

## **NPD in patients with, and at risk of ARDS -**

### **Supplemental Materials**

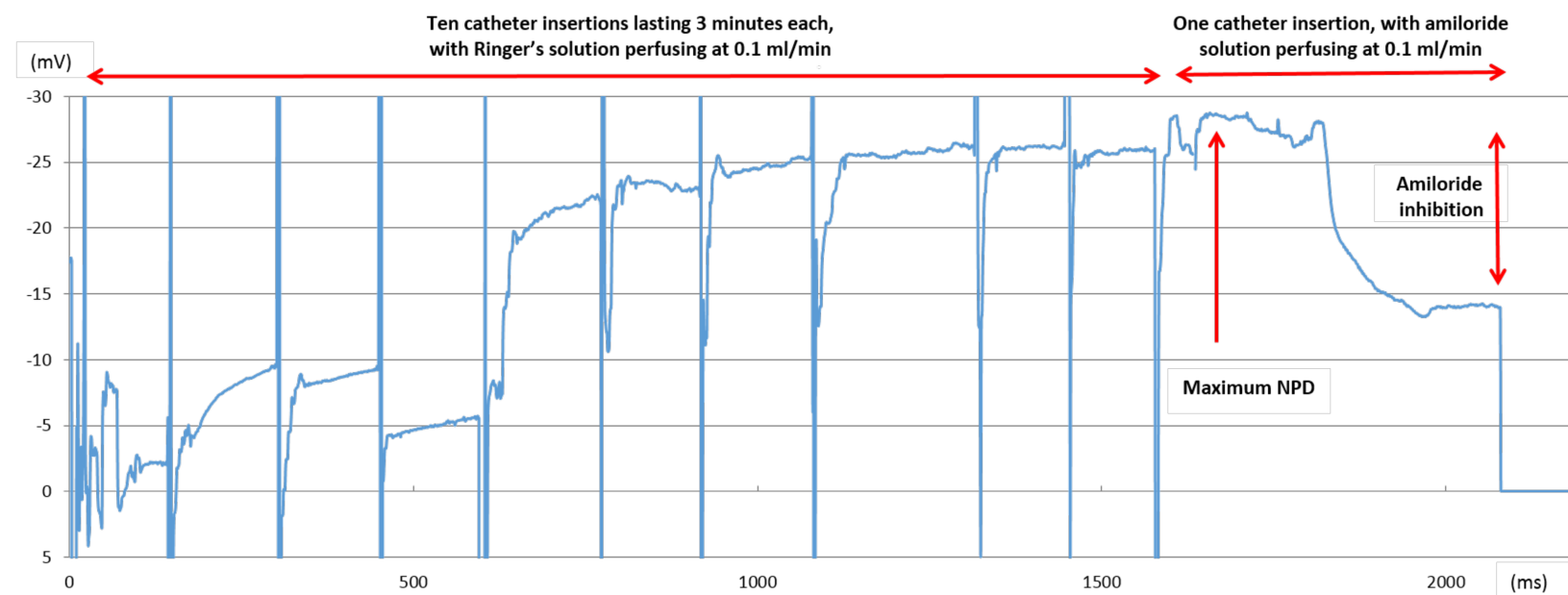
#### **1. Nasal potential difference measurement technique**

NPD was measured using a standardized technique,<sup>1</sup> but with two important modifications – catheter re-insertion and a low flow rate of the perfusing fluid.<sup>2</sup> Only the Na<sup>+</sup> specific components of a typical NPD reading were performed: maximum NPD and amiloride response. Electrode offset (<2mV) and skin PD (>-30mV) were confirmed at the start and end of each reading.

The exploring electrode consisted of an end-holed double lumen catheter (Marquat Génie Biomédical, France) with one lumen filled with electrocardiogram electrode cream (Signacreme, Parker Laboratories, USA) and connected to a high impedance voltmeter (LoganSinclair Scientific, England) via a disposable Ag Ag/Cl electrode (neuroline cup, Ambu, Denmark). The reference electrode consisted of a similar electrode secured over an area of abraded skin on the forearm. Skin abrasion was achieved with Nuprep gel (Weaver and Company, USA). The perfusate was Ringer's solution (Baxter, Europe), which has an approximate osmolality of 309 mOsm/l, pH 5.0 to 7.5 and electrolyte composition, in mmol/l, of Na<sup>+</sup> 147, K<sup>+</sup> 4, Ca<sup>++</sup> 2.25, Cl<sup>-</sup> 155.5. A second solution containing 100 µM amiloride in Ringer's solution was used to measure the ENaC sensitive component of the NPD value.

The NPD catheter was placed in the nostril not containing medical devices such as a temperature probe or nasogastric tube. If no devices were present, NPD was briefly measured in both nostrils and the side of higher initial value was chosen for the full reading. Perfusion was commenced with Ringer's solution at 0.1 ml/min; the catheter was passed along the floor of the nose and fixed with transpore tape (3M, UK) at the site of most negative NPD. After 3 minutes the catheter was withdrawn and immediately reinserted, again locating the site of most negative NPD, usually the previous location the catheter had

been sited. This process was repeated for 30 minutes totalling 10 insertions. On the eleventh, and final insertion, freshly prepared amiloride solution was perfused at 0.1 ml/min and continued for 5 minutes or longer until a stable new value had been reached. (Figure S1). The difference between the highest stable NPD during the eleventh catheter insertion, immediately prior to amiloride perfusion, and the new lowest stable NPD during amiloride perfusion was taken as the amiloride response.



### Figure S1: Example NPD reading

Ten catheter insertions are used to ensure stimulation of the nasal epithelium and a maximum response is obtained. In this instance, the maximum nasal potential difference (mNPD) is also the highest NPD during the 11<sup>th</sup> catheter insertion, from which the amiloride response is determined. If the mNPD occurs earlier in the reading, then the amiloride response will be calculated as the difference between the highest NPD during the 11<sup>th</sup> catheter insertion and the lowest stable NPD during the amiloride infusion. As the low perfusion flow rate results in a delay before the amiloride reaches the nasal epithelium, a clear lag time is seen between the commencement of the amiloride infusion and the decrease in NPD.

## 2. Pilot data investigating the effect of repeated NPD catheter insertion on NPD

### Introduction

Here we report a pilot study that was undertaken before the study reported in the main manuscript. The purpose of this pilot work was to establish NPD measurement technique and to determine the stability of repeated measurements in healthy volunteers.

### Methods

The local Ethics Committee approved the study and all subjects gave written informed consent. Only healthy, adult (18 or older), non-pregnant volunteers were included. NPD was measured using a modified standard technique.<sup>1</sup> The exploring electrode consisted of an end-holed double lumen catheter (Marquat Génie Médical, France) with one lumen filled with electrocardiogram (ECG) electrode cream (Signacreme, Parker Laboratories, USA) and connected to a high impedance voltmeter (Logan-Sinclair Scientific, England) via a Ag Ag/Cl electrode (Biosense Medical, UK). The reference electrode consisted of a second Ag Ag/Cl electrode, dipped in ECG cream, and secured with transpore tape (3M, UK) over an area of abraded skin on the forearm. Skin abrasion was achieved with Nuprep gel (Weaver and Company, USA) after the area had been cleansed with an alcohol wipe.

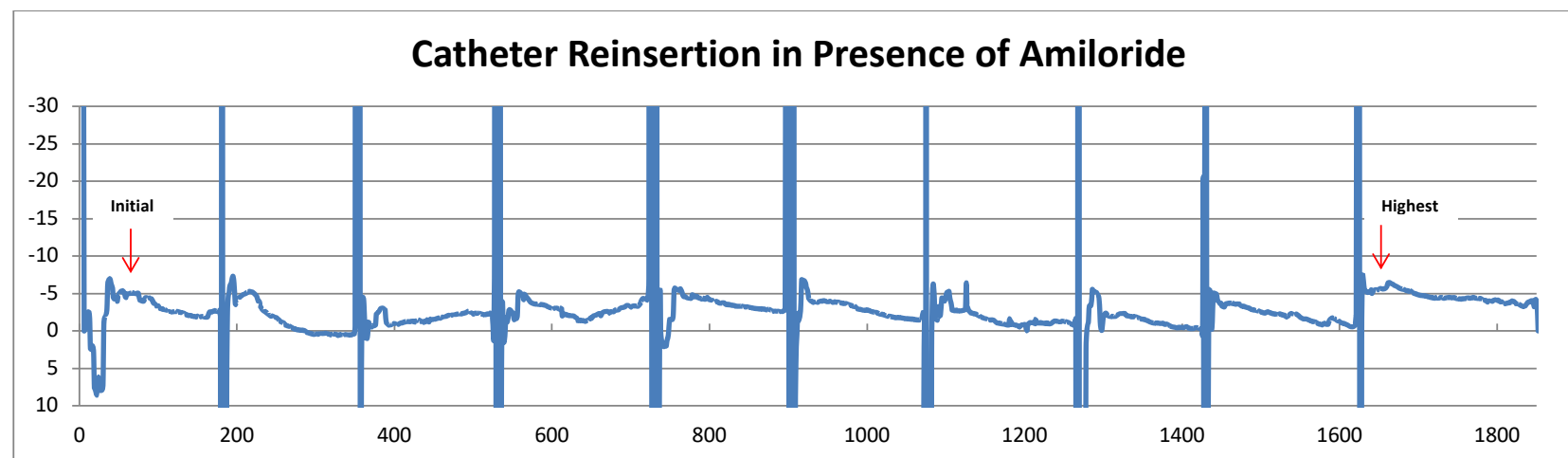
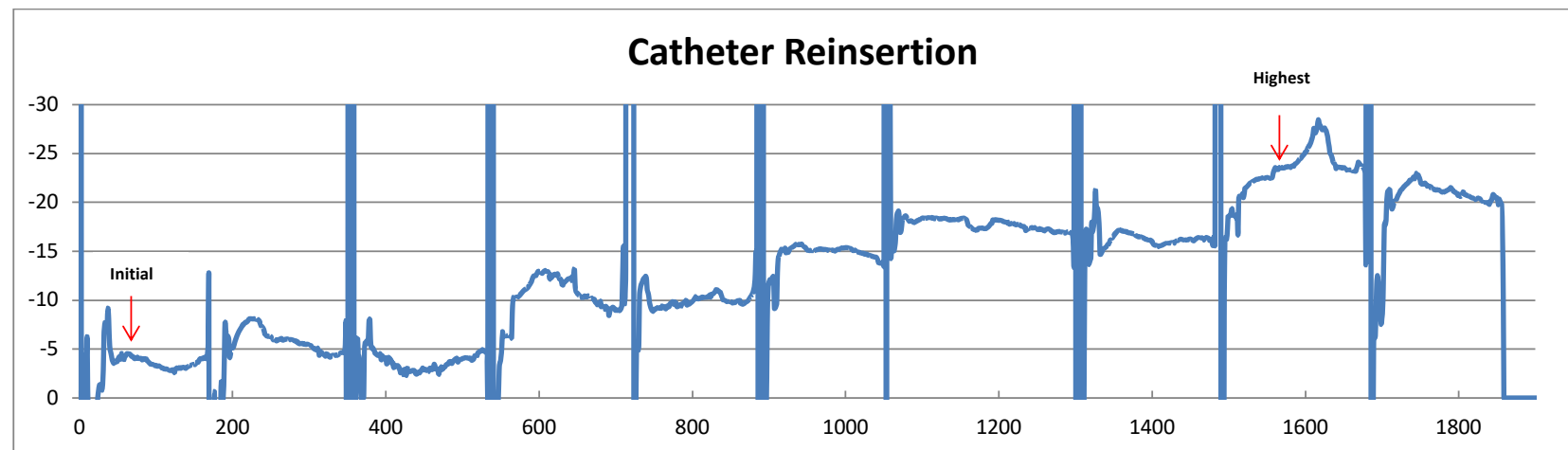
With the subject in a comfortable sitting position, perfusion with room temperature Ringer's solution was commenced at 0.1 ml/min and the catheter was passed along the floor of the nose. The site of most negative NPD was located, and the catheter fixed at this location using Transpore tape. Every three minutes the catheter was withdrawn and immediately reinserted to the site of most negative NPD. This was repeated 10 times. After 2 hours, the process was repeated using Ringer's solution containing 100  $\mu$ M amiloride. Electrode offset ( $< 2$ mV) and SPD ( $> -30$ mV) were confirmed at the start and end of each reading (Figures S2a and S2b). Only sections of the NPD reading which were stable were considered suitable for analysis, with stability being defined as the NPD changing by less than  $\pm 0.5$  mV over a 30 second period.

## Statistical Analysis

Data were analysed using Graphpad Prism version 5.01 (GraphPad Software, San Diego, USA). After testing for normality with the Kolmogorov–Smirnov test, paired t tests were used to compare the initial and maximum NPD values for both sets of readings. A p value of <0.05 was considered statistically significant.

## Results

Five healthy subjects, four male and one female, with an age of 29.8 (2.8) years, took part in this study. In the presence of Ringer's solution, the initial NPD was -13.0 (10.7) mV, which increased to -27.1 (8.2) mV following repeated catheter insertion, an increase in maximum NPD of -14.2 mV (95% CL: -8.8, -19.6;  $p=0.002$ ). When the study was repeated in the presence of amiloride no increase in NPD was observed (mean difference 2.4; 95% CL: -1.6, 6.3;  $p=0.17$ ).



**Figures S2a and S2b: Effect of catheter reinsertion on maximum nasal potential difference**

Graphs are recorded from the same subject 2 hours apart.

### 3. Statistics

#### Prospective Sample Size Calculations

##### *Primary Endpoint:*

Subjects prone to the development of HAPE had a baseline NPD 8.2mV lower than subjects resistant to its development.<sup>3</sup> Control subjects had a standard deviation (SD) of 9.6 mV and the subjects prone to the development of HAPE had a SD of 5.8 mV. As the groups both contained 33 subjects, the pooled SD was 7.7 mV.

Sample size calculations to relate NPD measurement to development of and mortality from ARDS were performed with PASS software (NCSS LLC, Kaysville, UT, USA). Logistic models which predict the development of and mortality from ARDS were simplified in order to use this program.

To use NPD measurement to predict the development of ALI a sample size of 155 patients at risk for ALI was required to achieve a power of 80% when applying a test for the fit of the logistic model using a 5% significance test.

##### *Secondary Endpoints:*

To use NPD measurement to predict mortality from ARDS a sample size of 60 subjects with ARDS was required to achieve a power of 80% when applying a test for the fit of the logistic model using a 5% significance test.

15 subjects per group were required to detect a clinically significant difference of 8.2mV between groups, using a two-sided two-sample t-test with a 5% significance level, with 80% power. These calculations were performed using the computer program nQuery Advisor version 6.01 (Statistical Solutions Ltd., Boston, MA, USA).

## Statistical Analysis

Statistical analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria). The capacity of NPD to predict outcome was assessed with the function ROC from the R package DiagnosisMed version 0.2.3 (Brasil P, DiagnosisMed: Diagnostic test accuracy evaluation for medical professionals). The optimum cut-off point was determined according to the maximum Youden Index. Binary logistic regression was performed using the R package “logistf: Firth's bias reduced logistic regression”. Comparative analyses were determined using permutation tests with bootstrapping to estimate the standard error of the mean (SEM) and 95% confidence limits (CL). Bootstrapping was undertaken using functions one.boot and two.boot from R package simpleboot version 1.1-3 (Peng RD, Simpleboot: simple bootstrap routines). Bias-corrected and accelerated confidence limits were obtained using function boot.ci from R package boot version 1.3-2 (Canty A, Ripley B, boot: Bootstrap R (S-Plus) Functions).<sup>4</sup> The one-sample and two-sample permutation tests were generated using functions onetPermutation and twotPermutation from R package DAAG version 1.06 (Maindonald J, Braun WJ, DAAG: Data analysis and graphics data and functions). The permutation test for one-way analysis of variance was done using function aovp from R package lmPerm (Wheeler B, lmPerm: permutation tests for linear models). A p-value <0.05 was considered to be statistically significant.

Unless otherwise stated, results are presented as mean (standard deviation), with bootstrapped standard error of the mean (SEM) and/or 95% CL where appropriate, apart from the correlations, which are presented as Pearson correlation coefficient with 95% CL. A p-value of <0.05 was considered statistically significant.

## References

1. Middleton PG, Geddes DM, Alton EW. Protocols for in vivo measurement of the ion transport defects in cystic fibrosis nasal epithelium. *Eur Respir J* 1994; **7**(11): 2050-6.
2. Southern KW, Noone PG, Bosworth DG, Legrys VA, Knowles MR, Barker PM. A modified technique for measurement of nasal transepithelial potential difference in infants. *J Pediatr* 2001; **139**(3): 353-8.
3. Sartori C, Duplain H, Lepori M, et al. High altitude impairs nasal transepithelial sodium transport in HAPE-prone subjects. *Eur Respir J* 2004; **23**(6): 916-20.
4. Davison A, Hinkey D. Bootstrap methods and their applications. Cambridge: Cambridge University Press; 1997.