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Title: Insights into the variability of Nasal Potential Difference, a biomarker of CFTR activity

Article Type: Original Article

Keywords: Nasal potential difference, Biomarker, Cystic Fibrosis

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Abstract: Abstract

Background: Nasal potential difference (NPD) is used to evaluate CFTR function in vivo.

We aimed to evaluate the intrasubject and intersubject variability of NPD measurements.

Methods: We reviewed NPD tracings of 116 patients with CF enrolled in the placebo arm of a multicenter study. Patients carried at least one nonsense mutation and underwent repeated NPD tests every 16 weeks. NPD parameters included basal potential difference (basal PD), inhibition of sodium absorption by amiloride (Δ Amiloride), chloride (Cl⁻) transport in response to a Cl⁻-free solution (Δ Low Cl⁻), isoproterenol (Δ Isoproterenol), the sum of Δ Low Cl⁻ and Δ Isoproterenol (Δ Low Cl⁻-Isoproterenol) and ATP (Δ ATP).

Results: Basal PD and Δ Amiloride displayed the highest variabilities, mainly stemming from intercenter and intrasubject effect. Δ Low Cl⁻, Δ Isoproterenol and Δ Low Cl⁻-Isoproterenol demonstrated a large intrasubject variability but a smaller intersubject variability. The intrasubject measurement variability for Δ Low Cl⁻-Isoproterenol, was within ± 7.2 mV with 95% probability. It was greater in patients reporting ongoing pulmonary exacerbations.

Conclusions: The large intercenter variability of basal PD and Δ Amiloride highlights the operator-dependent aspect of these measurements. A difference greater than 7.2 mV in Δ Low Cl⁻-Isoproterenol in a given patient on CFTR modulator can be attributed, with 95% probability, to a treatment effect rather than to the variability inherent in the measurement.

29 August 2019

Dear Editor,

We would like to thank the reviewers for their thorough review of our manuscript. We carefully addressed the Editor's and reviewers' comments and suggestions and believe this revised version of the manuscript has been significantly improved. The answers to the reviewers' comments and suggestions can be found below. All co-authors have reviewed and approved the revised version of the manuscript.

My co-authors and I thank you for considering this manuscript for publication in the *J of Cystic Fibrosis* and I look forward to hearing from you regarding the submission. Please do not hesitate to contact me if you have any queries or concerns.

Sincerely,

On behalf of my co-authors, I Sermet-Gaudelus

A handwritten signature in black ink, consisting of a stylized 'I' followed by a horizontal line and a vertical line that curves at the bottom.

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A handwritten signature in black ink, consisting of a stylized 'I' followed by a large, looped 'S' and a trailing flourish.

Point by point response

Editor

Your data are of interest, but a few things need to be clarified:

The study included North American centres and there was involvement of the North American NPD core in this study; please provide evidence that they chose not to be part of the author ship of this study.

We thank the Editor for this request for clarification. The clinical trial indeed included centers from US and Canada. The study presented here is part of the methodological analysis plan of the European Clinical Trial Network, Nasal Potential Difference being a biomarker used in clinical trial. Material Transfer Agreement was made between PTC Therapeutics and members of the CTN. Therefore, only European investigators were part of this manuscript.

NPD measurements in patients with stop mutations are usually quite flat; a bit more discussion of how genotypes affected this would be warranted.

We agree with the editor that non sense mutations are usually associated with non functional CFTR. However some mutations can be associated with residual CFTR function.

This can be explained by (i) persistence of the target mRNA because of defective nonsense mediated decay or alternative splicing (Sharma et al; PLoS Genetics 2018; Hinzpeter et al. PLoS Genetics 2010); (ii) reinitiation of CFTR synthesis at alternative Methionine codons, downstream the initial one (Ramalho et al. Cell Physiol Biochem 2009) (iii) mutations located in the terminal part of CFTR generating truncated partially functional protein (Pranke et al; Open ERJ 2018). Finally, the second allele, if carrying a mutation associated with residual function, may be associated with some chloride transport.

We therefore reviewed mutations associated with a total repolarization after low chloride solution < - 5 mV, which is the cut-off generally used in clinical trials.

Among the 24 Nasal Potential difference tests resulting in a total Chloride conductance < - 5 mV

- 3 were associated with mutations resulting in alternative splicing such as 3849-10kB (n=2) and E831X (n=1)
- 1 was associated with possible reinitiation downstream M1 (E60X)
- 1 was associated with wide spectrum mutation (D1152H)
- 19 were associated with F508del, which may be associated with residual function because of trafficking of some protein to the apical membrane

Therefore, in this study, at least in 4 cases, a set of nonsense mutations could be associated with some residual functional expression. In the other cases, the alternative allele F508del might be the cause of some residual function.

This point has been added in the discussion.

A threshold for 40 mmol/L for the CF diagnosis is listed; is that a typo?

Yes , i apologize this is a typo. This has been corrected.

Reviewer #1: This study by Kyrilli et al. presents an analysis on quarterly repeated NPD measurements in a large number of patients enrolled in the placebo arm of a multi center drug trial. The paper is generally well written and the data are highly relevant for the use of NPD as a biomarker of CFTR function in future drug trials. Of special interest is the intra-subject variability of repeated measurements of NPD. However, the influence of pulmonary exacerbations deserves a more detailed analysis. Further, the authors should compare their results with other biomarkers of CFTR function (i.e. sweat Cl- and ICM) that have been used to assess the effect of CFTR modulators.

Comments

1. *Were any of the included patients pancreatic sufficient?*

We thank the reviewer for this question. In the data base, obtained by transfer agreement from PTC Therapeutics, information of pancreatic status was not given. To answer to this question, we considered the allele not carrying the nonsense mutation, and found 2 mutations associated with pancreatic sufficiency : R117H (1 patient), D1152H (1 patient). In these 2 cases, the patients were probably pancreatic sufficient.

2. *Please include the unit for BMI and sweat chloride in Table 1.*

This has been corrected/

3. *Why are sweat chloride values only available for 92 of 116 patients?*

We do not have any explanation for this. As stated above, we got the data base from PTC therapeutics. There were missing data.

3. *Please comment on the total number of 447 visits as the number of visits does not add up to this number.*

We thank the reviewer for asking this. The total number of visits (and NPD) was 447. The patients had 4 NPD in the clinical trial protocol. However, 14 patients had a 5th NPD test recorded in the data base, the reason of which is unclear. To give a harmonized interpretation of the data, we decided not

to consider these additional visits, and therefore the analysis was done on 433 NPD/visits. We however also performed the analysis on the full set of data (447) and this changed minimally the results. This has been corrected in Table 2.

4. What is the added value of Figure 2 in addition to Table 2?

Indeed, we agree with the reviewer that this is a graphic representation of Table 2. This Figure has been put as Supplemental now, but kept as we think that this is very visual.

5. The results of the NPD measurements should be discussed in the context of other biomarkers of CFTR function such as sweat chloride and intestinal current measurement (ICM) that have been used recently to assess improvement of CFTR function by CFTR modulator therapy in patients with CF.

We thank the reviewer for the suggestion. Indeed, CFTR activity can be measured generally in or ex vivo by 3 different surrogate biomarkers, e.g. Cl^- sweat concentration, changes induced by CFTR agonists in nasal potential difference and current measurement on rectal biopsies. However, comparative studies show that these three biomarkers are not interchangeable. This is because they reflect CFTR activity differently : sweat test assess Cl^- reabsorption along the sweat duct; NPD is an indirect measurement of transepithelial voltage reflecting relative changes in ion conductance while Short Circuit Current is a direct measurement of active transepithelial ion transport. This point has been added in the discussion.

6. As the intrasubject variability seems to be influenced by pulmonary exacerbations. What would be the measurement error of total chloride conductance if only visits without pulmonary exacerbations are included in the analysis.

We thank the reviewer for asking this question. In order to assess the impact of pulmonary exacerbations on total chloride conductance variability, we determined the measurement error in the patients who never had any exacerbations during the protocol (75 patients, 272 NPD tests) and in those who had at least 1 exacerbation during the clinical trial (41 patients, 161 NPD). In the first group (non-infected patients), the measurement error was 6.34 (corresponding to a variance of 10.47), whereas in the second group (infected patients) the measurement error was 8.55 (corresponding to a variance of 19.04). This clearly shows that the variability of the measurement is increased by bronchial exacerbation. This point has been added in the result.

7. Were there any topical treatments applied to the nasal mucosa in these patients? Especially, were there changes in the topical treatment before the visits during pulmonary exacerbations? The possible influence of these treatments on the intrasubject variability should be discussed.

We fully agree that this is an important question. We however do not have these data and can not answer to this question.

Reviewer #2: Thank you for reviewing this excellent manuscript on a very important aspect of NPD. All information and results are presented clearly and in depth.

Minor comments:

The reference list ended with reference 29, but in the manuscript reference up to 33 are mentioned. Please check and correct. The reference are not ordered as they appear in the paper: On page 11 reference n 24 was introduced and on page 6 the next reference starts with 32. Reference 25 appear on page 7. Please check and correct.

We very much apologize. This has been corrected.

The important references 11 and 24 should be made public available at a public available website or as an online supplement.

We absolutely agree. Those SOP are available on demand.

Highlights

- Using nasal potential difference in proof of concept trials requires knowledge of its variability.
- We reviewed NPD tracings of 116 patients with CF enrolled in the placebo arm of a multicenter study.
- .A difference greater than -7.2 mV of the total Chloride transport can be attributed, with 95% probability to a treatment effect.

Insights into the variability of Nasal Potential Difference, a biomarker of CFTR activity

Spyridoula Kyrialli ^{1*}, Theophraste Henry ^{2*}, Michael Wilschanski ³, Isabelle Fajac^{4,5,6}, Jane C Davies ^{7,8}, Jean-Philippe Jais ^{2,6#} and Isabelle Sermet-Gaudelus ^{1,6,9#}

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Authors' contributions

SK analysed the data and wrote the manuscript. THJ and JPJ made the statistical analysis. MW, IF and JCD participated in data analysis and writing of the manuscript. ISG designed the study, analysed the data and wrote the manuscript.

Running title: Nasal Potential Difference variability

Word count: 2996

This article has an online data supplement.

Abstract

Background: Nasal potential difference (NPD) is used to evaluate CFTR function *in vivo*.

We aimed to evaluate the intrasubject and intersubject variability of NPD measurements.

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Conclusions: The large intercenter variability of basal PD and Δ Amiloride highlights the operator-dependent aspect of these measurements. A difference greater than 7.2 mV in Δ Low Cl^- -Isoproterenol in a given patient on CFTR modulator can be attributed, with 95% probability, to a treatment effect rather than to the variability inherent in the measurement.

Word count: 227

Key words: Nasal potential difference, Biomarker, Cystic Fibrosis

Highlights

- Using nasal potential difference in proof of concept trials requires knowledge of its variability.
- We reviewed NPD tracings of 116 patients with CF enrolled in the placebo arm of a multicenter study.
- A difference greater than 7.2 mV of the total Chloride transport can be attributed, with 95% probability to a treatment effect.

Introduction

Cystic Fibrosis (CF) is the most frequent life-limiting autosomal disease in the Caucasian population. It is a channelopathy caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, which acts as a cAMP chloride (Cl⁻) channel after activation by protein kinase A (PKA). The typical phenotype includes chronic sinopulmonary disease, pancreatic exocrine insufficiency, salt loss syndrome and congenital bilateral atresia of the vas deferens (1). The diagnosis is based on a positive newborn screen test, characteristic phenotype, and/or a family history of CF in conjunction with a sweat Cl⁻ concentration > 60 mmol/L and/or identification of two CF causing mutations and/or identification of CFTR dysfunction (1). Nonetheless, CF is not always a clear-cut diagnosis (2, 3). Clinicians encounter individuals with clinical features suggestive of CF, but who do not meet the current diagnostic criteria. CF diagnosis remains equivocal in subjects with intermediate sweat chloride results (30-59 mmol/L) and identification of only one or no CF-causing mutations. In these cases, CFTR functional assays may provide further helpful information (4-6). Identification of a CFTR functional defect, e.g; increased ENaC-mediated sodium (Na⁺) absorption, and minimal/ absent Cl⁻ secretion in response to cAMP is strongly supportive of CF or CFTR-related disorder (2-3, 6)

Assessment of CFTR function can be evaluated *in vivo* by the nasal potential difference test (NPD) (7). It is now considered a diagnostic criterion for CF in the European consensus guidelines and a contributory test by the North American and the French national diagnosis consensus to clarify the diagnosis in patients with 'questionable' CF (2,3) and to confirm CFTR activity restoration in proof of concept studies, (8-10).

However, employing NPD in proof of concept multicenter trials requires knowledge of its variability to determine the reliability of this biomarker as a surrogate endpoint for CFTR activity. This led to collaborative efforts to standardize the test as much as possible (11-18). Studies assessing the repeatability and the precision of the measurement as well as the intrasubject and the intersubject variability of this test are lacking. Yaakov and Leonard reported a low within-subject repeatability in single centers (19-20). But if NPD is to be useful as a surrogate marker of CFTR function in proof of concept studies, its variability needs to be assessed in a multicenter context.

The aim of the present study was to evaluate the variability of NPD measurements in CF patients based on NPD tests performed in the placebo arm of a trial of PTC124, an orally bioavailable, small-molecule drug intended to promote ribosomal readthrough of mRNA

containing a premature stop-codon. It was tested in a phase 3, multicenter, double-blind, placebo-controlled study in patients carrying at least one nonsense mutation (21).

We investigated the intrasubject, intersubject and intercenter variability of NPD measurements among patients enrolled in the placebo arm of this study. We also assessed the impact on the variability of different factors potentially influencing CFTR function including age, sex, genotype and pulmonary exacerbation.

2. Material and methods

2.1. Study design

The data set was obtained from the placebo arm of the phase 3 study aiming to test the efficacy and the tolerability of PTC124 administered for 48 weeks in CF patients carrying stop-codon mutations (21,22). The patients were ≥ 6 years of age, had a sweat chloride concentration ≥ 60 mmol/L and carried 2 CF causing mutations with at least one stop-codon mutation. The primary endpoint of the study was the relative change in % predicted FEV₁ from baseline to week 48. The secondary endpoint was the rate of pulmonary exacerbations defined according to Fuchs criteria (23). Tertiary criteria of the study included NPD test and sweat chloride concentrations performed every 16 weeks.

2.2. NPD measurements

NPD was measured according to standardized operating procedures and with centrally made solutions (11,24). Pharmacodynamic explorations were based on PD changes after the following superfusions: 100 μ mol amiloride in Ringer's saline solution (Δ Amiloride), 100 μ mol amiloride in Cl⁻-free solution to drive CFTR-mediated chloride secretion (Δ Low Cl⁻), 10 μ mol isoproterenol in the chloride-free solution to stimulate cAMP-dependent CFTR-related Cl⁻ transport (Δ Isoproterenol). The sum of the latter 2 responses (Δ Low Cl⁻-Isoproterenol) served as an index of CFTR function. Adenosine triphosphate (Δ ATP) was superfused at the end of the test as a positive control. Signs and symptoms of pulmonary exacerbation were collected at the time of NPD test.

2.3. Statistical analysis

Values from NPD tests were extracted for both nostrils and reported as the mean value of both nostrils. NPD measurements distribution characteristics were based on their mean/SD. Correlation between variables was assessed by the Pearson correlation coefficient. To study NPD measurement variability, we first estimated the variability within the same subject over time. Variability was further decomposed into intrasubject variability and interindividual variability, the latter encompassing the intersubject and intercenter variability. To explore the

sources of variability of NPD measurements, linear mixed effect models were fitted considering the center and the subject as random effects and the other measurable factors as fixed effects. Statistical significance of these different effects was assessed by comparing the nested models including or not the evaluated effect based on the log-likelihood ratio statistical test. Random effects estimates were expressed as the variance of the evaluated NPD measurement, representing the sum of random components, e.g., intersubject, intercenter, and residual (or intrasubject) variance (or variability). They were expressed as percentage (%) of the total variance. The 95% credibility interval of intrasubject measurement 'error', e.g the variability inherent to the measurement for the same subject, was defined as $\pm 1.96 \times \text{SD}$ of the intrasubject variance.

To explore the relationship between intrasubject variability of the NPD measurements and clinical factors, SDs of NPD measurements (NPD-SD) were computed for each subject on whom all 4 NPD tests were available. NPD-SD values for each parameter were then regressed with clinical variables using a linear regression model.

All statistical computations were performed with the R statistical environment V3.4.1 (R Foundation for Statistical Computing, Vienna Austria) and the lme4 package. P-values were considered as statistically significant when ≤ 0.05 .

3. Results

3.1. Study population

238 CF patients were enrolled in the study and 118 were allocated to the placebo arm. Two subjects presenting out of range NPD data were eliminated from our data set (1 tracing with $\Delta \text{Low Cl}^-$ at -90 mV, 1 tracing with ΔATP at -130 mV). Sixteen patients prematurely withdrew from the study because of low compliance or side effects. The remaining 100 patients provided NPD tests at all time points (**Figure 1**). The trial was performed in 36 CF centers between August 2009 and November 2011. Three centers enrolled > 30 subjects, 13 enrolled between 10-30 subjects and 15 enrolled < 10 subjects. The baseline clinical and demographic characteristics of the placebo group patients are shown in **Table 1**. The subjects had a mean age of 23.3 years (range 8 to 53 years). Thirty nine of the 116 patients (33.6 %) were ≤ 18 years old. Sweat Cl^- concentrations were all above 60 mmol/L, apart from 2 cases, described in **Supplemental material**. Fifteen patients (13%) carried nonsense mutations on both alleles and 80 patients (69%) were compound heterozygotes with a p.Phe.508del (F508del) mutation. The two most common nonsense mutations, W1282X (c.3846G>A) and

G542X (c.1585-1G>A) were present in 32.2% and 31% of the population respectively. Other stop-codon mutations present in at least 2 patients are listed in the **Supplementary Table 1**.

3.2. NPD measurements' characteristics

447 tracings from 116 subjects were considered reliable, according to standardized scoring (24). Characteristics of the measurements are shown in **Table 2** and **Supplemental Figure**. The distribution of the different parameters is presented in **Figure 2**.

At study inclusion, all patients displayed an abnormal NPD test as assessed by a response to Cl⁻-free-Isoproterenol solution ≤ -5 mV, a cut-off previously reported in different studies (25). The other NPD parameters showed typical features of CFTR dysfunction: the basal PD was below -30 mV, in relation to hyperactivity of the ENaC channel demonstrated by an increased response to amiloride (Δ Amiloride > 15 mV) (25, 26). However, the range of NPD measurements within the whole data set was large, as reported in **Table 2**; e.g., Δ amiloride ranged from 0 and 80 mV and Δ Low Cl⁻-Isoproterenol between -11 and 17 mV.

As expected, there was a strong negative correlation between the basal PD and Δ Amiloride ($R = -0.88$, $R^2 = 0.77$) (**Supplementary Table 2**). No correlation was observed between measurements related to Cl⁻ channel transport (either Δ Low Cl⁻ or Δ Isoproterenol) and those related to Na⁺ transport (either basal PD or Δ Amiloride).

3.3. NPD measurements' variability

Basal PD, Δ Low Cl⁻, Δ Isoproterenol and Δ low Cl⁻-Isoproterenol did not change significantly between visits ($p > 0.05$) (**Table 2**, **Supplemental Figure**).

To study the NPD measurements' variability, we considered the variance of the mean difference in the NPD data between visits, which represents the variability of the measurements. The components of variability for each measurement were expressed by the corresponding variance estimate. The total variability is the sum of intrasubject (or residual) variability and intersubject variability. Intersubject variability can be further decomposed into intercenter and intrinsic intersubject variability. This allows a calculation of the percentage of each variance component (intrasubject, intercenter, and intersubject) from the total variance of each measurement (**Table 3**). Basal PD and Δ Amiloride displayed the highest variabilities mainly stemming from intercenter and intrasubject components, each accounting for more than ~40% of the total variance. Δ Low Cl⁻ and Δ Isoproterenol demonstrated considerably less variability. The intercenter variability was negligible (4% and 8% of the total variance) whereas intrasubject variability accounted for ~80% of the variability.

The intrasubject measurement variability, representing the inherent error of the measurement with 95% probability, was within ± 7.2 mV for the total Cl^- conductance (Δ low Cl^- Isoproterenol) and reached ± 17.5 mV for Δ Amiloride (**Table 4**).

3.4. Factors of variability

We investigated the impact of factors potentially influencing the CFTR function such as age, sex, genotype and pulmonary exacerbation at the time of the NPD measurements. **Supplementary Table 3** shows that no significant association was found between any of these factors and the mean values of the different NPD components. We then examined whether these factors would influence the intrasubject variability of the measurements. We also studied whether a low number of patients (<10) enrolled per center might influence the variability of NPD, assuming that this might reflect the level of training of the operator. The requested outcome was the coefficient of regression between the SD of each measurement and the different covariates. Only the 100 patients who undertook all 4 NPD tests were considered in order to obtain a correct estimation of the SD.

As shown in **Table 5**, the presence of a pulmonary exacerbation at the time of the NPD test statistically significantly increased the variabilities of the Δ Isoproterenol and the total Cl^- conductance ($p=0.02$ and 0.0006 respectively) compared to those without any exacerbation at the time of NPD tests. This is reflected by the fact that the measurement error of the total Cl^- conductance was increased to -8.5 in the 41 patients who experienced at least 1 exacerbation during the clinical trial whereas it was decreased to 6.34 in the 75 patients who never experienced a pulmonary infection. A low number of patients enrolled (<10) increased the variability of basal PD and Δ Isoproterenol. Finally, other factors decreased the variability but have to be interpreted with caution because of borderline p value: older age diminished basal PD variability; men displayed a lower Δ Amiloride variability, and the presence of a nonsense mutation on both alleles decreased the variability of the Δ Isoproterenol, whereas the Δ Low Cl^- stayed uninfluenced by the genotype.

4. Discussion

This work represents the first meticulous, large-scale, multicentre study extensively analysing the inter- and intrasubject variability of NPD tests, as well as the impact of different clinical factors on the variability.

Variability of the different measurements

This group of patients carrying 2 CF causing mutations including a nonsense mutation demonstrated the expected NPD values (15-20, 21, 25, 26). As a whole, there was no

significant difference between visits for basal PD, low Cl^- and isoproterenol secretion. $\Delta\text{Amiloride}$ and ΔATP decreased significantly at the 4th visit, the reason of which is not clear. Endpoints linked to the Na^+ transport (basal PD and $\Delta\text{Amiloride}$) demonstrated important intrasubject and interindividual variabilities. The percentage of the total variance attributed to the intercenter variability for these two parameters was high, likely illustrating the operator-dependent aspect of these two measurements. In contrast, the $\Delta\text{Low Cl}^-$ and $\Delta\text{Isoproterenol}$ displayed a much more important inherent biological intrasubject variation with lower intersubject variability as well as intercenter variability, the latter observations being partly explained by the smaller dynamic range. Importantly, the sum $\Delta\text{low Cl}^-$ -Isoproterenol was the most reliable parameter with the smallest variance. This means that this parameter should be the criteria used to assess CFTR function change in CFTR modulator proof of concept studies.

Our results for intrasubject variabilities are in the same range of those of Standaert and Leonard who also reported high variability for maximal PD (respectively 6.8 and 12 SD, *versus* 10.9 SD (i.e., the square root of the total variance in our study) and $\Delta\text{Amiloride}$ (respectively 12.1 and 18.6 SD *versus* 8.9 SD in our study) but a lower variability for the sum $\Delta\text{Low Cl}^-$ -Isoproterenol (respectively 6.3 and 2.9 SD *versus* 3.7 in our study) (18, 20).

Factors influencing variability

Several factors appear to contribute to the variability of NPD measures, despite the efforts to standardize this measurement in this study.

As the same operator performed the measurements in a given center, the intercenter variability may reflect different levels of operator skill and technique. This notion is further strengthened by a greater variability in basal PD in centres with fewer recruits and in children, generally more challenging as test participants. On the other hand, the intersubject variability may be influenced by the patients' genotype. In this study, genotypes associated with total Cl^- secretion lower than -5 mV included mutations associated with alternative splicing such as E831X, reinitiation of CFTR downstream the initiation codon, such as E60X, or the presence of a wide spectrum mutation on the second allele such as D1152H (27). Other factors including pollution, concomitant nasal epithelial inflammation or pulmonary exacerbation and probably more subtle physiological variations such as estrogen variation during menstrual variation may also account for this variability (28)..

Inflammation has already been reported to influence Na^+ and Cl^- transport in cell experiments (29). Given the fact that the intrasubject variability of the $\Delta\text{Isoproterenol}$ and total Cl^-

conductance was significantly increased for the patients with pulmonary exacerbations, we could hypothesize that the inflammatory epithelial remodeling due to infection results in the increase of the variability of these NPD measurements. However, lack of data on upper airway inflammation, nor on topical medication before the tests during exacerbations, 2 factors which may affect nasal epithelial integrity and therefore NPD measures, makes this conclusion incomplete. The genotype was the second covariate that influenced the intrasubject variability of Δ Isoproterenol, which is a direct measure of CFTR activity. A nonsense mutation on both alleles was associated with a lower intrasubject variability, probably because they are associated with a total absence of Cl^- channel function, in contrast to heterozygous stop-codon genotype which may result in some residual function. Finally, females displayed a higher variability of Δ Amiloride which may be related to hormonal changes.

Limitations of the study

The current study has a few limitations. The fact that the study included centers with patients of different genetic background potentially unbalanced the results and influenced the intercenter variability. The design of the study did not allow evaluation of the intrasubject repeatability of NPD tests, which should normally be performed within a short term period. To our knowledge, this was studied only by Leonard et al who reported a large measurement error, a poor precision and a low within-subject repeatability of nasal PD measurements performed 3-5 days apart (20). Therefore, the intrasubject variability in our study encompasses the systematic technical error and any real, inherent, biological fluctuations. The absence of a control group of healthy subjects does not allow to evaluate whether an overlap exists in the average values or the variabilities of the measurements of CF patients when compared to healthy subjects. Finally, the statistical evaluation of factors accounting for variability may be jeopardized by the high number of tests and should be assessed in another set of data.

Measurement error and threshold for interpretation

Based on the intrasubject variabilities, we were able to calculate the intrasubject variability (i.e., the measurement error) for each parameter. These values can be used as cut-offs in case of testing novel drugs in order to assess the CFTR activity modulation. For instance, the measurement error for the Δ low Cl^- -Isoproterenol was estimated, with 95% probability, at ± 7.2 mV. Thus, an absolute difference greater than -7.2 mV in the Δ low Cl^- -Isoproterenol response observed in a CFTR modulator clinical trial can be attributed, with 95% probability, to a correcting effect of the treatment tested rather than to the inherent measurement error

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itself. The same goes for the Na⁺ conductance parameters, but with larger measurement errors, (± 21.4 mV for the basal PD and ± 17.4 mV for Δ Amiloride).

Our cut-offs are higher than those for the Δ Low Cl⁻-Isoproterenol of 5 mV usually used in clinical trials and that reported in the study of Leonard at 5.7 mV, but similar to that of Yaakov and when applying similar calculations to the reported SD measurements (19, 20, 30).

Importantly, these cut-offs are clearly higher than the values reported for VX-770 in CF patients with G551D mutation which demonstrated a difference of 3.7 mV in the Δ Low Cl⁻-Isoproterenol between the placebo and VX-770/150 mg group and a 5.1 mV difference between the placebo and the VX-770/250 mg group (10). This clearly indicates that establishing a biologically relevant effect by NPD (and not only statistically) in proof of concept studies requires a large correction effect to overcome the intrasubject variability.

Those results have to be compared with other biomarkers of CFTR function such as sweat chloride and intestinal current measurement (ICM) that have been used recently to assess improvement of CFTR function by CFTR modulator therapy in patients with CF (31, 32)..

Comparative studies show that these three biomarkers are not interchangeable. This is because they reflect CFTR activity differently: sweat test assess Cl⁻ reabsorption along the sweat duct; NPD is an indirect measurement of transepithelial voltage reflecting relative changes in ion conductance while Short Circuit Current is a direct measurement of active transepithelial ion transport. As NPD, ICM also shows significant variability at the individual level.

Conclusion

Our data demonstrate that the total Cl⁻ transport in response to β -adrenergic stimulation, displays the lowest variability and is the most reliable NPD parameter for monitoring between-test changes in proof of concept clinical trials of CFTR modulators. Its large intrasubject variation results in a large measurement error of 7.2 mV. .NPD test should not be performed when a subject is experiencing a pulmonary exacerbation. Minimizing test variability is an important goal which should be addressed in further standardization processes.

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Conflict of interest statements

Dr. Wilschanski reports grants from Vertex therapeutics, during the conduct of the study; .

Dr. Davies reports other from PTC Therapeutics, during the conduct of the study; other from Aligpharma AS, other from Bayer AG, other from Boehringer Ingelheim Pharma GmbH & Co. KG, other from Galapagos NV, other from ImevaX GmbH, other from Nivalis Therapeutics, Inc., other from ProQR Therapeutics III B.V., other from Proteostasis Therapeutics, INC., other from PTC Therapeutics International Limited, other from Raptor Pharmaceuticals, Inc, other from Vertex Pharmaceuticals (Europe) Limited, other from Enterprise, other from Novartis, other from Pulmocide, other from Flatley, other from Nivalis Therapeutics Inc., grants from CF Trust, other from Teva, outside the submitted work; .

Dr. Sermet-Gaudelus reports grants from Vertex Therapeutics, other from Vertex Therapeutics, other from Eloxx , other from PTC Therapeutics, other from PRO-QR, outside the submitted work; .

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Figure legends

Figure 1

Study population showing the number of patients enrolled in the study and the number of patients allocated to the PTC124 and the placebo arm. 2 subjects with aberrant NPD tracings were excluded from the study. Except 16 subjects who prematurely withdrew from the study, the remaining 100 subjects underwent NPD tests at 4 consecutive visits.

Figure 2

Distribution of different NPD endpoints at all visits

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Table 1. Patient demographic and clinical characteristics

n	116
Age, years *	23.35 (9.2)
Sex, male *	59 (50.9)
BMI (kg/m ²)*	20.69 (3)
Predicted FEV ₁ (%) *	60.29 (15)
Sweat Chloride (mM/l)(n=92) *	97.05 (14.7)
F508del on at least 1 allele **	80 (69%)
W1282X on at least 1 allele **	37 (32%)
G542X on at least 1 allele **	36 (31%)
Nonsense mutation on both alleles **	15 (13%)

Results are expressed either as mean (standard deviation)* or as number of patients (percentage of the population) **

BMI = Body Mass Index ; FEV₁ = Forced Expiratory Volume in 1 second, expressed as percentage predicted of normal values

Table 2. Characteristics of NPD measurements.

Measurements (mV)	Mean of 4 visits n=433	Visit 1 n=116	Visit 2 n=112	Visit 3 n=105	Visit 4 n=100	p-value*
Basal PD	-46.3 (16.8)	-48 (17.4)	-47.3 (17.2)	-45 (17)	-44.7 (16)	0.11
Δ Amiloride	27.5 (12.7)	29.2 (13.2)	27.7 (12.3)	26.9 (13.9)	25.8 (11.7)	0.03
Δ Low Cl ⁻	1.7 (3.7)	1.7 (3.4)	1.4 (3.6)	1.4 (4.1)	2.2 (3.8)	0.28
Δ Isoproterenol	0.03 (2.7)	0.3 (2.8)	-0.12 (2.6)	0.1 (2.8)	-0.2 (2.8)	0.41
Δ Low Cl ⁻ -Isoproterenol	1.7 (4.1)	2 (3.3)	1.2 (3.8)	1.5 (4.6)	2.03 (4.8)	0.34
Δ ATP	-18.8 (11.3)	-19.6 (12.2)	-18.6 (10.8)	-19.7 (11.6)	-16.7 (10.6)	0.01

Results are expressed as mean (standard deviation)

*Significance of the difference between mean visit values was assessed by testing the “visit” effect in a linear mixed effect model including subject and center as random effects.

Table 3. Intrasubject, intersubject and intercenter variability of NPD measurements

Variability	Total variance*	Intrasubject variance (% of total)	Intersubject variance (% of total)	p**	Intercenter variance (% of total)	p***
Basal PD	351	116 (33)	54 (15)	$<2.2 \cdot 10^{-16}$	181 (52)	$5 \cdot 10^{-11}$
Δ Amiloride	197	80 (40)	25 (13)	$<2.2 \cdot 10^{-16}$	92 (47)	$2 \cdot 10^{-10}$
Δ Low Cl^-	14	11 (78)	2.5 (18)	$<2.2 \cdot 10^{-16}$	0.5 (4)	0.66
Δ Isoproterenol	7.7	6.5 (84)	0.6 (8)	0.002	0.6 (8)	0.41
Δ low Cl^- Isoproterenol	17.4	13.9 (80)	2.4 (14)	0.00002	1.1 (6)	0.34
Δ ATP	133	56 (42)	44 (33)	$<2.2 \cdot 10^{-16}$	33 (25)	$2 \cdot 10^{-5}$

*The total variance (or variability) is equal to the sum of its three components (intersubject, intercenter, intrasubject which is the residual variance). The components of variance are expressed by the corresponding variance estimates for each measurement and their percentage of the total variance. The corresponding p-value < 0.05 means that the source of variability is present and differs significantly from 0.

**The test for intersubject variability component was performed by comparison of a fixed effect model (without random component) and a model including subject ID as random effect

*** The test for intercenter variability component was performed by comparison of a model including subject ID as random effect and a model including both subject ID and center ID as random effects.

Table 4. Measurement error of different NPD measurements.

Measurements	Measurement error (mV)
Basal PD	± 21.1
Δ Amiloride	± 17.5
Δ low Cl^-	± 6.5
Δ Isoproterenol	± 4.9
Δ low Cl^- -Isoproterenol	± 7.2
Δ ATP	± 14.7

Measurement error is calculated as ± 1.96 intrasubject SD.

Table 5. Impact of different covariates on intrasubject variability of NPD endpoints

Measurements Coefficient* p-value	Age**		Sex***		Nonsense mutation on both alleles****		Pulmonary exacerbations		< 10 patients by center	
Basal PD	-0.11	0.04	-1.32	0.19	-2.69	0.06	1.5	0.15	3.33	0.02
Δ Amiloride	-0.04	0.27	-1.75	0.03	-2.04	0.08	1.04	0.22	2.14	0.07
Δ Low Cl ⁻	-0.009	0.56	-0.36	0.2	-0.53	0.2	0.39	0.18	0.42	0.3
Δ Isoproterenol	-0.02	0.13	0.04	0.88	-0.79	0.04	0.66	0.02	0.89	0.02
Δ Low Cl ⁻ -Isoproterenol	-0.02	0.16	-0.02	0.94	-0.82	0.07	1.1	0.0006	0.53	0.24
Δ ATP	0.02	0.64	-1.33	0.09	-1.1	0.33	0.26	0.75	0.52	0.65

*Coefficient of regression between SD of NPD measurements and each covariate. SD is given by the square root of the respective variance. A negative value of the coefficient means a decrease in variability. The value of the coefficient allows to calculate the p value.

** Age is expressed per year

*** Reference group is female

**** Reference group is nonsense mutation in a single allele

Figure 1

Figure 2

Supplementary Material

[Click here to download Supplementary Material: NPD_supplemental_SERMET_R1_Clean.docx](#)

Supplementary Material

[Click here to download Supplementary Material: figure Supplemental_SERMET.pptx](#)

Insights into the variability of Nasal Potential Difference, a biomarker of CFTR activity

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Authors' contributions

SK analysed the data and wrote the manuscript. THJ and JPJ made the statistical analysis. MW, IF and JCD participated in data analysis and writing of the manuscript. ISG designed the study, analysed the data and wrote the manuscript.

Running title: Nasal Potential Difference variability

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This article has an online data supplement.

Abstract

Background: Nasal potential difference (NPD) is used to evaluate CFTR function *in vivo*.

We aimed to evaluate the intrasubject and intersubject variability of NPD measurements.

Methods: We reviewed NPD tracings of 116 patients with CF enrolled in the placebo arm of a multicenter study. Patients carried at least one nonsense mutation and underwent repeated NPD tests every 16 weeks. NPD parameters included basal potential difference (basal PD), inhibition of sodium absorption by amiloride (Δ Amiloride), chloride (Cl^-) transport in response to a Cl^- -free solution (Δ Low Cl^-), isoproterenol (Δ Isoproterenol), the sum of Δ Low Cl^- and Δ Isoproterenol (Δ Low Cl^- -Isoproterenol) and ATP (Δ ATP).

Results: Basal PD and Δ Amiloride displayed the highest variabilities, mainly stemming from intercenter and intrasubject effect. Δ Low Cl^- , Δ Isoproterenol and Δ Low Cl^- -Isoproterenol demonstrated a large intrasubject variability but a smaller intersubject variability. The intrasubject measurement variability for Δ Low Cl^- -Isoproterenol, was within ± 7.2 mV with 95% probability. It was greater in patients reporting ongoing pulmonary exacerbations.

Conclusions: The large intercenter variability of basal PD and Δ Amiloride highlights the operator-dependent aspect of these measurements. A difference greater than 7.2 mV in Δ Low Cl^- -Isoproterenol in a given patient on CFTR modulator can be attributed, with 95% probability, to a treatment effect rather than to the variability inherent in the measurement.

Word count: 227

Key words: Nasal potential difference, Biomarker, Cystic Fibrosis

Highlights

- Using nasal potential difference in proof of concept trials requires knowledge of its variability.
- We reviewed NPD tracings of 116 patients with CF enrolled in the placebo arm of a multicenter study.
- A difference greater than 7.2 mV of the total Chloride transport can be attributed, with 95% probability to a treatment effect.

Introduction

Cystic Fibrosis (CF) is the most frequent life-limiting autosomal disease in the Caucasian population. It is a channelopathy caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, which acts as a cAMP chloride (Cl⁻) channel after activation by protein kinase A (PKA). The typical phenotype includes chronic sinopulmonary disease, pancreatic exocrine insufficiency, salt loss syndrome and congenital bilateral atresia of the vas deferens (1). The diagnosis is based on a positive newborn screen test, characteristic phenotype, and/or a family history of CF in conjunction with a sweat Cl⁻ concentration > 60 mmol/L and/or identification of two CF causing mutations and/or identification of CFTR dysfunction (1). Nonetheless, CF is not always a clear-cut diagnosis (2, 3). Clinicians encounter individuals with clinical features suggestive of CF, but who do not meet the current diagnostic criteria. CF diagnosis remains equivocal in subjects with intermediate sweat chloride results (30-59 mmol/L) and identification of only one or no CF-causing mutations. In these cases, CFTR functional assays may provide further helpful information (4-6). Identification of a CFTR functional defect, e.g; increased ENaC-mediated sodium (Na⁺) absorption, and minimal/ absent Cl⁻ secretion in response to cAMP is strongly supportive of CF or CFTR-related disorder (2-3, 6)

Assessment of CFTR function can be evaluated *in vivo* by the nasal potential difference test (NPD) (7). It is now considered a diagnostic criterion for CF in the European consensus guidelines and a contributory test by the North American and the French national diagnosis consensus to clarify the diagnosis in patients with 'questionable' CF (2,3) and to confirm CFTR activity restoration in proof of concept studies, (8-10).

However, employing NPD in proof of concept multicenter trials requires knowledge of its variability to determine the reliability of this biomarker as a surrogate endpoint for CFTR activity. This led to collaborative efforts to standardize the test as much as possible (11-18). Studies assessing the repeatability and the precision of the measurement as well as the intrasubject and the intersubject variability of this test are lacking. Yaakov and Leonard reported a low within-subject repeatability in single centers (19-20). But if NPD is to be useful as a surrogate marker of CFTR function in proof of concept studies, its variability needs to be assessed in a multicenter context.

The aim of the present study was to evaluate the variability of NPD measurements in CF patients based on NPD tests performed in the placebo arm of a trial of PTC124, an orally bioavailable, small-molecule drug intended to promote ribosomal readthrough of mRNA

containing a premature stop-codon. It was tested in a phase 3, multicenter, double-blind, placebo-controlled study in patients carrying at least one nonsense mutation (21).

We investigated the intrasubject, intersubject and intercenter variability of NPD measurements among patients enrolled in the placebo arm of this study. We also assessed the impact on the variability of different factors potentially influencing CFTR function including age, sex, genotype and pulmonary exacerbation.

2. Material and methods

2.1. Study design

The data set was obtained from the placebo arm of the phase 3 study aiming to test the efficacy and the tolerability of PTC124 administered for 48 weeks in CF patients carrying stop-codon mutations (21,22). The patients were ≥ 6 years of age, had a sweat chloride concentration ≥ 60 mmol/L and carried 2 CF causing mutations with at least one stop-codon mutation. The primary endpoint of the study was the relative change in % predicted FEV₁ from baseline to week 48. The secondary endpoint was the rate of pulmonary exacerbations defined according to Fuchs criteria (23). Tertiary criteria of the study included NPD test and sweat chloride concentrations performed every 16 weeks.

2.2. NPD measurements

NPD was measured according to standardized operating procedures and with centrally made solutions (11,24). Pharmacodynamic explorations were based on PD changes after the following superfusions: 100 μ mol amiloride in Ringer's saline solution (Δ Amiloride), 100 μ mol amiloride in Cl⁻-free solution to drive CFTR-mediated chloride secretion (Δ Low Cl⁻), 10 μ mol isoproterenol in the chloride-free solution to stimulate cAMP-dependent CFTR-related Cl⁻ transport (Δ Isoproterenol). The sum of the latter 2 responses (Δ Low Cl⁻-Isoproterenol) served as an index of CFTR function. Adenosine triphosphate (Δ ATP) was superfused at the end of the test as a positive control. Signs and symptoms of pulmonary exacerbation were collected at the time of NPD test.

2.3. Statistical analysis

Values from NPD tests were extracted for both nostrils and reported as the mean value of both nostrils. NPD measurements distribution characteristics were based on their mean/SD. Correlation between variables was assessed by the Pearson correlation coefficient. To study NPD measurement variability, we first estimated the variability within the same subject over time. Variability was further decomposed into intrasubject variability and interindividual variability, the latter encompassing the intersubject and intercenter variability. To explore the

sources of variability of NPD measurements, linear mixed effect models were fitted considering the center and the subject as random effects and the other measurable factors as fixed effects. Statistical significance of these different effects was assessed by comparing the nested models including or not the evaluated effect based on the log-likelihood ratio statistical test. Random effects estimates were expressed as the variance of the evaluated NPD measurement, representing the sum of random components, e.g., intersubject, intercenter, and residual (or intrasubject) variance (or variability). They were expressed as percentage (%) of the total variance. The 95% credibility interval of intrasubject measurement 'error', e.g. the variability inherent to the measurement for the same subject, was defined as $\pm 1.96 \times \text{SD}$ of the intrasubject variance.

To explore the relationship between intrasubject variability of the NPD measurements and clinical factors, SDs of NPD measurements (NPD-SD) were computed for each subject on whom all 4 NPD tests were available. NPD-SD values for each parameter were then regressed with clinical variables using a linear regression model.

All statistical computations were performed with the R statistical environment V3.4.1 (R Foundation for Statistical Computing, Vienna Austria) and the lme4 package. P-values were considered as statistically significant when ≤ 0.05 .

3. Results

3.1. Study population

238 CF patients were enrolled in the study and 118 were allocated to the placebo arm. Two subjects presenting out of range NPD data were eliminated from our data set (1 tracing with Δ Low Cl^- at -90 mV, 1 tracing with Δ ATP at -130 mV). Sixteen patients prematurely withdrew from the study because of low compliance or side effects. The remaining 100 patients provided NPD tests at all time points (**Figure 1**). The trial was performed in 36 CF centers between August 2009 and November 2011. Three centers enrolled > 30 subjects, 13 enrolled between 10-30 subjects and 15 enrolled < 10 subjects. The baseline clinical and demographic characteristics of the placebo group patients are shown in **Table 1**. The subjects had a mean age of 23.3 years (range 8 to 53 years). Thirty nine of the 116 patients (33.6 %) were ≤ 18 years old. Sweat Cl^- concentrations were all above 60 mmol/L, apart from 2 cases, described in **Supplemental material**. Fifteen patients (13%) carried nonsense mutations on both alleles and 80 patients (69%) were compound heterozygotes with a p.Phe.508del (F508del) mutation. The two most common nonsense mutations, W1282X (c.3846G>A) and

G542X (c.1585-1G>A) were present in 32.2% and 31% of the population respectively. Other stop-codon mutations present in at least 2 patients are listed in the **Supplementary Table 1**.

3.2. NPD measurements' characteristics

447 tracings from 116 subjects were considered reliable, according to standardized scoring (24). Characteristics of the measurements are shown in **Table 2** and **Supplemental Figure**. The distribution of the different parameters is presented in **Figure 2**.

At study inclusion, all patients displayed an abnormal NPD test as assessed by a response to Cl^- -free-Isoproterenol solution ≤ -5 mV, a cut-off previously reported in different studies (25). The other NPD parameters showed typical features of CFTR dysfunction: the basal PD was below -30 mV, in relation to hyperactivity of the ENaC channel demonstrated by an increased response to amiloride (Δ Amiloride > 15 mV) (25, 26). However, the range of NPD measurements within the whole data set was large, as reported in **Table 2**; e.g., Δ amiloride ranged from 0 and 80 mV and Δ Low Cl^- -Isoproterenol between -11 and 17 mV.

As expected, there was a strong negative correlation between the basal PD and Δ Amiloride ($R = -0.88$, $R^2 = 0.77$) (**Supplementary Table 2**). No correlation was observed between measurements related to Cl^- channel transport (either Δ Low Cl^- or Δ Isoproterenol) and those related to Na^+ transport (either basal PD or Δ Amiloride).

3.3. NPD measurements' variability

Basal PD, Δ Low Cl^- , Δ Isoproterenol and Δ low Cl^- -Isoproterenol did not change significantly between visits ($p > 0.05$) (**Table 2**, **Supplemental Figure**).

To study the NPD measurements' variability, we considered the variance of the mean difference in the NPD data between visits, which represents the variability of the measurements. The components of variability for each measurement were expressed by the corresponding variance estimate. The total variability is the sum of intrasubject (or residual) variability and intersubject variability. Intersubject variability can be further decomposed into intercenter and intrinsic intersubject variability. This allows a calculation of the percentage of each variance component (intrasubject, intercenter, and intersubject) from the total variance of each measurement (**Table 3**). Basal PD and Δ Amiloride displayed the highest variabilities mainly stemming from intercenter and intrasubject components, each accounting for more than $\sim 40\%$ of the total variance. Δ Low Cl^- and Δ Isoproterenol demonstrated considerably less variability. The intercenter variability was negligible (4% and 8% of the total variance) whereas intrasubject variability accounted for $\sim 80\%$ of the variability.

The intrasubject measurement variability, representing the inherent error of the measurement with 95% probability, was within ± 7.2 mV for the total Cl^- conductance (Δ low Cl^- -Isoproterenol) and reached ± 17.5 mV for Δ Amiloride (**Table 4**).

3.4. Factors of variability

We investigated the impact of factors potentially influencing the CFTR function such as age, sex, genotype and pulmonary exacerbation at the time of the NPD measurements. **Supplementary Table 3** shows that no significant association was found between any of these factors and the mean values of the different NPD components. We then examined whether these factors would influence the intrasubject variability of the measurements. We also studied whether a low number of patients (<10) enrolled per center might influence the variability of NPD, assuming that this might reflect the level of training of the operator. The requested outcome was the coefficient of regression between the SD of each measurement and the different covariates. Only the 100 patients who undertook all 4 NPD tests were considered in order to obtain a correct estimation of the SD.

As shown in **Table 5**, the presence of a pulmonary exacerbation at the time of the NPD test statistically significantly increased the variabilities of the Δ Isoproterenol and the total Cl^- conductance ($p=0.02$ and 0.0006 respectively) compared to those without any exacerbation at the time of NPD tests. This is reflected by the fact that the measurement error of the total Cl^- conductance was increased to -8.5 in the 41 patients who experienced at least 1 exacerbation during the clinical trial whereas it was decreased to 6.34 in the 75 patients who never experienced a pulmonary infection. A low number of patients enrolled (<10) increased the variability of basal PD and Δ Isoproterenol. Finally, other factors decreased the variability but have to be interpreted with caution because of borderline p value: older age diminished basal PD variability; men displayed a lower Δ Amiloride variability, and the presence of a nonsense mutation on both alleles decreased the variability of the Δ Isoproterenol, whereas the Δ Low Cl^- stayed uninfluenced by the genotype.

4. Discussion

This work represents the first meticulous, large-scale, multicentre study extensively analysing the inter- and intrasubject variability of NPD tests, as well as the impact of different clinical factors on the variability.

Variability of the different measurements

This group of patients carrying 2 CF causing mutations including a nonsense mutation demonstrated the expected NPD values (15-20, 21, 25, 26). As a whole, there was no

significant difference between visits for basal PD, low Cl^- and isoproterenol secretion. Δ Amiloride and Δ ATP decreased significantly at the 4th visit, the reason of which is not clear. Endpoints linked to the Na^+ transport (basal PD and Δ Amiloride) demonstrated important intrasubject and interindividual variabilities. The percentage of the total variance attributed to the intercenter variability for these two parameters was high, likely illustrating the operator-dependent aspect of these two measurements. In contrast, the Δ Low Cl^- and Δ Isoproterenol displayed a much more important inherent biological intrasubject variation with lower intersubject variability as well as intercenter variability, the latter observations being partly explained by the smaller dynamic range. Importantly, the sum Δ low Cl^- -Isoproterenol was the most reliable parameter with the smallest variance. This means that this parameter should be the criteria used to assess CFTR function change in CFTR modulator proof of concept studies.

Our results for intrasubject variabilities are in the same range of those of Standaert and Leonard who also reported high variability for maximal PD (respectively 6.8 and 12 SD, *versus* 10.9 SD (i.e., the square root of the total variance in our study) and Δ Amiloride (respectively 12.1 and 18.6 SD *versus* 8.9 SD in our study) but a lower variability for the sum Δ Low Cl^- -Isoproterenol (respectively 6.3 and 2.9 SD *versus* 3.7 in our study) (18, 20).

Factors influencing variability

Several factors appear to contribute to the variability of NPD measures, despite the efforts to standardize this measurement in this study.

As the same operator performed the measurements in a given center, the intercenter variability may reflect different levels of operator skill and technique. This notion is further strengthened by a greater variability in basal PD in centres with fewer recruits and in children, generally more challenging as test participants. On the other hand, the intersubject variability may be influenced by the patients' genotype. In this study, genotypes associated with total Cl^- secretion lower than -5 mV included mutations associated with alternative splicing such as E831X, reinitiation of CFTR downstream the initiation codon, such as E60X, or the presence of a wide spectrum mutation on the second allele such as D1152H (27). Other factors including pollution, concomitant nasal epithelial inflammation or pulmonary exacerbation and probably more subtle physiological variations such as estrogen variation during menstrual variation may also account for this variability (28)..

Inflammation has already been reported to influence Na^+ and Cl^- transport in cell experiments (29). Given the fact that the intrasubject variability of the Δ Isoproterenol and total Cl^-

conductance was significantly increased for the patients with pulmonary exacerbations, we could hypothesize that the inflammatory epithelial remodeling due to infection results in the increase of the variability of these NPD measurements. However, lack of data on upper airway inflammation, nor on topical medication before the tests during exacerbations, 2 factors which may affect nasal epithelial integrity and therefore NPD measures, makes this conclusion incomplete. The genotype was the second covariate that influenced the intrasubject variability of Δ Isoproterenol, which is a direct measure of CFTR activity. A nonsense mutation on both alleles was associated with a lower intrasubject variability, probably because they are associated with a total absence of Cl^- channel function, in contrast to heterozygous stop-codon genotype which may result in some residual function. Finally, females displayed a higher variability of Δ Amiloride which may be related to hormonal changes.

Limitations of the study

The current study has a few limitations. The fact that the study included centers with patients of different genetic background potentially unbalanced the results and influenced the intercenter variability. The design of the study did not allow evaluation of the intrasubject repeatability of NPD tests, which should normally be performed within a short term period. To our knowledge, this was studied only by Leonard et al who reported a large measurement error, a poor precision and a low within-subject repeatability of nasal PD measurements performed 3-5 days apart (20). Therefore, the intrasubject variability in our study encompasses the systematic technical error and any real, inherent, biological fluctuations. The absence of a control group of healthy subjects does not allow to evaluate whether an overlap exists in the average values or the variabilities of the measurements of CF patients when compared to healthy subjects. Finally, the statistical evaluation of factors accounting for variability may be jeopardized by the high number of tests and should be assessed in another set of data.

Measurement error and threshold for interpretation

Based on the intrasubject variabilities, we were able to calculate the intrasubject variability (i.e., the measurement error) for each parameter. These values can be used as cut-offs in case of testing novel drugs in order to assess the CFTR activity modulation. For instance, the measurement error for the Δ low Cl^- -Isoproterenol was estimated, with 95% probability, at ± 7.2 mV. Thus, an absolute difference greater than -7.2 mV in the Δ low Cl^- -Isoproterenol response observed in a CFTR modulator clinical trial can be attributed, with 95% probability, to a correcting effect of the treatment tested rather than to the inherent measurement error

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itself. The same goes for the Na^+ conductance parameters, but with larger measurement errors, (± 21.4 mV for the basal PD and ± 17.4 mV for Δ Amiloride).

Our cut-offs are higher than those for the Δ Low Cl^- -Isoproterenol of 5 mV usually used in clinical trials and that reported in the study of Leonard at 5.7 mV, but similar to that of Yaakov and when applying similar calculations to the reported SD measurements (19, 20, 30).

Importantly, these cut-offs are clearly higher than the values reported for VX-770 in CF patients with G551D mutation which demonstrated a difference of 3.7 mV in the Δ Low Cl^- -Isoproterenol between the placebo and VX-770/150 mg group and a 5.1 mV difference between the placebo and the VX-770/250 mg group (10). This clearly indicates that establishing a biologically relevant effect by NPD (and not only statistically) in proof of concept studies requires a large correction effect to overcome the intrasubject variability.

Those results have to be compared with other biomarkers of CFTR function such as sweat chloride and intestinal current measurement (ICM) that have been used recently to assess improvement of CFTR function by CFTR modulator therapy in patients with CF (31, 32)..

Comparative studies show that these three biomarkers are not interchangeable. This is because they reflect CFTR activity differently: sweat test assess Cl^- reabsorption along the sweat duct; NPD is an indirect measurement of transepithelial voltage reflecting relative changes in ion conductance while Short Circuit Current is a direct measurement of active transepithelial ion transport. As NPD, ICM also shows significant variability at the individual level.

Conclusion

Our data demonstrate that the total Cl^- transport in response to β -adrenergic stimulation, displays the lowest variability and is the most reliable NPD parameter for monitoring between-test changes in proof of concept clinical trials of CFTR modulators. Its large intrasubject variation results in a large measurement error of 7.2 mV. .NPD test should not be performed when a subject is experiencing a pulmonary exacerbation. Minimizing test variability is an important goal which should be addressed in further standardization processes.

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Conflict of interest statements

Dr. Wilschanski reports grants from Vertex therapeutics, during the conduct of the study; .

Dr. Davies reports other from PTC Therapeutics, during the conduct of the study; other from Aligpharma AS, other from Bayer AG, other from Boehringer Ingelheim Pharma GmbH & Co. KG, other from Galapagos NV, other from ImevaX GmbH, other from Nivalis Therapeutics, Inc., other from ProQR Therapeutics III B.V., other from Proteostasis Therapeutics, INC., other from PTC Therapeutics International Limited, other from Raptor Pharmaceuticals, Inc, other from Vertex Pharmaceuticals (Europe) Limited, other from Enterprise, other from Novartis, other from Pulmocide, other from Flatley, other from Nivalis Therapeutics Inc., grants from CF Trust, other from Teva, outside the submitted work; .

Dr. Sermet-Gaudelus reports grants from Vertex Therapeutics, other from Vertex Therapeutics, other from Eloxx , other from PTC Therapeutics, other from PRO-QR, outside the submitted work; .

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Figure legends

Figure 1

Study population showing the number of patients enrolled in the study and the number of patients allocated to the PTC124 and the placebo arm. 2 subjects with aberrant NPD tracings were excluded from the study. Except 16 subjects who prematurely withdrew from the study, the remaining 100 subjects underwent NPD tests at 4 consecutive visits.

Figure 2

~~Mean value of each NPD measurement at the 4 different visits. V1 to V4 refers to the number of visits. n refers to the number of patients with NPD at each visit. Basal PD, Δ Low CF, Δ Isoproterenol and Δ Low CF-Isoproterenol remained stable at all time points, in contrast to Δ Amiloride, and ATP response which slightly differed between the 4 visits.~~

Figure 2

Distribution of different NPD endpoints at all visits

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