measured in a total of 44 nasal cavities with a sophisticated method [34]. The authors inserted a thermocouple into the nasal cavity under direct visualisation and measured the temperature during inspiration and expiration at two sites, site 1 the nasal vestibule and site 2 across the head of the inferior turbinate. During inspiration the mucosal temperature was 29.6°C at site 1, which was not significantly different from 30.0°C at site 2. During expiration the mucosal temperature was 33.7°C at site 1 and significantly lower than 34.1°C at site 2 [34]. Although our participants usually held their breath in the middle of a breathing cycle, the results are well in accordance with these data and can best be compared with the mucosal temperature at site 1. In our study we analysed healthy individuals and found no abnormal CBP at the different temperatures. It has been suggested that CBP in PCD with extremely stiff beating and recovery strokes (CCDC39/40 mutations) or reduced proximal bending (DNAH11 mutation) can better be evaluated at 25°C [4], but analysis of CBF below 37°C might also risk PCD misdiagnosis [35]. In this study CBF and CBP were measured in healthy individuals, while changes in the surrounding conditions such as temperature, pH or time ex vivo might have more effect on mutated cilia, such as different PCD variants. Our own data and the published data from the literature indicate that the physiological temperature on the nasal mucosa is ~30–34°C. According to these results, the most appropriate temperature at which to measure the CBF is 32°C and not 25°C or 37°C as previously suggested.

Conflict of interest: None declared.

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