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**Title: Nasal potential measurements on the nasal floor
and under the inferior turbinate: does it matter?**

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3 **Title : Nasal potential measurements on the nasal floor and under the inferior turbinate : does it**
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5 **matter ?**
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49 **Abbreviated title :**

50 Nasal potential measurements : nasal floor vs turbinate
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55 **Keywords :**

56 Cystic fibrosis, diagnosis
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For Peer Review

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3 1 **Nasal potential measurements on the nasal floor and under the inferior turbinate: does it matter ?**
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8 3 **Aim: Measurement of nasal potential difference (NPD) is increasingly used as diagnostic test for cystic**
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10 4 **fibrosis (CF) and for *in vivo* evaluation of treatments aimed at correcting the defective function of the**
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12 5 **CFTR-protein. Several methods are used to measure NPD. This study explores the influence of the site**
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14 6 **of measurement and compares NPD results obtained on the nasal floor and under the inferior**
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16 7 **turbinate**

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19 8 **Methods: NPD was measured in 34 CF, 26 heterozygote and 61 control subjects. In every subject**
20
21 9 **measurements were taken simultaneously under the inferior turbinate in one nostril, and on the nasal**
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23 10 **floor in the other nostril. Criteria for interpretable tracings were predefined. Repeat measurements**
24
25 11 **were done in 57 persons.**

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27
28 12 **Results: More interpretable tracings were obtained under the turbinate (120/124) than on the nasal**
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30 13 **floor (109/124), $p=0.015$. Within each subject group, mean values obtained were similar for maximal**
31
32 14 **basal potential, response to amiloride and total chloride response. Both techniques discriminate well**
33
34 15 **between CF and controls. Repeatability was similar with both methods: mean differences between 2**
35
36 16 **measurements approximated zero for most values.**

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39 17 **Also after correction for different number of interpretable tracings, simulation of sample size**
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41 18 **calculation for use in CFTR corrective trials was slightly in favour of measurements obtained on the**
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43 19 **nasal floor.**

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46 20 **Conclusion: NPD measurements under the inferior turbinate and on the nasal floor have similar**
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48 21 **discriminative power for diagnostic use. Measurements under the turbinate result in a slightly higher**
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50 22 **proportion of interpretable tracings but sample size calculation slightly favours the nasal floor method.**
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3 23 **Introduction :**
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5 24 Most patients with CF have a typical clinical picture of multi-organ disease, and the diagnosis is
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8 25 confirmed by high sweat chloride concentration. In addition, genetic testing shows 2 *CFTR* mutations¹.
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10 26 However, a small number of patients have an inconclusive sweat test and only one or even no mutation
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12 27 found after genetic analysis. For these patients, further assessment of *CFTR* function is indicated^{2,3}.
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16
17 29 The initial pathophysiological step leading to the multi-organ clinical manifestations of cystic fibrosis (CF)
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19 30 is dysfunction of a single protein, the Cystic Fibrosis Transmembrane Regulator (*CFTR*). The protein is a c-
20
21 31 AMP activated chloride channel, located at the apical membrane of the respiratory epithelia. In the
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23
24 32 airways, absence or dysfunction of the *CFTR* protein leads to decreased chloride secretion and increased
25
26 33 sodium absorption. The latter is caused by loss of inhibition of the epithelial sodium channel (ENaC) by
27
28 34 the absent or dysfunctional *CFTR*⁴. This disturbed salt transport results in a dehydrated surface liquid
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31 35 layer and viscous secretions, defective mucociliary transport, chronic infection and inflammation and
32
33 36 eventually lung destruction and respiratory insufficiency⁵.
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38 38 *CFTR* function can be assessed *in vivo* by measurement of transepithelial nasal potential difference
39
40 39 (NPD). The electrical potential difference between the surface and the subepithelial layer arises from ion
41
42 40 transport through the epithelia⁶. This potential is measured *in vivo* between a surface electrode placed
43
44 41 on the nasal epithelium and an electrode in connection with the subcutaneous space. Under basal
45
46 42 conditions, net absorption of sodium through the ENaC channel results in a negative basal potential at
47
48 43 the epithelial surface. Addition of amiloride to the perfusion fluid inhibits ENaC, making the surface
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50 44 potential less negative. Perfusion with a chloridefree solution promotes chloride secretion through *CFTR*,
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52 45 thereby generating a more negative surface potential. Adding isoprenalin to the perfusion fluid further
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54 46 activates protein-kinase A regulated chloride channels, mainly *CFTR*, leading to a more negative
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3 47 epithelial surface. Basal potential and response to amiloride are indirect measurements of sodium
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5 48 transport through the mucosa, and the changes in potentials induced by perfusion with a chloridefree
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8 49 solution and isoproterenol mainly reflect the chloride transport through CFTR. Perfusion of ATP activates
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10 50 alternative chloride channels, and results in mucosal hyperpolarization independent of CFTR (figure 1).
11
12 51 Compared to controls, CF patients have defective chloride current and an increased sodium absorption
13
14 52 through the epithelium, resulting in a more negative airway lumen at baseline and no significant
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17 53 response during perfusion of chloridefree solutions, and a strongly reduced response to isoprenalin⁷
18
19 54 (figure 1). These abnormalities are present throughout the respiratory epithelium, from nose to bronchi
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21 55 and are specific to CF, as they were not found in other lung diseases such as non-CF bronchiectasis⁸.
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23
24 56 Introduced in the 80's, NPD has been a tool in exploring the ion transport defect in CF, well before the
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26 57 *CFTR* gene or protein were identified. This test has been proposed as a diagnostic tool to identify
27
28 58 patients with atypical presentation⁹⁻¹¹ and is included in diagnostic guidelines and algorithms^{1,3}. In
29
30 59 longitudinal evaluations, the test result remains the same in individuals retested over time¹². In cross-
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33 60 sectional evaluation, the degree of impairment of ion transport correlates with the clinical picture in CF
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35 61 and related disorders¹³. Therefore, improvement in ion transport could be a potential substitute
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38 62 endpoint for clinical improvement. NPD measurement has been used as primary outcome parameter in
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40 63 phase 2 trials looking for proof of concept for therapies aimed at correction of the defective CFTR ion
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42 64 transport^{14,15}.

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47 66 However, the lack of standardisation of measurement technique has been pointed out^{16,17}. Differences in
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49 67 NPD method may affect test results. The first published protocol for the measurement of NPD assessed
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51 68 the characteristics of the nasal potential in normal subjects and in CF patients^{6,7}. Nasal potential was
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54 69 most negative at the inferior surface of the turbinate, where the highest proportion of ciliated cells was
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56 70 found. Therefore, this location was proposed as the optimal site of measurement¹⁸. Because of the
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3 71 difficulty in learning this technique, a modified approach was proposed using a larger exploring catheter
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5 72 placed on the nasal floor^{8,19}. Both protocols are still used, often with adaptations, according to the
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8 73 experience of the operator and availability of disposables. To the best of our knowledge, no study
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10 74 directly compared measurements on the nasal floor with measurements under the inferior turbinate.
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14 76 **Aim of the study:**

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17 77 This study explores the difference between measurements of transepithelial nasal potential made
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19 78 simultaneously on the nasal floor and under the nasal turbinate. The proportion of successful tracings,
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21 79 the ability to discriminate between CF and control patients, and the number of patients needed to
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24 80 evaluate changes in CFTR function was assessed. Also, inpatient variability was determined for both
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26 81 methods.
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5 83 **Methods :**
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8 84 Nasal potential difference was measured simultaneously under the nasal turbinate and on the nasal floor
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10 85 following the methods previously published^{18,19}. To minimize movement, the subject sits upright with his
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12 86 chin on a headrest. The head is in flexion to allow the perfused solutions to drip out of the nose.

14 87 A PE90 tubing exploring bridge is inserted under the inferior turbinate under direct visualisation by a
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17 88 nasal speculum plus a frontal light. In the opposite nostril, a modified 8 Fr Foley bladder catheter is
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19 89 inserted along the nasal floor, close to the septum, with a side hole facing down. Both exploring bridges
20
21 90 are connected to an Ag/AgCl electrode through a continuous perfusion with Ringer solution (NaCl 8,58
22
23 91 g/l, CaCl₂.2H₂O 0,33 g/l, KCl 0.3 g/l, K₂HPO₄ 0.42 g/l, KH₂PO₄ 0.05 g/l, MgCl₂.6H₂O 0.24 g/l) at 0.1
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25 92 ml/minute. The reference bridge is a 23 G subcutaneous needle flushed with identical Ringer solution,
26
27 93 connected to an Ag/AgCl electrode. Two high impedance voltmeters (Knick Portamess®, Elektronische
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29 94 Meßgeräte, Berlin, Germany) connected to a computer recorded the potential at a frequency of 1 Hz.
30
31 95 The nasal mucosae is perfused with different solutions through a PE50 tubing attached to the PE90
32
33 96 tubing with a silicone sheath (under the turbinate), or via the second channel of the Foley catheter (nasal
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35 97 floor) (figure 1, panel c).

38 98 Sequence of test procedures was standardized as follows: maximal basal potential at the inferior surface
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40 99 of the inferior turbinate was measured, followed by the maximal basal potential on the nasal floor in the
41
42 100 other nostril. The Foley catheter is then inserted to the place of maximal potential, and secured with
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44 101 tape on the nose. The procedure is repeated in the other nostril under the turbinate, with the PE50/PE90
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46 102 catheter. Continuous recording is done during perfusion with Ringers solution (solution 1 at 5 ml/min)
47
48 103 during 1-3 minutes, until a stable value is reached (<1 mV drift over 30 seconds). Sequential perfusion is
49
50 104 then started with Ringers + amiloride 100 µM (solution 2), chloridefree sodium gluconate solution (Na
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52 105 gluconate 32.26 g/l, Ca gluconate 0.97 g/l, K gluconate 0.95 g/l, K₂HPO₄ 0.42 g/l, KH₂PO₄ 0.05 g/l,
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3 106 MgSO₄.7H₂O 0.30 g/l) + amiloride 100 μM (solution 3), chloridefree solution + amiloride 100 μM +
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5 107 isoprenaline 10 μM (solution 4), each at 5 ml/minute during 3 minutes and until a stable value is reached.
6
7 108 The final chloridefree solution containing amiloride, isoprenaline and ATP 100 μM (solution 5) is
8
9 109 perfused during 1 to 2 minutes and until a peak value is reached. The perfusion line passes through a
10
11 110 thermostatic bath set up at a temperature of 43°C, to heat the solutions to 35-37°C, measured at the tip
12
13 111 of the exploring catheters. The amiloride response is the potential difference between measurement at
14
15 112 the end of perfusion with solution 1 and the end of solution 2. Total chloride response is the difference
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17 113 between potential at the end of perfusion with solution 2 and at the end of solution 4. ATP response is
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19 114 the potential difference at the end of perfusion of solution 4 and the peak potential during solution 5,
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21 115 and is used as positive control. Ratio between amiloride response and total chloride response is
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23 116 calculated as $e^{(\text{total chloride response}/\text{amiloride response})^{10}}$. *TransPD* is the difference between potential at the end of
24
25 117 perfusion with solution 1 and at the end of perfusion with solution 4 (figure 1, panel a and b)
26
27 118 At the end of the measurement, position of the catheters is checked visually. For quality control, skin
28
29 119 potential and offset of the electrodes and of the closed circuit are measured at the start and the end of
30
31 120 the procedure. Only skin potentials more negative than -30 mV and electrode or circuit offsets below 5
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33 121 mV are allowed. Tracings were discarded if the response to amiloride and total chloride response were
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35 122 both less negative than -5 mV ('flat tracings') or if technical problems occurred (drift, large artefacts,
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37 123 position of the catheter changed during the procedure).
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41 125 Patient preference for one of the methods was assessed by a visual analogue scale. Nasal potential
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43 126 measurement was not done during periods of exacerbation of nasal symptoms. Nasal medication other
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45 127 than irrigations with normal saline and topical steroids were not allowed in the 24 hours preceding the
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47 128 test. All tests were performed by two experienced operators. The study was approved by the IRB of the
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49 129 University Hospital of Leuven, and informed consent was obtained from all participants.
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5 131 Nasal potential difference was measured in 61 non-smoking healthy subjects, without significant
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7 132 respiratory or upper airway morbidity (median age 30 years, range 20-57), 37 CF patients (median age 18
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9 133 years, range 10-35) and 26 known or obligate heterozygotes (siblings or parents of CF patients with
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11 134 median age 43 years, range 8-56). All but one of the CF patients carried two severe *CFTR* mutations, with
12
13 135 the exception of one patients compound heterozygote for G551R/3849+10 kB. The latter had a sweat
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15 136 chloride of 30 mEq/l, while the other patients had a median sweat chloride of 105 (range 86 to 124
16
17 137 mEq/l). In 41 control patients and 16 CF patients, a second measurement was done after a median delay
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19 138 of 57 days.
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26 140 SPSS 15 (SPSS Inc) was used for statistical analysis. Number of successful tracings with each method is
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28 141 compared with McNemar's test, difference of proportion of successful tracings between operators with
29
30 142 Fisher's exact test. Patient preference for one of the methods was assessed with a chi square test. NPD
31
32 143 variables are presented as median and interquartile range (IQR). Differences between the controls,
33
34 144 heterozygotes and CF subjects were assessed pairwise with the Mann-Whitney U test. Difference
35
36 145 between paired measurements on the nasal floor and under the nasal turbinate were assessed by the
37
38 146 Wilcoxon test, as were differences between repeated measurements, together with the Bland and
39
40 147 Altman method²⁰. The ability to discriminate between normal subjects and CF patients was assessed
41
42 148 with the area under the curve(AUC) of the ROC-curves²¹. Power calculations were done using Statistica 8
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44 149 (Statsoft, Inc), with a power of 80%, 2-tailed, p<0.05. Linear regression was used to explore trends over
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49 150 time.
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3 151 **Results**
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8 153 Fifteen of 124 floor tracings (first measurement in each subject) were not interpretable, 13 because of a
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10 154 'flat tracing', two because of a technical problem. Under the turbinate, 4 of 124 tracings were not
11
12 155 interpretable. Two tracings were discarded because of a technical problem and two because of catheter
13
14 156 displacement to the medial part of the nose. Less tracings taken from the inferior turbinate were
15
16
17 157 rejected ($p=0.015$). The proportion of rejected tracings was similar for both operators.
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19 158
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21 159 To compare values obtained with each technique, only paired acceptable measurements were assessed.
22
23 160 Median and IQR of NPD values are given in table 1. No significant differences were found between values
24
25 161 measured on the nasal floor and under the nasal turbinate, with only a slightly more negative maximal
26
27 162 potential in heterozygotes and a trend towards a more negative maximal potential under the inferior
28
29 163 nasal turbinate in CF patients. CF patients had significantly different mean values for all NPD parameters
30
31 164 compared to control subjects and heterozygotes. Significant difference between heterozygotes and
32
33 165 controls were only found for ATP response ($p=0.023$) and $e^{(\text{total chloride response}/\text{amiloride response})}$ ($p=0.048$)
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35 166 measured under the turbinate. There was no consistent trend over time in the measurements, and no
36
37 167 systematic difference between operators.
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42 168
43
44 169 Ability to discriminate between CF and control subjects was examined with ROC curves and optimal cut-
45
46 170 offs were calculated separately for each measurement technique. $e^{(\text{total chloride response}/\text{amiloride response})}$ and
47
48 171 *transPD* discriminate best. Cut-offs were similar for both methods, with a cut-off of 0.75 for $e^{(\text{total chloride}$
49
50 172 $\text{response}/\text{amiloride response})}$, and 7.5 mV for *transPD* resulting in a sensitivity and a specificity of 100% . Area
51
52 173 under the curve (AUC) of the ROC curve for total chloride response (figure 2) was 0.99, both on the nasal
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54 174 floor and under the nasal turbinate ($p=0.72$), making this parameter also very suitable for differentiating
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3 175 CF and control subjects. Using the classical cut-off of -5 mV, sensitivity and specificity were 94.6 % and
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5 176 97.1% under the nasal turbinate, and 94.1 % and 98.2 % on the nasal floor. To reach a sensitivity of
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7
8 177 100%, the cut-off for total chloride response would be -8 mV for the floor (with a specificity of 82.1 %)
9
10 178 and -10 mV for the turbinate (with a specificity of 78.5 %).

11
12 179
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14 180 To assess repeatability, the measurement was repeated in 41 controls and 16 CF patients, resulting in
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16
17 181 two acceptable tracings in 34 controls and 14 CF patients for the nasal floor and 38 controls and 16 CF
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19 182 patients under the turbinate. Median and IQR of differences between first and second measurement are
20
21 183 given in Table 1. Correlation between first and second measurement was adequate to excellent, with a
22
23 184 Spearman r coefficient for total chloride response of 0.71 ($p < 0.001$) for the nasal floor and 0.78
24
25 185 ($p < 0.001$) for the turbinate. Assessment of repeatability for total chloride response between the first and
26
27 186 second measurement by the Bland and Altman method is shown in figure 3. There was no correlation
28
29 187 between time elapsed between measurements and differences in NPD values obtained. In 8 control
30
31 188 subjects, measurements were repeated 4 times over 4 to 8 days. Values obtained for total chloride
32
33 189 response are shown in figure 4. Mean coefficient of variation of the total chloride response was similar
34
35 190 with both methods (37 % on the nasal floor and 41 % under the inferior turbinate).

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39 192 In 43 evaluated subjects, measurement was perceived as more convenient on the nasal floor by 17
40
41 193 subjects, under the turbinate by 11, and 15 reported no difference between techniques ($p = 0.5$).

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43 194
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45 195 Total chloride response means and standard deviations in controls and CF subjects were used to
46
47 196 calculate the number of patients needed to include in each arm of a placebo controlled study with a
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49 197 CFTR modulator. Power calculations were assuming correction of chloride response to 10, 35, 50 or 100
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51 198 % of the values observed in control subjects with power of 80% at an alpha-level of 0.05 (Table 2).

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200 **Discussion**

201 Recently, measurement of transepithelial nasal potential difference has gained interest because of its
202 potential to evaluate responses to CFTR modulators *in vivo*. This study aimed at exploring the influence
203 of the site of the measurement in the nostril as one of the major methodological differences between
204 techniques in use. The setup was identical for both techniques, except for the different site of
205 measurement and catheter. Main results are that mean NPD values were similar with both techniques,
206 and the power to discriminate between controls and CF subjects was equivalent.

207 Measurements on the floor of the nose resulted in a small but significant increase in non-interpretable
208 tracings, mainly because of more frequent tracings with low potential changes (amplitude of both
209 response to amiloride and total chloride response less than 5 mV). Low total chloride response is
210 suggestive of CF, and a cut-off of 5 mV has been chosen to differentiate control patients from CF, based
211 on earlier publications¹⁷. However, if both the amiloride response and the total chloride response are
212 weak, the latter is possibly the result of an overall poor mucosal ion transport or a poor contact of the
213 electrode with the mucosa, and not of a defective CFTR protein. Therefore, we chose to consider such
214 tracings with weak potential changes as non-interpretable. Further supporting this approach,
215 hyperpolarization during perfusion with ATP was also low (less than 5 mV) in all of these 'flat' tracings.
216 The reasons for more frequent flat tracings from the nasal floor could be diverse. The nasal floor is more
217 exposed to temperature changes, dry air or chemical irritants. The proportion of ciliated cells is lower in
218 mucosae from the nasal floor (around 50%) than at the inferior surface of the turbinate (75%)⁶.
219 Therefore, reduction of the number of 'functional' ciliated cells below a critical number leading to
220 changes in NPD is likely to occur earlier on the nasal floor.

221
222 If nasal potential difference is to be used in clinical studies of CFTR modulators, efforts should be made
223 towards using the endpoints with the highest ability to detect changes in CFTR function. A few trials

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3 224 evaluated the effect of CFTR modulators by their effect on nasal potential. Applying topical gentamicin
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5 225 on the nasal epithelium, mean total chloride response increased from 0 ± 3.6 to -5 ± 2.7 mV in treated CF
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7 226 patients harbouring a nonsense mutation¹⁵. This suggests correction in treated CF subjects to
8
9 227 approximately 40 % of the total chloride response in control subjects (-12 ± 7 mV)¹⁵. The number of
10
11 228 patients to be included in a placebo controlled study of a CFTR modulator would be slightly lower if
12
13 229 measurements were done on the nasal floor, even after correction for the higher number of non-
14
15 230 acceptable tracings (Table 2).

16
17 231 Patients' and physicians' preference should be taken into account. For measurements under the
18
19 232 turbinate, more technical skills are needed to visualize the turbinate and to place the catheter at the
20
21 233 right location in the nose. Displacement of the catheter is also more likely while securing the catheter to
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23 234 the nose. None of the techniques is accessible without experience and learning time. Our centre has
24
25 235 several years of experience measuring NPD on the nasal floor. More recently, measurement under the
26
27 236 turbinate was introduced to anticipate participation to international studies. More than 20
28
29 237 measurements per technique were performed by each operator before the start of the study, to ensure
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31 238 expertise in both techniques. Measured values and success rate did not change over time, showing
32
33 239 absence of a 'learning effect' during the data collection.

34
35 240 This head-to-head comparison illustrates that simultaneous measurements taken from the nasal floor
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37 241 and from the inferior turbinate do not differ much. Mean NPD values are similar, as is their
38
39 242 discriminating power between CF and controls and their repeatability. From the patients' perspective,
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41 243 both techniques cause similar discomfort. Measurement taken from the nasal floor have the advantages
42
43 244 of an easier technique, less risk of catheter displacement during the test, and a lower number of patients
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45 245 to include in trials of CFTR modulators, but more frequent flat tracings could obscure this advantage.
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47 246 Measurements from the inferior turbinate benefit from a higher rate of interpretable tracings, which
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49 247 could improve the data analysis for studies of CFTR modulators, as flat tracings are more difficult to
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248 define in CF patients than in controls. Incorrect placement of the catheter is more likely to occur,
249 especially in non-experienced hands, resulting in increased variability of measurements.
250

For Peer Review

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7
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254 **Legends to the figures :**

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259 Figure 1: NPD tracings in a healthy (panel a) and CF (panel b) subject. CF subjects have a more

260 negative basal PD than healthy persons. Their PD shows a larger change after

261 perfusion of the nasal mucosa with the sodium channel blocking agent amiloride and

262 very little change when CFTR chloride channel secretion is stimulated with a low

263 chloride solution or isoproterenol in the presence of amiloride. ATP, a known

264 stimulant of alternative chloride channels induces a large but transient change in PD.

265 See text for more details.

266 Panel c shows the setup for the nasal PD measurement. The reference bridge made of

267 a 23G needle (a) is inserted subcutaneously on the forearm, flushed with Ringers

268 solution in contact with a Ag/AgCl electrode (b). The exploring bridge is a PE90

269 tubing for measurement under the turbinate or a 8Fr Foley catheter for floor

270 measurements (c), inserted along the nasal mucosa and perfused at a speed of 0,1

271 ml/min with Ringers solution in contact with the second Ag/AgCl electrode (d). Both

272 Ag/AgCl electrodes are connected to a high impedance voltmeter (e) and a laptop

273 computer (f) for data acquisition. Perfusion pumps (g) push the solutions through a

274 PE50 tubing inserted next to the PE90 tubing or 8 Fr Foley catheter in the exploring

275 bridge. The PE50 tubing passes through a thermostatic bath (h) to warm the solutions

276 just before perfusion to 35-37°C measured at the tip of the exploring catheter.

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3 278 Figure 2: Left panel: Individual values for total chloride response measured on the nasal floor and
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5 279 under the inferior turbinate in controls and in CF subjects. A line is drawn at the
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7 280 cutoff of 5 mV. Right panel: ROC-curve of total chloride response for discrimination
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9 281 between control subjects and CF patients, using measurements from the nasal floor
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11 282 (dashed line) or from the inferior turbinate (full line)
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17 284 Figure 3: Left : Bland and Altman plot showing limits of agreement between the first and the
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19 285 second measurement for total chloride response on the nasal floor (left) and under the
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21 286 inferior turbinate (right) in controls and CF patients
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24 287 Right : comparison of total chloride response in the first and second measurement on
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26 288 the nasal floor (closed symbols) and under the inferior turbinate (open symbols) for
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28 289 control subjects (circles) and CF patients (triangles)
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34 291 Figure 4: Total chloride response measured on 4 occasions in 8 control subjects on the nasal floor
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36 292 (left) and under the inferior turbinate (right)
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3 1 **Nasal potential measurements on the nasal floor and under the inferior turbinate: does it matter ?**
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8 3 **Aim: Measurement of nasal potential difference (NPD) is increasingly used as diagnostic test for cystic**
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10 4 **fibrosis (CF) and for *in vivo* evaluation of treatments aimed at correcting the defective function of the**
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12 5 **CFTR-protein. Several methods are used to measure NPD. This study explores the influence of the site**
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14 6 **of measurement and compares NPD results obtained on the nasal floor and under the inferior**
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16 7 **turbinate**

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19 8 **Methods: NPD was measured in 34 CF, 26 heterozygote and 61 control subjects. In every subject**
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21 9 **measurements were taken simultaneously under the inferior turbinate in one nostril, and on the nasal**
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23 10 **floor in the other nostril. Criteria for interpretable tracings were predefined. Repeat measurements**
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25 11 **were done in 57 persons.**

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28 12 **Results: More interpretable tracings were obtained under the turbinate (120/124) than on the nasal**
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30 13 **floor (109/124), $p=0.015$. Within each subject group, mean values obtained were similar for maximal**
31
32 14 **basal potential, response to amiloride and total chloride response. Both techniques discriminate well**
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34 15 **between CF and controls. Repeatability was similar with both methods: mean differences between 2**
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36 16 **measurements approximated zero for most values.**

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39 17 **Also after correction for different number of interpretable tracings, simulation of sample size**
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41 18 **calculation for use in CFTR corrective trials was slightly in favour of measurements obtained on the**
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43 19 **nasal floor.**

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46 20 **Conclusion: NPD measurements under the inferior turbinate and on the nasal floor have similar**
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48 21 **discriminative power for diagnostic use. Measurements under the turbinate result in a slightly higher**
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50 22 **proportion of interpretable tracings but sample size calculation slightly favours the nasal floor method.**
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3 23 **Introduction :**
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5 24 Most patients with CF have a typical clinical picture of multi-organ disease, and the diagnosis is
6 confirmed by high sweat chloride concentration. In addition, genetic testing shows 2 *CFTR* mutations¹.
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10 26 However, a small number of patients have an inconclusive sweat test and only one or even no mutation
11 found after genetic analysis. For these patients, further assessment of *CFTR* function is indicated^{2,3}.
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17 29 The initial pathophysiological step leading to the multi-organ clinical manifestations of cystic fibrosis (CF)
18 is dysfunction of a single protein, the Cystic Fibrosis Transmembrane Regulator (*CFTR*). The protein is a c-
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21 AMP activated chloride channel, located at the apical membrane of the respiratory epithelia. In the
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23 31
24 32 airways, absence or dysfunction of the *CFTR* protein leads to decreased chloride secretion and increased
25 sodium absorption. The latter is caused by loss of inhibition of the epithelial sodium channel (ENaC) by
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27 33
28 34 the absent or dysfunctional *CFTR*⁴. This disturbed salt transport results in a dehydrated surface liquid
29 layer and viscous secretions, defective mucociliary transport, chronic infection and inflammation and
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31 35
32 eventually lung destruction and respiratory insufficiency⁵.
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38 38 *CFTR* function can be assessed *in vivo* by measurement of transepithelial nasal potential difference
39 (NPD). The electrical potential difference between the surface and the subepithelial layer arises from ion
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41 40 transport through the epithelia⁶. This potential is measured *in vivo* between a surface electrode placed
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43 41 on the nasal epithelium and an electrode in connection with the subcutaneous space. Under basal
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45 42 conditions, net absorption of sodium through the ENaC channel results in a negative basal potential at
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47 43 the epithelial surface. Addition of amiloride to the perfusion fluid inhibits ENaC, making the surface
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49 44 potential less negative. Perfusion with a chloridefree solution promotes chloride secretion through *CFTR*,
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51 45 thereby generating a more negative surface potential. Adding isoprenalin to the perfusion fluid further
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53 46 activates protein-kinase A regulated chloride channels, mainly *CFTR*, leading to a more negative
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3 47 epithelial surface. Basal potential and response to amiloride are indirect measurements of sodium
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5 48 transport through the mucosa, and the changes in potentials induced by perfusion with a chloridefree
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7 49 solution and isoproterenol mainly reflect the chloride transport through CFTR. Perfusion of ATP activates
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10 50 alternative chloride channels, and results in mucosal hyperpolarization independent of CFTR (figure 1).
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12 51 Compared to controls, CF patients have defective chloride current and an increased sodium absorption
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14 52 through the epithelium, resulting in a more negative airway lumen at baseline and no significant
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17 53 response during perfusion of chloridefree solutions, and a strongly reduced response to isoprenalin⁷
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19 54 (figure 1). These abnormalities are present throughout the respiratory epithelium, from nose to bronchi
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21 55 and are specific to CF, as they were not found in other lung diseases such as non-CF bronchiectasis⁸.
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24 56 Introduced in the 80's, NPD has been a tool in exploring the ion transport defect in CF, well before the
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26 57 CFTR gene or protein were identified. This test has been proposed as a diagnostic tool to identify
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28 58 patients with atypical presentation⁹⁻¹¹ and is included in diagnostic guidelines and algorithms^{1,3}. In
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30 59 longitudinal evaluations, the test result remains the same in individuals retested over time¹². In cross-
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33 60 sectional evaluation, the degree of impairment of ion transport correlates with the clinical picture in CF
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35 61 and related disorders¹³. Therefore, improvement in ion transport could be a potential substitute
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38 62 endpoint for clinical improvement. NPD measurement has been used as primary outcome parameter in
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40 63 phase 2 trials looking for proof of concept for therapies aimed at correction of the defective CFTR ion
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42 64 transport^{14,15}.

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47 66 However, the lack of standardisation of measurement technique has been pointed out^{16,17}. Differences in
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49 67 NPD method may affect test results. The first published protocol for the measurement of NPD assessed
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51 68 the characteristics of the nasal potential in normal subjects and in CF patients^{6,7}. Nasal potential was
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53 69 most negative at the inferior surface of the turbinate, where the highest proportion of ciliated cells was
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56 70 found. Therefore, this location was proposed as the optimal site of measurement¹⁸. Because of the

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3 71 difficulty in learning this technique, a modified approach was proposed using a larger exploring catheter
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5 72 placed on the nasal floor^{8,19}. Both protocols are still used, often with adaptations, according to the
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8 73 experience of the operator and availability of disposables. To the best of our knowledge, no study
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10 74 directly compared measurements on the nasal floor with measurements under the inferior turbinate.
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14 76 **Aim of the study:**

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17 77 This study explores the difference between measurements of transepithelial nasal potential made
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19 78 simultaneously on the nasal floor and under the nasal turbinate. The proportion of successful tracings,
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21 79 the ability to discriminate between CF and control patients, and the number of patients needed to
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24 80 evaluate changes in CFTR function was assessed. Also, inpatient variability was determined for both
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26 81 methods.
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5 83 **Methods :**
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8 84 Nasal potential difference was measured simultaneously under the nasal turbinate and on the nasal floor
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10 85 following the methods previously published^{18,19}. To minimize movement, the subject sits upright with his
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12 86 chin on a headrest. The head is in flexion to allow the perfused solutions to drip out of the nose.

14 87 A PE90 tubing exploring bridge is inserted under the inferior turbinate under direct visualisation by a
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16 88 nasal speculum plus a frontal light. In the opposite nostril, a modified 8 Fr Foley bladder catheter is
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18 89 inserted along the nasal floor, close to the septum, with a side hole facing down. Both exploring bridges
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20 90 are connected to an Ag/AgCl electrode through a continuous perfusion with Ringer solution (NaCl 8,58
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22 91 g/l, CaCl₂.2H₂O 0,33 g/l, KCl 0.3 g/l, K₂HPO₄ 0.42 g/l, KH₂PO₄ 0.05 g/l, MgCl₂.6H₂O 0.24 g/l) at 0.1
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24 92 ml/minute. The reference bridge is a 23 G subcutaneous needle flushed with identical Ringer solution,
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26 93 connected to an Ag/AgCl electrode. Two high impedance voltmeters (Knick Portamess®, Elektronische
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28 94 Meßgeräte, Berlin, Germany) connected to a computer recorded the potential at a frequency of 1 Hz.
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30 95 The nasal mucosae is perfused with different solutions through a PE50 tubing attached to the PE90
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32 96 tubing with a silicone sheath (under the turbinate), or via the second channel of the Foley catheter (nasal
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34 97 floor) (figure 1, panel c).

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36 98 Sequence of test procedures was standardized as follows: maximal basal potential at the inferior surface
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38 99 of the inferior turbinate was measured, followed by the maximal basal potential on the nasal floor in the
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40 100 other nostril. The Foley catheter is then inserted to the place of maximal potential, and secured with
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42 101 tape on the nose. The procedure is repeated in the other nostril under the turbinate, with the PE50/PE90
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44 102 catheter. Continuous recording is done during perfusion with Ringers solution (solution 1 at 5 ml/min)
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46 103 during 1-3 minutes, until a stable value is reached (<1 mV drift over 30 seconds). Sequential perfusion is
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48 104 then started with Ringers + amiloride 100 µM (solution 2), chloridefree sodium gluconate solution (Na
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50 105 gluconate 32.26 g/l, Ca gluconate 0.97 g/l, K gluconate 0.95 g/l, K₂HPO₄ 0.42 g/l, KH₂PO₄ 0.05 g/l,
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3 106 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.30 g/l) + amiloride 100 μM (solution 3), chloridefree solution + amiloride 100 μM +
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5 107 isoprenaline 10 μM (solution 4), each at 5 ml/minute during 3 minutes and until a stable value is reached.
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7 108 The final chloridefree solution containing amiloride, isoprenaline and ATP 100 μM (solution 5) is
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9 109 perfused during 1 to 2 minutes and until a peak value is reached. The perfusion line passes through a
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11 110 thermostatic bath set up at a temperature of 43°C, to heat the solutions to 35-37°C, measured at the tip
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13 111 of the exploring catheters. The amiloride response is the potential difference between measurement at
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15 112 the end of perfusion with solution 1 and the end of solution 2. Total chloride response is the difference
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17 113 between potential at the end of perfusion with solution 2 and at the end of solution 4. ATP response is
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19 114 the potential difference at the end of perfusion of solution 4 and the peak potential during solution 5,
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21 115 and is used as positive control. Ratio between amiloride response and total chloride response is
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23 116 calculated as $e^{(\text{total chloride response}/\text{amiloride response})^{10}}$. *TransPD* is the difference between potential at the end of
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25 117 perfusion with solution 1 and at the end of perfusion with solution 4 (figure 1, panel a and b)
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27 118 At the end of the measurement, position of the catheters is checked visually. For quality control, skin
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29 119 potential and offset of the electrodes and of the closed circuit are measured at the start and the end of
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31 120 the procedure. Only skin potentials more negative than -30 mV and electrode or circuit offsets below 5
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33 121 mV are allowed. Tracings were discarded if the response to amiloride and total chloride response were
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35 122 both less negative than -5 mV ('flat tracings') or if technical problems occurred (drift, large artefacts,
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37 123 position of the catheter changed during the procedure).
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41 125 Patient preference for one of the methods was assessed by a visual analogue scale. Nasal potential
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43 126 measurement was not done during periods of exacerbation of nasal symptoms. Nasal medication other
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45 127 than irrigations with normal saline and topical steroids were not allowed in the 24 hours preceding the
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47 128 test. All tests were performed by two experienced operators. The study was approved by the IRB of the
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49 129 University Hospital of Leuven, and informed consent was obtained from all participants.
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5 131 Nasal potential difference was measured in 61 non-smoking healthy subjects, without significant
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8 132 respiratory or upper airway morbidity (median age 30 years, range 20-57), 37 CF patients (median age 18
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10 133 years, range 10-35) and 26 known or obligate heterozygotes (siblings or parents of CF patients with
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12 134 median age 43 years, range 8-56). All but one of the CF patients carried two severe *CFTR* mutations, with
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14 135 the exception of one patients compound heterozygote for G551R/3849+10 kB. The latter had a sweat
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16 136 chloride of 30 mEq/l, while the other patients had a median sweat chloride of 105 (range 86 to 124
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18 137 mEq/l). In 41 control patients and 16 CF patients, a second measurement was done after a median delay
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20 138 of 57 days.
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26 140 SPSS 15 (SPSS Inc) was used for statistical analysis. Number of successful tracings with each method is
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28 141 compared with McNemar's test, difference of proportion of successful tracings between operators with
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30 142 Fisher's exact test. Patient preference for one of the methods was assessed with a chi square test. NPD
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32 143 variables are presented as median and interquartile range (IQR). Differences between the controls,
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34 144 heterozygotes and CF subjects were assessed pairwise with the Mann-Whitney U test. Difference
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36 145 between paired measurements on the nasal floor and under the nasal turbinate were assessed by the
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38 146 Wilcoxon test, as were differences between repeated measurements, together with the Bland and
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40 147 Altman method²⁰. The ability to discriminate between normal subjects and CF patients was assessed
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42 148 with the area under the curve(AUC) of the ROC-curves²¹. Power calculations were done using Statistica 8
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44 149 (Statsoft, Inc), with a power of 80%, 2-tailed, p<0.05. Linear regression was used to explore trends over
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3 151 **Results**
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8 153 Fifteen of 124 floor tracings (first measurement in each subject) were not interpretable, 13 because of a
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10 154 'flat tracing', two because of a technical problem. Under the turbinate, 4 of 124 tracings were not
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12 155 interpretable. Two tracings were discarded because of a technical problem and two because of catheter
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14 156 displacement to the medial part of the nose. Less tracings taken from the inferior turbinate were
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17 157 rejected ($p=0.015$). The proportion of rejected tracings was similar for both operators.
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21 159 To compare values obtained with each technique, only paired acceptable measurements were assessed.
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23 160 Median and IQR of NPD values are given in table 1. No significant differences were found between values
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25 161 measured on the nasal floor and under the nasal turbinate, with only a slightly more negative maximal
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27 162 potential in heterozygotes and a trend towards a more negative maximal potential under the inferior
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29 163 nasal turbinate in CF patients. CF patients had significantly different mean values for all NPD parameters
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31 164 compared to control subjects and heterozygotes. Significant difference between heterozygotes and
32
33 165 controls were only found for ATP response ($p=0.023$) and $e^{(\text{total chloride response}/\text{amiloride response})}$ ($p=0.048$)
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35 166 measured under the turbinate. There was no consistent trend over time in the measurements, and no
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37 167 systematic difference between operators.
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44 169 Ability to discriminate between CF and control subjects was examined with ROC curves and optimal cut-
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46 170 offs were calculated separately for each measurement technique. $e^{(\text{total chloride response}/\text{amiloride response})}$ and
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48 171 *transPD* discriminate best. Cut-offs were similar for both methods, with a cut-off of 0.75 for $e^{(\text{total chloride}$
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50 172 $\text{response}/\text{amiloride response})}$, and 7.5 mV for *transPD* resulting in a sensitivity and a specificity of 100%. Area
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52 173 under the curve (AUC) of the ROC curve for total chloride response (figure 2) was 0.99, both on the nasal
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54 174 floor and under the nasal turbinate ($p=0.72$), making this parameter also very suitable for differentiating
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3 175 CF and control subjects. Using the classical cut-off of -5 mV, sensitivity and specificity were 94.6 % and
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5 176 97.1% under the nasal turbinate, and 94.1 % and 98.2 % on the nasal floor. To reach a sensitivity of
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8 177 100%, the cut-off for total chloride response would be -8 mV for the floor (with a specificity of 82.1 %)
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10 178 and -10 mV for the turbinate (with a specificity of 78.5 %).

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14 180 To assess repeatability, the measurement was repeated in 41 controls and 16 CF patients, resulting in
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17 181 two acceptable tracings in 34 controls and 14 CF patients for the nasal floor and 38 controls and 16 CF
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19 182 patients under the turbinate. Median and IQR of differences between first and second measurement are
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21 183 given in Table 1. Correlation between first and second measurement was adequate to excellent, with a
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23 184 Spearman r coefficient for total chloride response of 0.71 ($p < 0.001$) for the nasal floor and 0.78
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25 185 ($p < 0.001$) for the turbinate. Assessment of repeatability for total chloride response between the first and
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27 186 second measurement by the Bland and Altman method is shown in figure 3. There was no correlation
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29 187 between time elapsed between measurements and differences in NPD values obtained. In 8 control
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31 188 subjects, measurements were repeated 4 times over 4 to 8 days. Values obtained for total chloride
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33 189 response are shown in figure 4. Mean coefficient of variation of the total chloride response was similar
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35 190 with both methods (37 % on the nasal floor and 41 % under the inferior turbinate).

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39 192 In 43 evaluated subjects, measurement was perceived as more convenient on the nasal floor by 17
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41 193 subjects, under the turbinate by 11, and 15 reported no difference between techniques ($p = 0.5$).

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45 195 Total chloride response means and standard deviations in controls and CF subjects were used to
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47 196 calculate the number of patients needed to include in each arm of a placebo controlled study with a
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49 197 CFTR modulator. Power calculations were assuming correction of chloride response to 10, 35, 50 or 100
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51 198 % of the values observed in control subjects with power of 80% at an alpha-level of 0.05 (Table 2).

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200 **Discussion**

201 Recently, measurement of transepithelial nasal potential difference has gained interest because of its
202 potential to evaluate responses to CFTR modulators *in vivo*. This study aimed at exploring the influence
203 of the site of the measurement in the nostril as one of the major methodological differences between
204 techniques in use. The setup was identical for both techniques, except for the different site of
205 measurement and catheter. Main results are that mean NPD values were similar with both techniques,
206 and the power to discriminate between controls and CF subjects was equivalent.

207 Measurements on the floor of the nose resulted in a small but significant increase in non-interpretable
208 tracings, mainly because of more frequent tracings with low potential changes (amplitude of both
209 response to amiloride and total chloride response less than 5 mV). Low total chloride response is
210 suggestive of CF, and a cut-off of 5 mV has been chosen to differentiate control patients from CF, based
211 on earlier publications¹⁷. However, if both the amiloride response and the total chloride response are
212 weak, the latter is possibly the result of an overall poor mucosal ion transport or a poor contact of the
213 electrode with the mucosa, and not of a defective CFTR protein. Therefore, we chose to consider such
214 tracings with weak potential changes as non-interpretable. Further supporting this approach,
215 hyperpolarization during perfusion with ATP was also low (less than 5 mV) in all of these 'flat' tracings.

216 **The reasons for more frequent flat tracings from the nasal floor could be diverse. The nasal floor is more**
217 **exposed to temperature changes, dry air or chemical irritants. The proportion of ciliated cells is lower in**
218 **mucosae from the nasal floor (around 50%) than at the inferior surface of the turbinate (75%)⁶.**
219 **Therefore, reduction of the number of 'functional' ciliated cells below a critical number leading to**
220 **changes in NPD is likely to occur earlier on the nasal floor.**

221
222 If nasal potential difference is to be used in clinical studies of CFTR modulators, efforts should be made
223 towards using the endpoints with the highest ability to detect changes in CFTR function. A few trials

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3 224 evaluated the effect of CFTR modulators by their effect on nasal potential. Applying topical gentamicin
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5 225 on the nasal epithelium, mean total chloride response increased from 0 ± 3.6 to -5 ± 2.7 mV in treated CF
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7 226 patients harbouring a nonsense mutation¹⁵. This suggests correction in treated CF subjects to
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9 227 approximately 40 % of the total chloride response in control subjects (-12 ± 7 mV)¹⁵. The number of
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11 228 patients to be included in a placebo controlled study of a CFTR modulator would be slightly lower if
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13 229 measurements were done on the nasal floor, even after correction for the higher number of non-
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15 230 acceptable tracings (Table 2).

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17 231 Patients' and physicians' preference should be taken into account. For measurements under the
18
19 232 turbinate, more technical skills are needed to visualize the turbinate and to place the catheter at the
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21 233 right location in the nose. Displacement of the catheter is also more likely while securing the catheter to
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23 234 the nose. None of the techniques is accessible without experience and learning time. Our centre has
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25 235 several years of experience measuring NPD on the nasal floor. More recently, measurement under the
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27 236 turbinate was introduced to anticipate participation to international studies. More than 20
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29 237 measurements per technique were performed by each operator before the start of the study, to ensure
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31 238 expertise in both techniques. Measured values and success rate did not change over time, showing
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33 239 absence of a 'learning effect' during the data collection.

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35 240 This head-to-head comparison illustrates that simultaneous measurements taken from the nasal floor
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37 241 and from the inferior turbinate do not differ much. Mean NPD values are similar, as is their
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39 242 discriminating power between CF and controls and their repeatability. From the patients' perspective,
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41 243 both techniques cause similar discomfort. Measurement taken from the nasal floor have the advantages
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43 244 of an easier technique, less risk of catheter displacement during the test, and a lower number of patients
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45 245 to include in trials of CFTR modulators, but more frequent flat tracings could obscure this advantage.
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47 246 Measurements from the inferior turbinate benefit from a higher rate of interpretable tracings, which
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49 247 could improve the data analysis for studies of CFTR modulators, as flat tracings are more difficult to
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248 define in CF patients than in controls. Incorrect placement of the catheter is more likely to occur,
249 especially in non-experienced hands, resulting in increased variability of measurements.

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For Peer Review

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2
3 251 Acknowledgments :
4

5 252 The authors have no conflict of interests to disclose. We are grateful to Bram Verstockt for technical
6
7
8 253 assistance and data collection, and to all the trial participants.
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For Peer Review

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3 256 **Legends to the figures :**
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15 **Figure 1: NPD tracings in a healthy (panel a) and CF (panel b) subject. CF subjects have a more**
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17 **negative basal PD than healthy persons. Their PD shows a larger change after**
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19 **perfusion of the nasal mucosa with the sodium channel blocking agent amiloride and**
20 **very little change when CFTR chloride channel secretion is stimulated with a low**
21 **chloride solution or isoproterenol in the presence of amiloride. ATP, a known**
22 **stimulant of alternative chloride channels induces a large but transient change in PD.**
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24 **See text for more details.**
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31 268 **Panel c shows the setup for the nasal PD measurement. The reference bridge made of**
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33 269 **a 23G needle (a) is inserted subcutaneously on the forearm, flushed with Ringers**
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35 **solution in contact with a Ag/AgCl electrode (b). The exploring bridge is a PE90**
36 270 **tubing for measurement under the turbinate or a 8Fr Foley catheter for floor**
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38 271 **measurements (c), inserted along the nasal mucosa and perfused at a speed of 0,1**
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40 272 **ml/min with Ringers solution in contact with the second Ag/AgCl electrode (d). Both**
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42 273 **Ag/AgCl electrodes are connected to a high impedance voltmeter (e) and a laptop**
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44 274 **computer (f) for data acquisition. Perfusion pumps (g) push the solutions through a**
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46 275 **PE50 tubing inserted next to the PE90 tubing or 8 Fr Foley catheter in the exploring**
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48 276 **bridge. The PE50 tubing passes through a thermostatic bath (h) to warm the solutions**
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50 277 **just before perfusion.**
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3 280 Figure 2: Left panel: Individual values for total chloride response measured on the nasal floor and
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5 281 under the inferior turbinate in controls and in CF subjects. A line is drawn at the
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7 282 cutoff of 5 mV. Right panel: ROC-curve of total chloride response for discrimination
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9 283 between control subjects and CF patients, using measurements from the nasal floor
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11 284 (dashed line) or from the inferior turbinate (full line)
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17 286 Figure 3: Left : Bland and Altman plot showing limits of agreement between the first and the
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19 287 second measurement for total chloride response on the nasal floor (left) and under the
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21 288 inferior turbinate (right) in controls and CF patients
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24 289 Right : comparison of total chloride response in the first and second measurement on
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26 290 the nasal floor (closed symbols) and under the inferior turbinate (open symbols) for
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28 291 control subjects (circles) and CF patients (triangles)
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34 293 Figure 4: Total chloride response measured on 4 occasions in 8 control subjects on the nasal floor
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36 294 (left) and under the inferior turbinate (right)
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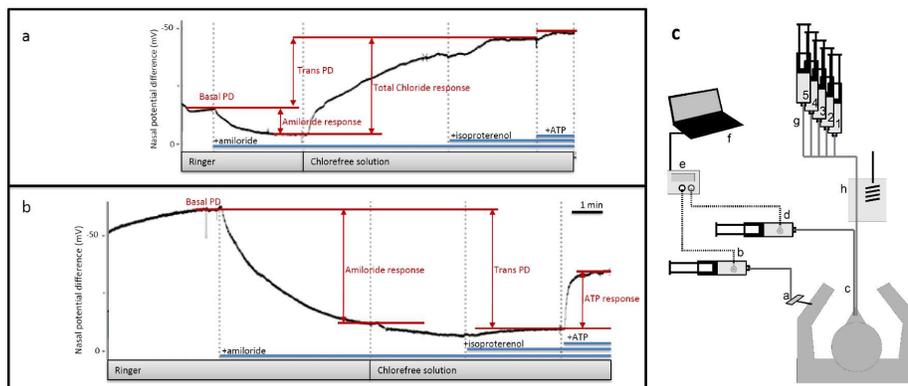
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	First measurement			Difference between first and second measurement			
	Floor		Turbinate	Floor		Turbinate	
Control patients	n=56			n=34		n=38	
Maximal potential (mV)	-16 (-20,5 ; -12)	-15,5 (-23 ; -12,5)	<i>p=0,697</i>	-0,5 (-3 ; 3,5)	<i>p=0,809</i>	-1,5 (-8,5 ; 3)	<i>p=0,184</i>
Amiloride response (mV)	7 (5 ; 9,5)	7 (5 ; 10)	<i>p=0,873</i>	-2 (-5 ; 1)	<i>p=0,045</i>	-1 (-3 ; 2)	<i>p=0,397</i>
Total chloride response (mV)	-14 (-18,5 ; -9)	-14,5 (-21 ; -10)	<i>p=0,586</i>	1 (-4 ; 5)	<i>p=0,607</i>	-0,5 (-5 ; 3)	<i>p=0,342</i>
ATP response (mV)	-5 (-8 ; -4)	-6 (-9 ; -4)	<i>p=0,309</i>	0,5 (-3 ; 4)	<i>p=0,876</i>	0 (-4 ; 3)	<i>p=0,348</i>
$e_{\text{(total chloride response/amiloride response)}}$	0,14 (0,07 ; 0,24)	0,13 (0,06 ; 0,26)	<i>p=0,928</i>	-0,03 (-0,12 ; 0,05)	<i>p=0,291</i>	-0,03 (-0,1 ; 0,04)	<i>p=0,133</i>
Transnasal PD (mV)	-7 (-10 ; -2)	-7 (-12 ; -3)	<i>p=0,444</i>	0 (-5 ; 4)	<i>p=0,614</i>	-1,5 (-6 ; 1)	<i>p=0,078</i>
CF	n=34			n=14		n=16	
Maximal potential (mV)	-41,5 (-49,5 ; -30,5)	-46,5 (-54,5 ; -36,5)	<i>p=0,516</i>	6 (-1 ; 24,5)	<i>p=0,060</i>	8 (-3 ; 22,25)	<i>p=0,023</i>
Amiloride response (mV)	28 (17 ; 39)	34,5 (20 ; 41)	<i>p=0,338</i>	-7 (-11 ; 8)	<i>p=0,362</i>	-9,5 (-22 ; 5)	<i>p=0,083</i>
Total chloride response (mV)	1 (0 ; 3)	2 (1 ; 5)	<i>p=0,258</i>	2 (-6 ; 3)	<i>p=0,728</i>	-0,5 (-5 ; 3,5)	<i>p=0,777</i>
ATP response (mV)	-9 (-16,5 ; -6,5)	-11 (-18,5 ; -8)	<i>p=0,048</i>	1 (-5 ; 3)	<i>p=1,000</i>	4 (-2 ; 10)	<i>p=0,154</i>
$e_{\text{(total chloride response/amiloride response)}}$	1,05 (1 ; 1,12)	1,07 (1,02 ; 1,19)	<i>p=0,411</i>	0,07 (-0,11 ; 0,16)	<i>p=0,397</i>	-0,04 (-0,18 ; 0,17)	<i>p=0,776</i>
Transnasal PD (mV)	29 (18 ; 40)	34,5 (22 ; 46)	<i>p=0,197</i>	-6,5 (-18 ; 10)	<i>p=0,396</i>	-8,5 (-26 ; 2,5)	<i>p=0,041</i>
Heterozygotes	n=23						
Maximal potential (mV)	-16 (-20,5 ; -12)	-18,5 (-22 ; -16)	<i>p=0,018</i>				
Amiloride response (mV)	7 (5 ; 9,5)	9 (5 ; 14)	<i>p=0,305</i>				
Total chloride response (mV)	-14 (-18,5 ; -9)	-10 (-26 ; -6)	<i>p=0,738</i>				
ATP response (mV)	-5 (-8 ; -4)	-8 (-12 ; -6)	<i>p=0,064</i>				
$e_{\text{(total chloride response/amiloride response)}}$	0,14 (0,07 ; 0,24)	0,3 (0,08 ; 0,41)	<i>p=0,144</i>				
Transnasal PD (mV)	-7 (-10 ; -2)	-4 (-11 ; 1)	<i>p=0,843</i>				

Table 1 : Median (IQR) NPD values measured on the nasal floor and under the inferior turbinate in normal subjects, CF patients and heterozygotes.

% correction of total chloride response	n of subjects in each arm	
	Floor	Turbinate
100 %	5	5
50%	14	16
35%	32	37
20%	95	114

Table 2 : Number of patients to be included in each arm of a placebo controlled trial of a CF disease modifying treatment, taking the different number of rejected tracings into account. Power calculations ($1-\beta$ at 80%, α at 0.05, 2-tailed) are based on mean and standard deviations of total chloride response in control subjects and CF patients with each method, assuming correction of total chloride response in CF patients to 20, 35, 50 and 100% of the values found in control subjects.



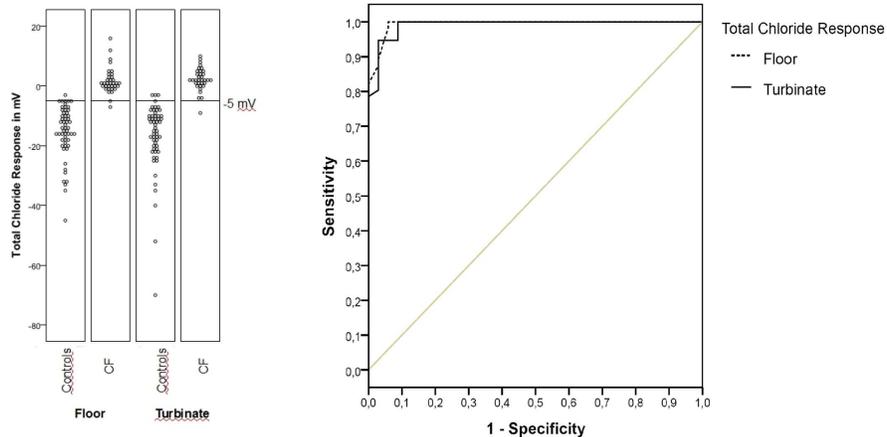
NPd tracings in a healthy (panel a) and CF (panel b) subject. CF subjects have a more negative basal PD than healthy persons. Their PD shows a larger change after perfusion of the nasal mucosa with the sodium channel blocking agent amiloride and very little change when CFTR chloride channel secretion is stimulated with a low chloride solution or isoproterenol in the presence of amiloride. ATP, a known stimulant of alternative chloride channels induces a large but transient change in PD.

See text for more details.

Panel c shows the setup for the nasal PD measurement. The reference bridge made of a 23G needle (a) is inserted subcutaneously on the forearm, flushed with Ringers solution in contact with a Ag/AgCl electrode (b). The exploring bridge is a PE90 tubing for measurement under the turbinate or a 8Fr Foley catheter for floor measurements (c), inserted along the nasal mucosa and perfused at a speed of 0,1 ml/min with Ringers solution in contact with the second Ag/AgCl electrode (d). Both Ag/AgCl electrodes are connected to a high impedance voltmeter (e) and a laptop computer (f) for data acquisition. Perfusion pumps (g) push the solutions through a PE50 tubing inserted next to the PE90 tubing or 8 Fr Foley catheter in the exploring bridge. The PE50 tubing passes through a thermostatic bath (h) to warm the solutions just before perfusion to 35-37°C measured at the tip of the exploring catheter.

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Left panel: Individual values for total chloride response measured on the nasal floor and under the inferior turbinate in controls and in CF subjects. A line is drawn at the cutoff of 5 mV. Right panel: ROC-curve of total chloride response for discrimination between control subjects and CF patients, using measurements from the nasal floor (dashed line) or from the inferior turbinate (full line)

635x323mm (96 x 96 DPI)

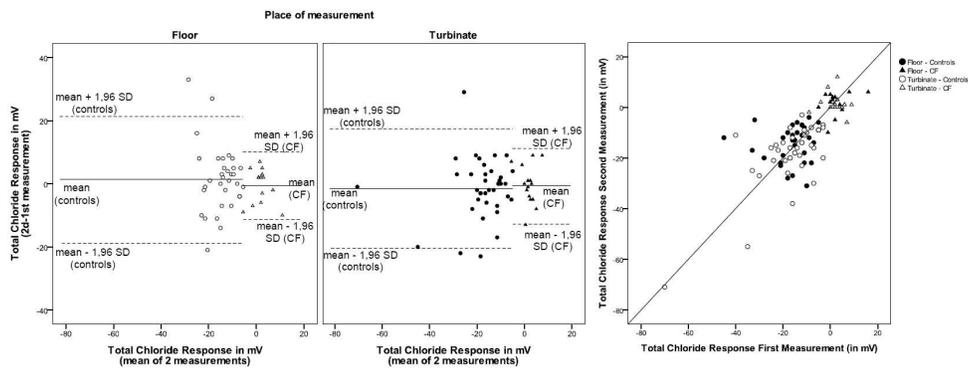
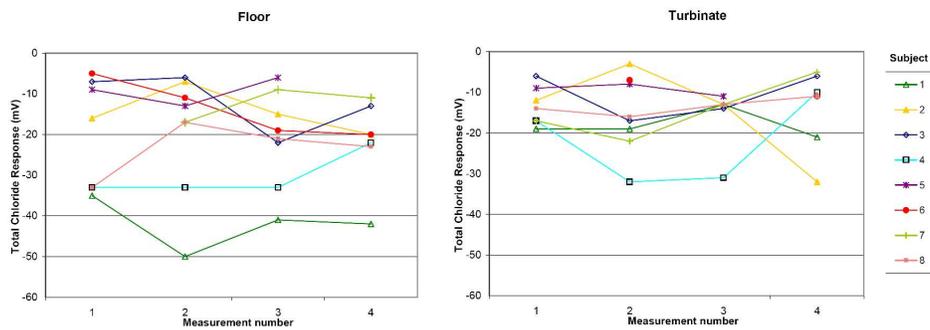


Figure 3: Left : Bland and Altman plot showing limits of agreement between the first and the second measurement for total chloride response on the nasal floor (left) and under the inferior turbinate (right) in controls and CF patients
 Right : comparison of total chloride response in the first and second measurement on the nasal floor (closed symbols) and under the inferior turbinate (open symbols) for control subjects (circles) and CF patients (triangles)

451x177mm (96 x 96 DPI)



Total chloride response measured on 4 occasions in 8 control subjects on the nasal floor (left panel) and under the inferior turbinate (right panel)
 993x347mm (96 x 96 DPI)

Peer Review

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3 Dear Dr. Vermeulen:
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5

6 Thank you for resubmitting Title : Nasal potential measurements on the nasal
7 floor and under the inferior turbinate : does it matter ? to Pediatric
8 Pulmonology.
9

10
11 Your manuscript has again been reviewed. I am pleased to report that your
12 paper has been provisionally accepted for publication in Pediatric Pulmonology
13 pending possible minor revision. One of the reviewers has the following
14 comments:
15

16 " Many thanks for addressing all the comments. I still think that Reviewer 1
17 comment 2 needs to be addressed With all due respect to the Bland Altman graph
18 (I have used this myself) I still think that the graph of chloride response
19 with both turbinate and floor results ON THE SAME FIGURE is really convincing
20 and should be in the final version "
21

22 Please consider these remarks and if you wish to change the figure then you
23 can submit a revision of the paper marked R2. If not please let me know and I
24 will send the current version on to the publisher.
25

26 Please respond within 30 days.
27
28

29 Sincerely,
30

31 Victor Chernick, MD
32 Editor-in-Chief
33 Pediatric Pulmonology
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35 ***
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38 Instructions for resubmission
39

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43 manuscript title. Click on "Create a Revision." A new window will open with
44 the reviewers comments and a box to enter your response. From this point,
45 complete the revision submission by following all the steps. Please respond in
46 a point-by-point fashion to each and every suggestion of the reviewers.
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49 discuss your work in the context of the most recent research.
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52 manuscript. Instead, you should revise your manuscript in a word processing
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54 and one "marked-up" copy.
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57 manuscript in the marked-up version. The clean version should be in all black
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59 the saved file titles contain "R2." For example, save your clean version as
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11 MUST NOT be embedded in the main text of the document. Only TIF or EPS files
12 are appropriate for figures. Tables should be uploaded as either DOC or RTF
13 files.
14

15 The following items should be included with your revision:
16

- 17
18 a) clean version of manuscript
19 b) marked up version of manuscript
20 c) point by point response to reviewers
21 d) figures (as EPS or TIF files) and tables (as DOC or RTF files), if
22 applicable
23
24

25 ***
26
27

28 Reviewers' Comments to Author:
29

30 Reviewer: 1

31 Comments to the Author

32 Many thanks for addressing all the comments.

33 I still think that Reviewer 1 comment 2 needs to be addressed With all due
34 respect to the Bland Altman graph (I have used this myself) I still think that
35 the graph of chloride response with both turbinate and floor results ON THE
36 SAME FIGURE is really convincing and should be in the final version
37
38

39 Answer from the authors :

40
41 As suggested, both graphs give similar information, but the different
42 visualizations show different aspects of the comparison between the two
43 measurements. We thus included a third graph in the figure, as you'll see in
44 the second revision
45
46
47

48 Reviewer: 2

49 Comments to the Author

50 The questions have been answered extensively.
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