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Comparative Variability of Nasal Potential Difference Measurements in Human and Mice

Anissa Leonard[1,2*], Bob Lubamba[2*], Barbara Dhooghe[2], Sabrina Noel[2], Pierre Wallemacq[2], Patrick Lebecque[1], Teresinha Leal[1,2#]

[1]Pediatric Pulmonology & Cystic Fibrosis Unit, St Luc University Hospital, Brussels, Belgium
[2]Louvain Centre for Toxicology and Applied Pharmacology, Institut de Recherche Clinique et Expérimentale, Université Catholique de Louvain, Brussels, Belgium
Email: [#]teresinha.leal@uclouvain.be

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ABSTRACT

Background: Nasal potential difference (NPD) test has long been used to assist in the diagnosis of Cystic Fibrosis (CF) and more recently as an outcome measure in clinical trials of new CF therapies. This test has also been adapted to the mouse nose. **Objectives:** We aimed at evaluating variability of the NPD measurements in CF patients displaying two severe CFTR mutations and in sex-matched healthy controls. NPD recorded from F508del-CF and normal wild-type mice were also compared. **Methods and Results:** In each setting, tests were performed by a single qualified operator. In the clinical setting, the latest standardized operation protocol of the CF foundation was followed. A total of 80 tracings were obtained from 10 patients (23.2 y; range 14 to 32) and 10 healthy subjects (34 y; range 24 to 53), each tested twice, in both nostrils. Two CF and two controls were excluded from the statistical data analysis due to the presence of a single non interpretable NPD tracing (4/80, 5%). To achieve equal sample size, tests were obtained from 8 CF mice and normal wild-type. Comprehensive multivariate analysis of paired data showed a good reproducibility of NPD parameters in the clinical and the preclinical setting; lower variability was observed in mice. However, 95% repeatability limits of NPD parameters were large indicating a large measurement error, poor precision and low within-subject repeatability. In both settings, chloride secretion was shown to be the most reproducible and repeatable parameter. **Conclusion:** In human as in mice, NPD showed good reproducibility but limited within-subject repeatability.

Keywords: Animal Models; CFTR; Cystic Fibrosis; Nasal Potential Difference

1. Introduction

Electrical potential difference (PD) across the mucous nasal epithelia has been used for more than two decades to assess cystic fibrosis (CF) transmembrane conductance regulator (CFTR) activity and to assist in the diagnosis of CF [1]. As a multiphase test performed under continuous perfusion [2,3], nasal PD allows functional dissection of CFTR and epithelial sodium channel (ENaC) at the nasal mucosa, explored as a representative sample of distal airways [1]. Implementation of the nasal PD test into CF centers around the world has been challenging as the technique is delicate and it requires dedicated equipment and supplies and trained skilled operators. To explore the presence in CF of ion transport abnormalities, CF research teams had to develop inventive in-house setups of the test. As a result, operating protocols have critically diverged among CF centers.

The nasal PD test has also proved to be helpful for the differential diagnosis of atypical CF and CFTRopathies other than CF [4,5]. More recently, the test has been used as an outcome measure in clinical trials of new CF therapies to assess therapeutic modulation of the basic CFTR defect [6-16]. Therefore, an urgent need of standardizing the test has emerged. Substantial efforts have been devoted by the CF Foundation Therapeutics-Therapeutics Development Network (CFFT-TDN) and by the Clinical Trial Network of the European Cystic Fibrosis Society (ECFS-CTN) to perfect a standardized operating protocol (SOP) to be adopted by CF centers worldwide. Adoption of a nasal PD SOP is expected to reduce real-time recording artifacts, to improve quality of data [17] and also to minimize inter-site variability. Studies on variability of the test under these optimized conditions are missing.

The nasal PD test has been adapted to the mouse nose [18] which has allowed investigating the potential of new CF therapies in preclinical settings [19-21]. Translation of data into clinical practice deals with obvious phenotypic differences between mouse and human CF disease

[*]Equal contributors.
[#]Corresponding author.

but also with variability of the nasal PD test in both clinical and preclinical settings. This study was designed to evaluate comparative variability of the nasal PD test in patients with CF and in healthy control subjects as well as in CF mice homozygous for the clinically relevant F508del mutation [22] and in normal homozygous wild-type mice from the corresponding background.

2. Material and Methods

2.1. Clinical Dataset

2.1.1. Study Subjects

The Local Ethics Committee approved the study and informed written consent was obtained from all participants. Ten young adult patients with CF, pancreatic insufficiency and clinical stable lung disease, and 10 healthy non-smoking subjects with no apparent respiratory morbidity took part in the study. CF was diagnosed by typical respiratory and gastrointestinal symptoms, elevated sweat chloride concentrations and the presence of two CFTR mutations. Prior to any nasal PD assessment, the operator ensured that patients and healthy subjects did not present any sign of upper airway infection within 2 weeks before study enrolment. Additional exclusion criteria were smoking, nasal polyps and previous nasal surgery.

2.1.2. Clinical Data

The following clinical data were collected: age, height, weight, sputum cultures and genotype analyses. Sweat test was carried out by pilocarpine iontophoresis according to Gibson and Cooke [23]. Quantitative analysis of sweat chloride and sodium was performed by coulometric titration and flame photometry, respectively. Pancreatic status was determined by fat or elastase contents in stool samples; pancreatic insufficiency wad defined as the need for supplemental pancreatic enzymes in patients with fecal fat >7% (wt/wt) of the amount of dietary fat or with fecal elastase <200 μg/g. Respiratory involvement was assessed by lung function testing on the day of first nasal PD measurements and values obtained were compared with those from a previous test performed 3 months ago to confirm stability (<10% difference from previous measure). Forced expiratory volume in 1s (FEV_1) and forced vital capacity (FVC) were expressed as percentage of the predicted value for age, sex and height [24]. Patients with persistent positive cultures of *Pseudomonas aeruginosa* from on average 2 sputum collections over at least 6 consecutive months were classified as chronically infected. Nutritional status was simply assessed by body mass index (BMI, ratio of body weight (kg) to height (m^2)). Genomic DNA extracted from circulating blood samples was used to perform CFTR gene mutation analyses by using a Luminex xTAG® 71 v2 kit [25].

2.1.3. Nasal PD Measurements

Nasal PDs were performed by a single operator (AL) according to the CFFT-TDN SOP. Briefly, nasal PD values were recorded at a high frequency (1 mHz) using a set of standardized equipment composed of an ISO-Z isolation head-stage (10-02020) coupled to a BMA-200 AC/DC bioamplifier (08-03000) and to an analog/digital converter (PowerLab 4/30) from ADInstruments (Colorado Springs, CO). Captured data was processed and scored using a dedicated client ADInstruments software (GLP Client V6). A pair of nonperfused calomel electrodes (13-620-79; Fisher Scientific, Waltham, MA) was connected to the head-stage unit; the measuring electrode was connected to a double-lumen end-hole nasal catheter (EU certificate 0337/B5/02; Marquat, Boissy Saint Leger, France). One lumen of the catheter was extemporarily filled with autoclaved warmed 3% agar/Ringer solution and the other lumen was used for perfusion of buffered solutions into the nose. The reference electrode was connected to a 23 G needle also filled with agar gel and inserted in the subcutaneous space in the forearm. Agar-filled nasal catheter and subcutaneous needle were attached to the calomel electrodes through a 3 M KCl bridge.

Basal values were initially recorded, under visual control, from 0.5 to 3.0 cm to the anterior tip of the inferior turbinate then the catheter was placed at the site of the most negative value. Solutions were perfused under a continuous 5 ml/min rate for a minimum of one minute up to five minutes until reaching a steady state value (±0.5 mV over 30 sec). Sequential perfusion was per- formed with 1) Ringer solution; 2) Ringer solution with 10^{-4} M amiloride; 3) Zero-chloride solution with 10^{-4} M amiloride; 4) Zero-chloride solution with 10^{-4} M amiloride and 10^{-5} M isoproterenol. Finally, 10^{-5} M ATP, an activator of alternative, non-CFTR dependent channels, was added to the solution containing isoproterenol, this served as a positive control of the test. Temperature of solutions, measured at the tip of the exploring catheter, was 36.9˚C. After testing the first nostril, lines were flushed out with perfused solutions in the reverse order for at least 30 seconds each in order to avoid possible cross-nostril contamination. To evaluate within-subject repeatability of measures, a nasal PD test was repeated, 3 - 5 days after the first test, in each participant. Low quality tracings (flat tracings, artifacts at solution changes, tracings with lots of wandering baseline, etc.) were excluded. Tracings were double read by the operator and a blind reviewer (TL) unaware of the first readings.

2.2. Mouse Dataset

CF mice homozygous for the F508del mutation in the

129/FVB outbred background [22] and their normal homozygous wild-type littermates were studied. Mouse age ranged from 3 - 4 months and weight from 20 - 30 g. The animals were housed at our Animal Care Facility following recommendations of the Federation of European Laboratory Animal Science Associations [26]. These studies and procedures were approved by the local Ethics Committee for Animal Welfare and conformed to the European Community regulations for animal use in research (CEE n° 86/609).

Nasal PD measurements in mice were recorded at a 1 Hz frequency, as described elsewhere [18-21]. In brief, a double lumen catheter was placed in a nasal passage, one lumen being used for perfusion of Ringer solutions, and the other one serving as measuring Ag/AgCl electrode (SLE Instruments, South Croydon, UK) connected to the positive terminal of a data memory high impedance ($>10^{12}$ Ω) voltmeter (Knick Portamess® 913, Elektronische Me *ßgeräte*, Berlin, Germany) through an electrode cream (Signa cream, Parker Labs, Fairfield, NJ) diluted 1:1/vol:vol in 3 M KCl. A needle inserted in the subcutaneous space in a hind leg served as reference bridge. Solutions were perfused at room temperature, at a constant rate of 12 µl/min in the same succession described in the clinical protocol, except for the ATP phase which was not tested in mice. Buffered solutions of identical compositions as those of the clinical setting were used; isoproterenol was replaced by forskolin.

2.3. Statistical Analysis

Descriptive statistics (mean ± SD) and tests of statistical significance were performed using SAS-JMP9 software (SAS Institute, Cary, NC, USA). Prior to statistical analysis, data were checked for normality of distributions (Shapiro-Wilk test). Comparisons between values obtained from right and left nostrils were performed by pairwise bivariate analysis and by Pearson's correlation after addressing concerns on potential outliers by applying a Mahalanobis discordance test, as needed. Before pooling together and averaging data from right and left nostrils, comparison of means was analyzed by using one-way analysis (ANOVA) and equality of variances was checked by posthoc homocedacity analysis by applying the Snedecor's F bilateral test. After checking normality of residuals, comparisons between repeated tests were performed by pairwise multivariate analysis of averaged right-left nostril values by applying the paired Student's *t* test; equality of variances was assessed by Welch test or nonparametric Wilcoxon test, as adequate. Between-test comparisons were also made by Bland-Altman reproducibility plots [27]. Comparative variability between groups was assessed by testing the null hypothesis that the ratio between variances of the two samples is equal to 1. Null hypothesis was rejected at $p < 0.05$.

3. Results

3.1. Clinical Dataset

A total of 80 nasal PD tracings were obtained from 10 patients and 10 healthy subjects tested twice, in quick succession, in both nostrils. Comprehensive statistical analysis of comparative variability of the test required 4 tracings of good quality from each participant. Four tracings (5%), 1 out of the 4 individual nasal PD tracings generated in two patients (numbered 9 and 10, **Table 1**) and in two healthy controls, were considered non interpretable because of a flat pattern or the presence of large artifacts. Even though the three other tracings obtained from these two patients and two controls were considered of good quality, the 4 corresponding participants were excluded from the statistical analysis of repeatability of the nasal PD test.

Table 1 summarizes clinical characteristics of CF patients. Median age at time of nasal PD tests in the 8 CF patients with 4 interpretable tests was 22.5 years (range: 14 to 32). The median age of the 8 healthy controls with interpretable tests was 34 years (range: 24 to 53). Sex distribution was identical in the selected patients and controls (5 females in each group). Sweat chloride and sodium concentrations in selected CF patients averaged 115 ± 5.6 and 106 ± 5.8 mmol/L, respectively. BMI reached a Z score of −0.5. All patients displayed severe class I or II mutations and were pancreatic insufficient.

Representative nasal PD tracings from a patient with CF and from a healthy subject are illustrated in **Figure 1**. From each tracing, the following three representative variables of the nasal PD test were extracted: 1) the maximally negative, most polarizing, stable basal PD value (PDmax); 2) the amiloride response, corresponding to the change in PD values recorded from the end to the beginning of perfusion with Ringer *plus* amiloride solution; and 3) the total chloride response (SumCl), corresponding to the change in PD values recorded from the end of perfusion with zero-chloride solution containing isoprenaline to the end of Ringer *plus* amiloride solution. As expected, CF patients displayed basal hyperpolarization, increased amiloride response and almost completely abolished SumCl. Additionally, CF patients showed more marked response to ATP (**Figure 1 (a)**).

There was no inter-reading disagreement between readings by the operator and the reviewer. Detailed individual values and means (SD) of PDmax, amiloride response and SumCl obtained from both nostrils of 8 patients with CF and 8 healthy controls subjects at two different measurements (test 1 and test 2) are depicted in **Figure 2**. Data from the same participant inside each group are indicated by the same colour code.

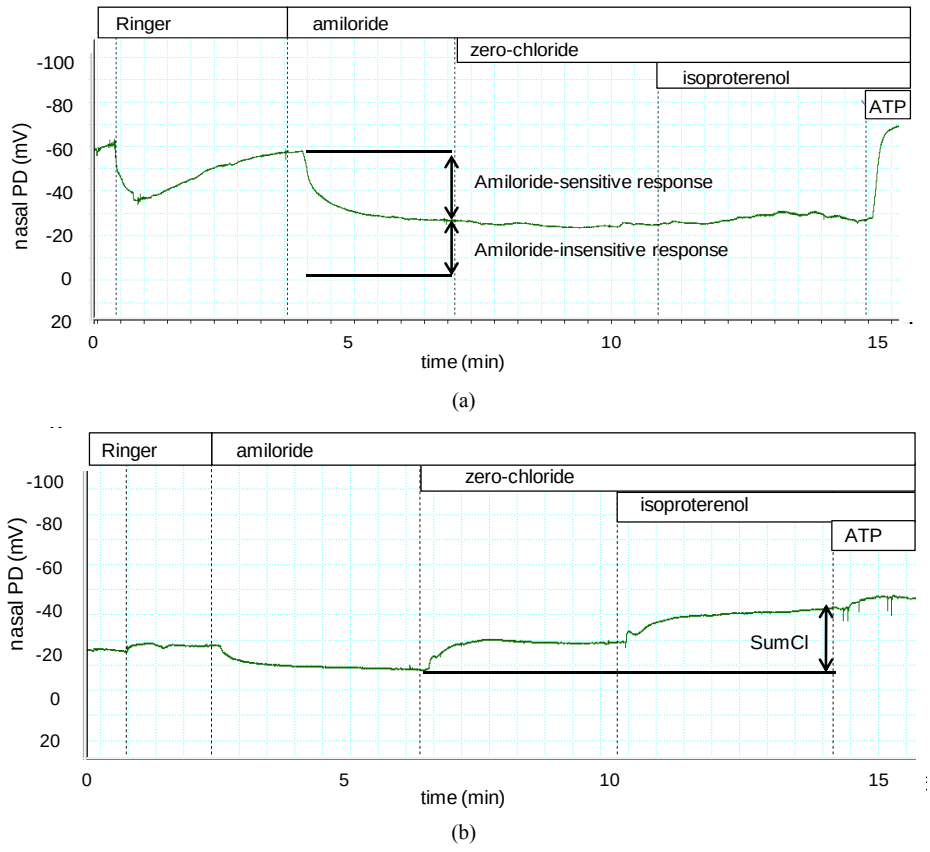


Figure 1. Representative tracings from a patient with CF (a) and a healthy control subject (b).

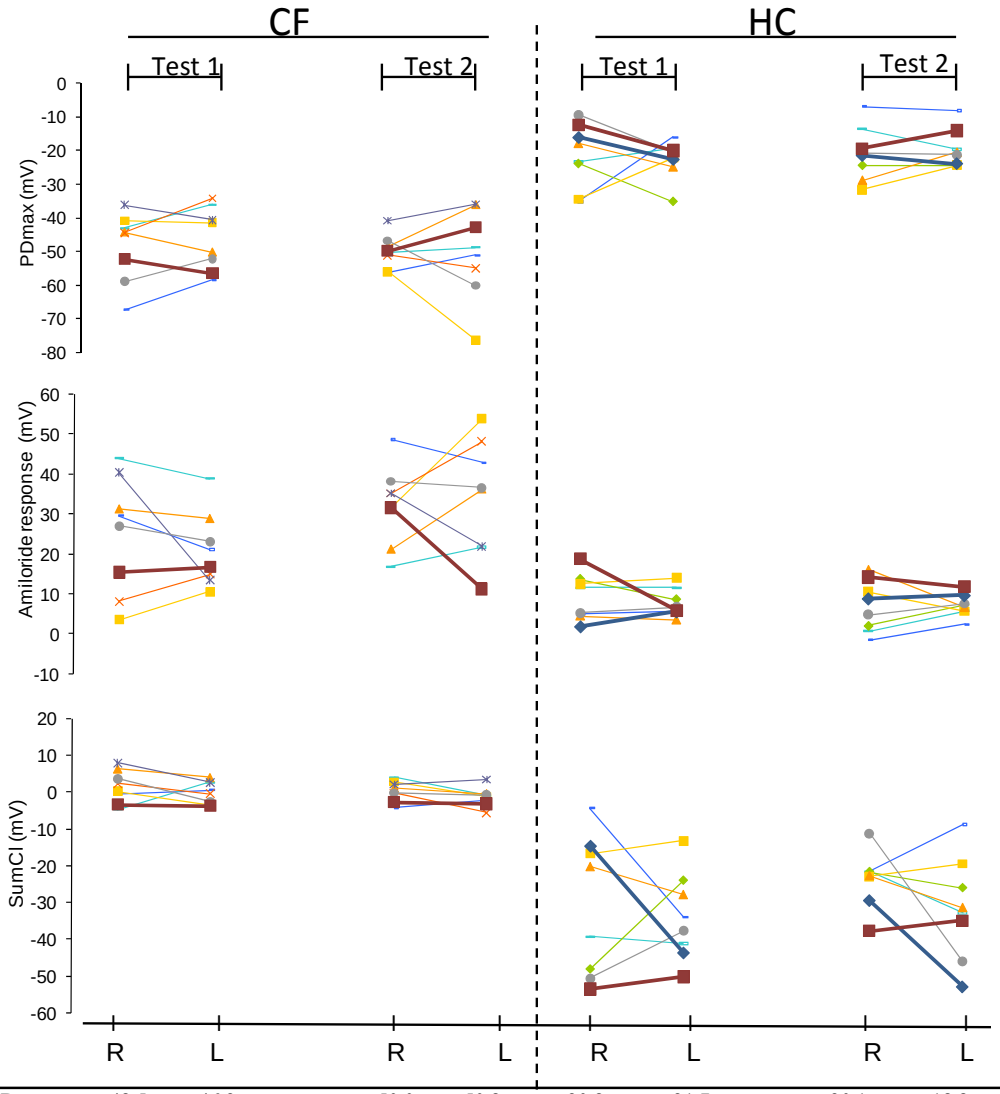
Table 1. Individual clinical and biological data of patients with CF.

Patient	Age years	Sweat test		Genotype	Sputum culture or throat swab	FEV_1 % pred	FVC % pred	BMI Z score
		Cl^-	Na^+					
		mmol/L						
1, F	14	120	97	F508del/1717-1G > A	Normal flora	101	103	−1.2
2, M	16	122	105	F508del/1717-1G > A	Normal flora	95	106	+0.3
3, F	23	110	104	F508del/N1303K	Normal flora	97	106	+1.6
4, M	17	119	109	F508del/2143delT	*A. fumigatus*	87	104	+0.7
5, F	15	111	110	F508del/1717-1G > A	Normal flora	116	112	−0.2
6, F	32	110	106	F508del/1717-1G > A	*P. aeruginosa*	50	96	−1.4
7, F	31	112	111	F508del/F508del	*P. aeruginosa*	61	87	+1.6
8, M	32	122	117	F508del/F508del	*P. aeruginosa*	75	94	−0.2
9, F	22	110	106	F508del/2183AA > G	Normal flora	89	102	−1.7
10, F	30	105	96	F508del/F508del	Normal flora	80	103	0

BMI = body mass index; FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity; F = female; M = male.

3.1.1. Good Right-Left Nostril Correspondence of Nasal PD Values

Nasal PD parameters obtained from CF and control groups showed a good correspondence between right to left nostril values (**Figures 3(a)-(c)**) with a Pearson's coefficient (upper-lower confidence interval) of 0.885 (0.769 - 0.944) for PDmax, of 0.872 (0.743 - 0.939) for amiloride response and of 0.850 (0.703 - 0.928) for SumCl. The differences between paired means obtained from the two nostrils in CF and nonCF were not different

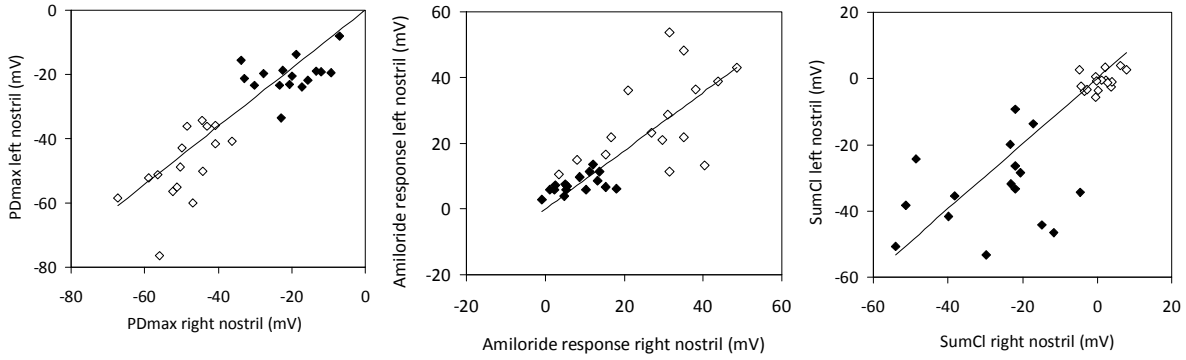


PDmax	-48.5	-46.2		-50.0	-50.8	-20.8	-21.7	-20.1	-18.8
	10.4	9.3		4.9	13.4	9.0	5.4	7.5	5.4
Amiloride response	24.9	20.9		32.2	34.0	9.1	7.7	7.0	7.2
	14.6	9.3		9.9	14.6	5.4	3.2	5.9	2.6
SumCl	1.5	-0.1		0.3	-1.4	-31.4	-34.4	-23.4	-31.9
	4.5	3.0		2.8	2.6	19.2	11.9	7.6	14.1

Figure 2. Individual and mean, SD values (mV) of maximal baseline (PDmax), amiloride response and total chloride response (SumCl) obtained from right (R) and left (L) nostrils of 8 patients with CF and 8 healthy control (HC) subjects at two different measurements (test 1 and 2). Data from the same individual from each group are indicated by the same colour code.

from zero for any variable (**Figure 3(d)**). This finding indicates the absence of significant cross contamination of the opposite nostril by amiloride or other components of perfused solutions.

Nasal PD values showed large data dispersion around means (**Figure 3(d)**). Accordingly, the coefficient of variation (CV), a normalized measure of dispersion calculated as the ratio of the standard deviation to the mean, ranged from 16% to 39% for PDmax and from 38% to 70% for amiloride response. In healthy subjects, the CV for SumCl ranged from 38% to 53%. The range of SumCl values in CF patients was narrower (−5.6 mV to +7.9 mV), contained positive and negative values for a mean value close to zero, thus precluding calculation of CV for this variable. Data were equally dispersed around the means with no significant difference between variances of values from right and left nostrils in the CF and the non CF groups (**Figure 3(d)**). As equality of sample



(a) (b) (c)

		Right nostril	Left nostril	Paired difference	P value for comparison of means	P value for comparison of variances
Patients with CF	PDmax	-49.2 ± 7.9	-48.5 ± 11.4	-0.7 ± 8.9	0.8371	0.1678
	Amiloride response	28.5 ± 12.6	27.4 ± 13.6	1.1 ± 12.8	0.8156	0.7670
	SumCl	0.9 ±3.6	-0.7 ± 2.8	1.6 ± 3.5	0.1691	0.3152
Healthy controls	PDmax	-20.4 ± 8.0	-20.3 ± 5.4	-0.2 ± 8.2	0.9431	0.1388
	Amiloride response	8.0 ± 5.6	7.4 ± 2.8	0.6 ± 4.8	0.6987	0.3920
	SumCl	-27.7 ± 14.6	-33.2 ± 12.7	5.5 ± 16.9	0.2626	0.5872

(d)

Figure 3. Comparison between right and left nostril values of maximal baseline (PDmax, a), amiloride response (b) and total chloride response (SumCl, c) variables obtained from repeated measurements in 8 patients with CF (open diamonds) and 8 healthy control subjects (closed diamonds). Table (d) illustrates means (±SD) of 16 individual values obtained from the right and left nostrils, the corresponding paired difference and the p values for comparisons of means (paired *t* test) and for comparisons of variances (Snedecor's test).

means and variances were adequately addressed, further statistical analyses consisting on assessment of repeatability of nasal PD measurements, averaged right-left values obtained from a given test were pooled together and averaged.

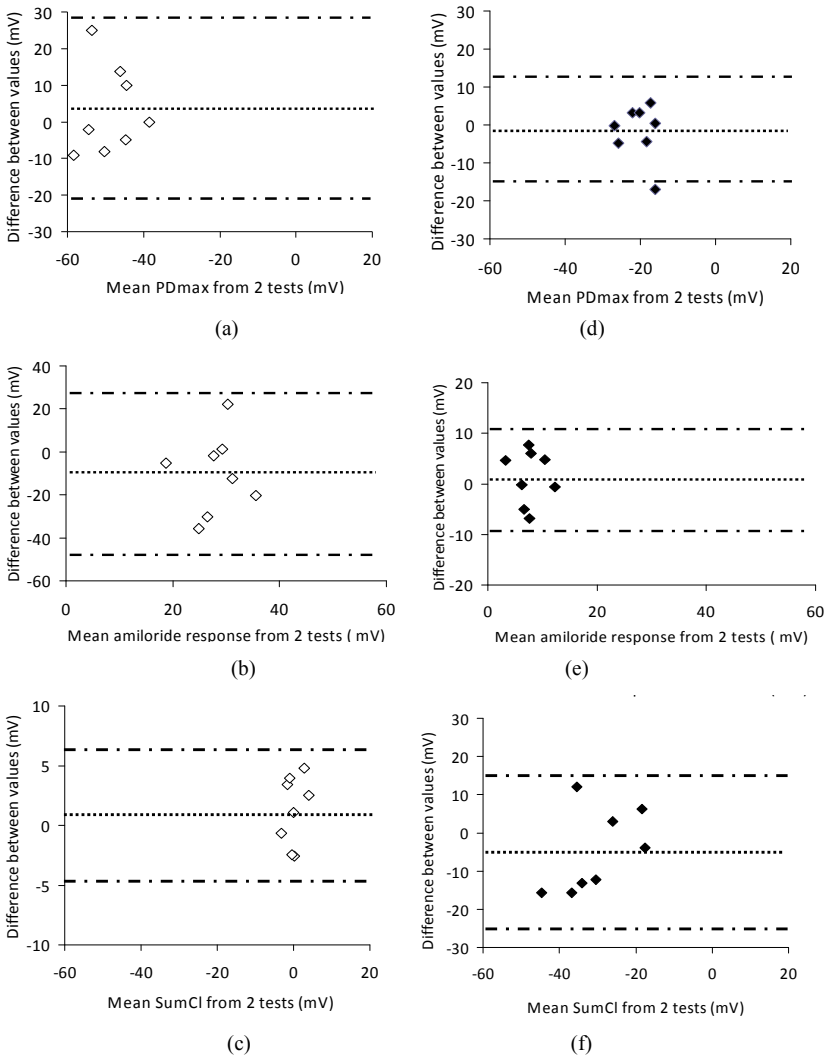
3.1.2. Good Agreement of Repeated Nasal PD Variables

Differences between repeated measurements for each individual were plotted against the means of corresponding values [27]. When examining repeatability of the nasal PD test, we expected the mean difference (bias difference) between repeated measurements to be zero. We also expected the difference between repeated measurements to be less than 1.96 SD for 95% of the paired observations. This corresponds to the 95% repeatability coefficient, and its upper and lower boundaries correspond to the repeatability limit or limit of agreement. As illustrated, all plotted values obtained in this study lied inside the 95% limits of agreement for each variable in both CF and controls (**Figures 4(a)-(f)**). As also illustrated, plotted values were similarly dispersed at the higher and at the low ends of the limits of agreement. Moreover, the averages of each variable were close to zero. These findings indicate a good within-subject agreement of repeated nasal PD measurements.

3.1.3. Lower Within-Subject Variability of Chloride Secretion in CF Patients

As illustrated in **Figure 4**, even though the difference between repeated measurements was globally lower than the estimated 95% repeatability limit, repeatability coefficients were large for all variables as compared to the corresponding mean values.

Between-group comparisons of the variability of the measurements showed that the SD of the paired difference of PDmax values obtained from repeated tests did not significantly differ between CF and controls (**Figure 4(g)**). However, the magnitude of the variability of the paired mean differences for the two other variables was significantly different between CF and controls (**Figure 4(g)**). In patients with CF, a relatively lower between-subject variability of chloride secretion and a higher corresponding variability of amiloride response were observed. The smallest SD (2.9) was found for CF SumCl. This finding indicates that the measurement error of



	Patients with CF	Healthy subjects	P value for comparison of means	P value for comparison of variances
PDmax	3.1 ± 12.0	-1.7 ± 7.2	0.3516	0.1983
Amiloride response	-10.3 ± 18.6	1.3 ± 5.3	0.2701	0.0023
SumCl	1.2 ± 2.9	-4.9 ± 10.9	0.0831	0.0038

(g)

Figure 4. Bland-Altman plots showing distribution of differences between repeated PDmax (a, d), amiloride response (b, e) and SumCl (c, f) values as a function of average of the corresponding values obtained from 8 patients with CF (a, b and c) and 8 healthy controls (d, e and f). Table (g) illustrates means (±SD) of paired differences obtained from two tests together with p values for comparisons of means (paired *t* test) and for comparisons of variances (Snedecor's test).

SumCl is estimated to be, with 95% probability, lower than ±5.7 (1.96 × SD) mV. The degree of variability was globally larger when data from the first investigated nostril only were analyzed (data not shown), instead of average of two nostril values, as presented here.

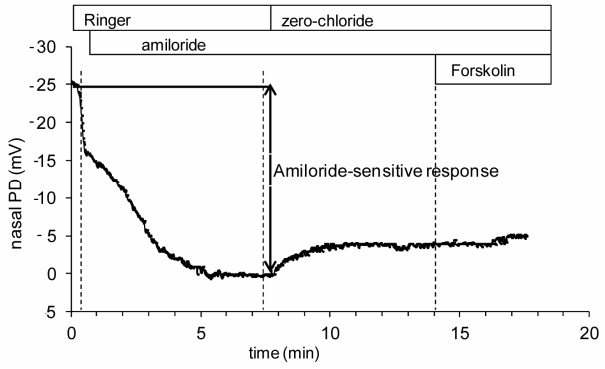
3.2. Mouse Dataset

In order to achieve equal sample size as that of the clinical dataset, nasal PD tests were obtained from 8 F508del homozygous mice and 8 normal homozygous wild-type mice from the same background. Each animal was tested twice within an interval of 3 - 5 days. Because of large cross-nostril contamination of the procedure in mice, a single nostril, always the same for the same animal, was explored. Statistical analysis of repeatability and between-group variability in mice was based on repeated

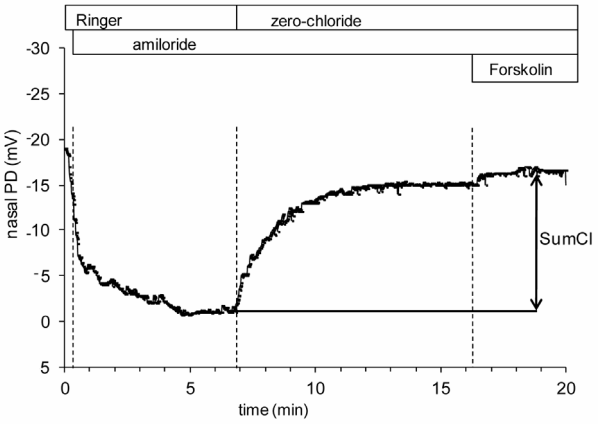
single nostril tracing tests from each animal. Nasal PD in mice was performed by a single operator (BL). Double-readings of tracings by a blinded reviewer (TL) did not show disagreement. All 32 tracings obtained from mice were considered of good quality.

Representative nasal PD tracings from an F508del-CF mouse and from a wild-type mouse are illustrated in **Figure 5**. As for the clinical dataset, the three representative variables of the nasal PD test, PDmax, amiloride response and SumCl, were extracted. CF mice showed typical ion transport abnormalities similar to those observed in patients, *i.e.* basal hyperpolarization, increased amiloride response and reduced SumCl.

Detailed individual values of PDmax, amiloride response and SumCl obtained from CF and wild-type animals at two different measurements (test 1 and test 2) are depicted in **Figure 6**. Data from the same animal inside each group are indicated by the same colour code. Mean (SD) values of variables, also shown in **Figure 6**, were significantly different between the two groups of animals and mean SumCl values were reduced by about 75% in F508del animals as compared with the wild-type group.

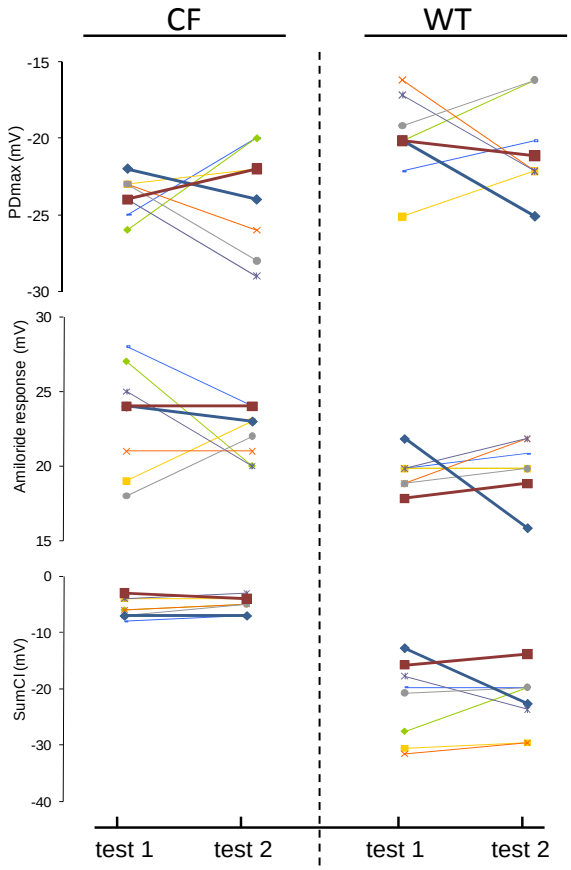


(a)



(b)

Figure 5. Representative tracings from a CF mouse homozygous for the F508del mutation (a) and a homozygous normal mouse from the same background (b).



PDmax	-23.8 1.3	-23.9 3.5	-19.9 2.8	-20.5 3.1
Amiloride response	23.3 3.6	22.1 1.6	19.8 1.2	20.0 1.9
SumCl	-5.6 1.8	-5.0 1.4	-21.4 7.1	-21.6 5.4

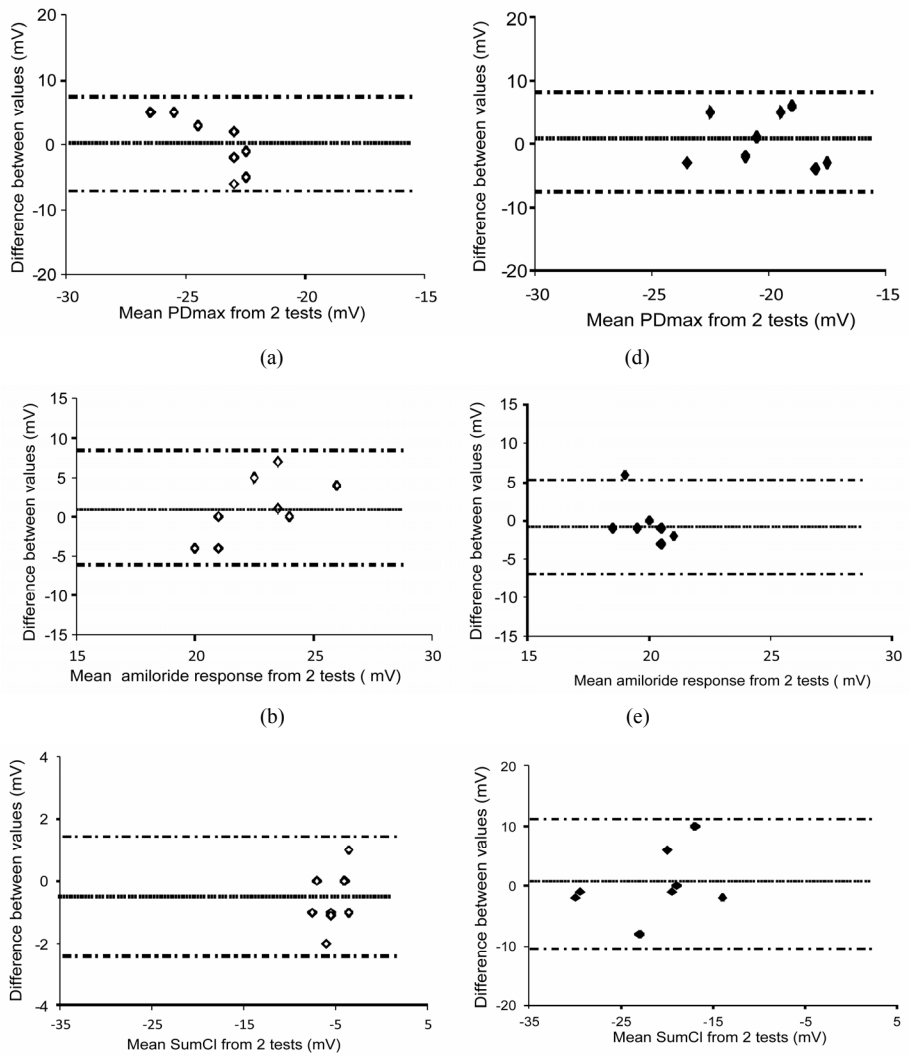
Figure 6. Individual and mean, SD values (mV) of maximal baseline (PDmax), amiloride response and total chloride response (SumCl) obtained from 8 CF mice homozygous for the F508del mutation and 8 normal homozygous mice from the same background (WT) at two different measurements (test 1 and 2). Data from the same animal from each group are indicated by the same colour code.

3.2.1. Good Agreement of Repeated Nasal PD Variables

Assessment of agreement between repeated measurements performed by Bland and Altman method (**Figures 7(a)-(f)**) showed that, in both CF and wild-type mice, plotted values for nasal PD variables lied inside the 95% limits of agreement and were similarly dispersed around the mean value that were close to zero. These findings indicate a good within-mouse agreement of repeated nasal PD measurements.

3.2.2. Lower Within-Mouse Variability of Chloride Secretion in CF

Differences between repeated values were lower than the



	F508del-CF mice	Wild-type mice	P value for comparison of means	P value for comparison of variances
PDmax	0.1 ± 4.3	0.6 ± 4.2	0.8166	0.9432
Amiloride response	1.1 ± 4.0	-0.3 ± 2.7	0.4375	0.3223
SumCl	-0.6 ± 0.9	0.3 ± 5.5	0.7862	<0.0001

(g)

Figure 7. Bland-Altman plots showing distribution of differences between repeated PDmax (a, d), amiloride response (b, e) and SumCl (c, f) values as a function of average of the corresponding values obtained from 8 CF mice homozygous for the F508del mutation (a, b and c) and 8 normal homozygous mice from the same background (d, e and f). Table (g) illustrates means (±SD) of paired differences obtained from two tests together with p values for comparisons of means (paired *t* test) and for comparesons of variances (Snedecor's test).

estimated 95% repeatability limits and that the repeatability coefficients were narrower as compared to the corresponding mean values. Between-group comparisons of the variability of the measurements showed that the SD of the paired difference of PDmax and amiloride response values obtained from two tests did not significantly differ between CF and controls (**Figure 7(g)**). However, the magnitude of the variability of the paired mean differences for the total chloride secretion was significantly different between CF and controls. In F508del-CF mice, a lower between-mouse variability of chloride secretion was observed. Indeed, the lowest SD (0.9) was

found for SumCl in CF mice. Thus, in CF mice, the measurement error of SumCl was estimated to be, with 95% probability, lower than ±1.7 (1.96 × SD) mV.

3.3. Comparison between Clinical and Preclinical Datasets

Comparative variability of nasal PD variables between the mouse and the human setting, in CF and in nonCF are shown side-by-side in **Figure 8(a)**. As illustrated, SDs of the paired differences between nasal PD variables were smaller in the preclinical setting (**Figure 8(a)**). Differences did not reach significance in healthy groups while significant levels were reached for all variables in the CF groups. These findings indicate that the nasal PD in the CF mouse model shows lower variability and greater repeatability than in human.

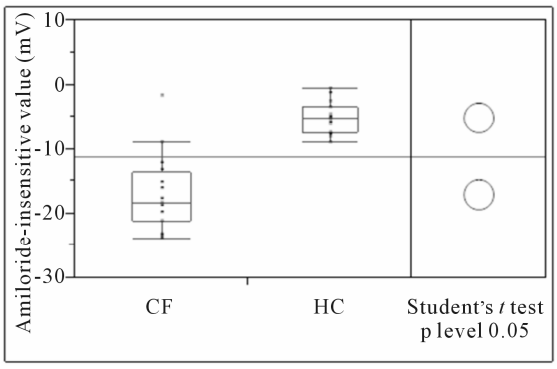
An interesting finding when comparing human to mouse nasal PD data appeared to be the relative degree of sensitivity to amiloride. A large residual amiloride-insensitive response, of similar magnitude of the amiloride-sensitive component, was measured in patients with CF (**Figure 1**), while in CF mice the amiloride-insensitive response was shown to be almost null (**Figure 5**). These findings were confirmed by mean comparisons between patients with CF and healthy controls (**Figure 8(b)**) and between F508del-CF mice and wild-type mice (**Figure 8(c)**).

4. Discussion

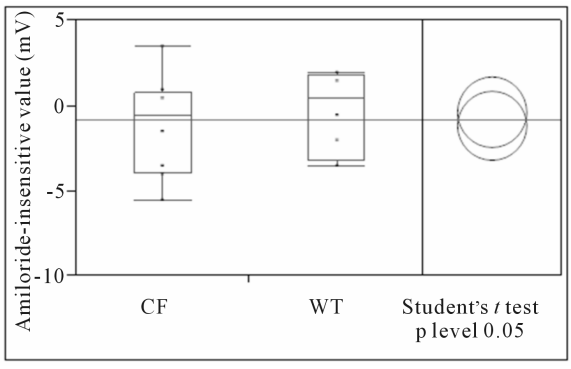
This work represents the first rigorous study of nasal PD variability in human and mice, as few CF reference centers worldwide do have experience with this delicate test in both settings. While practicing bench-to-bedside translational research, we have used nasal PD measurements to test therapeutic strategies that are expected to improve ion transport abnormalities at the mouse CF airways [19-21]. We have also taken part in clinical trials using nasal PD variables as measures of therapeutic efficacy of fundamental strategies in CF [12,15,16]. Beyond the confirmation of the high discriminative power of the

		Clinical setting	Preclinical setting	P value for comparison of means	P value for comparison of variances
CF	PDmax	3.1 ± 12.0	0.1 ± 4.3	0.9999	0.0142
	Amiloride response	-10.3 ± 18.6	1.1 ± 4.0	0.0827	0.0006
	SumCl	1.2 ± 2.9	-0.6 ± 0.9	0.1857	0.0074
nonCF	PDmax	-1.7 ± 7.2	0.6 ± 4.2	0.4367	0.1732
	Amiloride response	1.3 ± 5.3	-0.3 ± 2.7	0.4779	0.0963
	SumCl	-4.9 ± 10.9	0.3 ± 5.5	0.2576	0.0901

(a)



(b)



(c)

Figure 8. Between-setting differences in CF and nonCF of human and mouse nasal PD variables. Table (a) illustrates means (±SD) of paired differences of variables obtained from two tests together with *p* values for comparisons of means (paired *t* test) and for comparisons of variances (Snedecor's test). Panel (b) shows mean comparison of amiloride-insensitive sodium conductance between patients with CF (CF) and healthy controls (HC). Panel (c) shows mean comparison of amiloride-insensitive sodium conductance between F508del-CF mice (CF) and wild-type mice (WT). In box plots B and C, horizontal lines illustrate the 0.5th, 25th, 50th (median), 75th and 99.5th percentiles of the amiloride-insensitive nasal PD value. The horizontal line across the panel represents the mean of the study group. The degree of overlapping of comparison circles indicates differences between the two groups at a p level of 0.05, as analyzed by Student's *t* test. According to Grubb's discordance test, there are no outliers in the sample group data at a p level of 0.05.

nasal PD test to adequately differentiate between CF and nonCF in both human and mice, this study was designed to evaluate its comparative variability in the clinical and the preclinical setting. In the former, patients displaying classic CF disease with pancreatic insufficiency, two severe mutations and pathologic sweat test results were tested comparatively to sex-matched healthy subjects. In the latter, the clinically relevant mouse model of CF disease homozygous for the F508del mutation [22] and their normal homozygous littermates were used. Optimized conditions for recording and analyzing nasal PD data were assembled in both settings. In each setting, tests were performed by a single qualified operator and tracings were double-read by a blind reviewer. In the clinical setting, we used the new standardized equipment and setup, and we followed the standard operating protocol expected to reduce artifact frequency and to favor tracing stability and data reliability [17,28]. The proportion of non interpretable tracings (4/80, 5%) we obtained was in agreement with that previously reported (3% - 10%) [29]. Our data are also in agreement with Solomon's validation study [17] showing that the use of nonperfused agar-bridge electrodes is related to more hyperpolarized SumCl values (−24.2 ± 12.9 mV) as compared to those obtained upon using perfused bridges (−18.2 ± 12.9 mV).

The assumption of significant cross-nostril amiloride contaminating effect [30] has been raised as argument to explore a single nostril during the test [31,32]. By contrast, our results showing a good between-nostril correspondence rather support the notion that averaging data obtained from both nostrils contribute to strengthen data interpretation and to reduce data variability [33].

Based on the finding that plotted values in Bland-Altman analysis lied inside the 95% limits of agreement, our results would claim for a good reproducibility of nasal PD measurements. But the question that should be raised here is: is this enough to consider nasal PD as a robust test? In this work, we were concerned with modeling a narrow limit of agreement for each nasal PD variable, which would indicate good within-subject repeatability of the measures. The finding that large 95% repeatability coefficients were globally found indicate a large measurement error, a poor precision and a low within-subject repeatability of nasal PD measurements, particularly in the clinical setting. In human as in mice, the lowest variability was found for chloride secretion in CF pointing out SumCl as the most reliable nasal PD parameter for monitoring between-test changes in the case of therapeutic strategy expected to influence transepithelial chloride transport in CF. Accordingly, SumCl has been considered the most sensitive and specific indicator of CFTR-dependent ion transport and it has been included as an outcome endpoint in CF clinical trials aiming at rescuing CFTR function [6-16]. In agreement with our findings, if the difference between SumCl assessed under treatment in a given patient is greater, in absolute terms, than 5.7 mV of that recorded under baseline, then one can be 95% certain that the change is too large to be expected by the measurement error alone and a correcting effect of the drug treatment can be suspected. This value is somehow in agreement with the arbitrary cut-off of 5mV that has been used for SumCl in different clinical trials. If applying the estimation to Yaakov's [31] or to Simmonds' [32] work, the critical cut-off value should be revised to 8.6 mV, *i.e.* 1.96 SD (4.4) mV [31] or to 14.3 mV, *i.e.* 1.96 SD (7.36) [32]. The fact that in both studies [31,32] a single nostril was explored could, at least partly, contribute to explain differences with our data. As the repeatability coefficient in mice is lower, when testing a potential CFTR-correcting drug in mice, we could be 95% certain of a drug effect if the change observed in SumCl between two measurements in the same animal should be greater than 2 mV. As a function of larger 95% repeatability coefficients for PDmax and amiloride response, the expected changes in patients with CF would be greater than 23.5 (1.96 × 12.0) mV and 36.4 (1.96 × 18.6) mV, respectively. The expected cut-off for changes of PDmax and amiloride response in mice would be of similar magnitude and greater than 8.4 mV.

We showed here that amiloride response has the largest variability in patients while in another work [31], basal PD was considered as the most variable parameter. By the way, it has been well recognized that basal hyperpolarization and increased amiloride sensitivity both reflect exaggerated ENaC activity in CF. Even though the interplay between CFTR and ENaC is still not completely understood, convincing experimental data supporting the role of overactive ENaC function in CF disease have been brought by the development of a mouse model overexpressing the β-subunit of amiloride-sensitive ENaC protein [34,35]. Other sodium-coupled transport pathways, particularly in human, might contribute to the basal hyperpolarization across the airway epithelium. Indeed, a high fraction of amiloride-insensitive response remained in CF patients, but not in CF mice, suggesting that species-dependent factors may be involved. In mice, there is no apparent contribution of the amiloride-insensitive conductance as response to amiloride typically reached zero in CF as in non-CF mice. The nature of the amiloride-insensitve sodium conductances involved in the human nose and the possible functional interactions they might have with CFTR could not be determined in the present study.

Several factors could potentially contribute to the variability of nasal PD measures, despite the recent efforts to standardization and validation of the test in view to reduce artifact frequency and to improve reliability and reproducibility [17,28]. Modifiers genes [36] and

concomitant medications [37-42] have been recognized as potential sources of within-subject variability but they are not well studied. Lower variability was globally found in the mouse model. As in human, CF mice show characteristic transepithelial ion transport abnormalities [19-21,43,44]. The F508del mouse model used in this work represents a valuable tool to study pathophysiology of the disease and to test the efficacy and potential interest of new therapeutic strategies. Phenotypic differences in the expression of the disease with more severe gastrointestinal rather than lung manifestations, absence of concomitant medications, the obvious degree in complexity of genomes, together with the fact that the model is housed in privileged conditions with sanitary barriers might contribute to explain the lower variability observed in CF mice, particularly in SumCl for which the lowest SD (0.9) of differences between tests was showed.

In conclusion, our data demonstrate that, even being underwent under optimized conditions, nasal PD in human and in mice display good reproducibility but limited within-subject repeatability. The lowest measurement error was observed for SumCl confirming, taking into account the size of its measurement error, its place as an outcome endpoint in preclinical and clinical proof-of-concept trials. In practice, a difference between baseline and under treatment SumCl value larger than 5.7 mV in CF patients and 2.0 mV in F508del-CF mice would indicate 95% chance of a CFTR improving effect of the treatment tested.

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