

## Lack of a late asthmatic response after hypertonic saline challenge in children

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**ABSTRACT:** Hypertonic saline challenge has become a standardized method for measuring airway responsiveness. However, there is still uncertainty about the occurrence of a late asthmatic response. Therefore, the present study was designed to assess a possible late asthmatic response after hypertonic saline challenge in children.

Twenty-one children with mild to moderate bronchial hyperresponsiveness were studied. On days 1 and 2, forced expiratory volume in one second (FEV<sub>1</sub>) was measured hourly from 10:00 h to 22:00 h to assess diurnal variation of lung function. On the third study day, a hypertonic saline challenge was performed and FEV<sub>1</sub> was measured as on control days. The possibility of a late asthmatic response was tested by comparing FEV<sub>1</sub> levels up to 12 h after the challenge on the intervention day to FEV<sub>1</sub> levels on control days.

In no subjects were the FEV<sub>1</sub> values following the challenge found to be considerably below the individual mean of the control days. Furthermore, a nonparametric approach was applied for each child and the population looked into as a whole. Again, no late asthmatic response was detectable.

The results of this study suggest that in children with mild to moderate bronchial hyperresponsiveness a late asthmatic response does not occur 4–12 h after a 4.5% saline challenge.

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Hypertonic saline (HS) challenge has become a standardized method for measuring bronchial hyperresponsiveness (BHR) in epidemiological studies and for clinical purposes [1–3]. There is increasing evidence that the inhalation of a hypertonic aerosol creates a transient change in osmolarity of the airway surface fluid. Cell shrinkage and water loss from mucosa and submucosa seem to stimulate the production and release of endogenous substances from the epithelium and the mucosa [4, 5]. These substances act on the smooth muscle leading to contraction, activate other cells and act on blood vessels, which leads to changes in permeability and induction of airway wall oedema [6]. Mast cells, epithelial cells and nerves appear to play a key role [7–10]. The endogenous mediators released during inhalation of HS could potentially initiate an enhancement of the underlying inflammation of the airways to an extent such as to cause a late asthmatic response (LAR) and an increase of the underlying nonspecific BHR. Exercise-challenge, cold air challenge and isocapnic hyperventilation seem to involve similar mechanisms as HS in producing an early asthmatic response (EAR) *via* changes in osmolarity. However, the precise pathway is still a matter of debate and there is still controversy about the existence of an LAR in these challenges. Several studies have described the occurrence of an LAR following nonallergen challenges [11–13] but more studies have been unable to document an LAR [14–19].

To date, there is uncertainty about an LAR after inhalation of hypertonic aerosols [20] and no study has been

carried out in children. Therefore, the present study was designed to assess a possible LAR after HS challenge in children using statistical methods suggested in recently published recommendations [21].

### Materials and methods

#### Subjects

Twenty-five out of 75 children that had responded positively to a bronchial provocation test using 4.5% saline in an epidemiological study, which had been carried out 1 yr previously in Salzburg, Austria [3] agreed to be rechallenged and fulfilled the following inclusion criteria for taking part in the present study: a previous positive response to hypertonic saline challenge; an assessment of respiratory disease status by a paediatric respiratory physician; stable asthma with inhaled  $\beta_2$ -agonists on demand to control symptoms; baseline forced expiratory volume in one second (FEV<sub>1</sub>) >65% of predicted value; no inhaled corticosteroids or cromolyn during the last 2 months; no chest infection in the preceding 4 weeks; and the ability to perform reproducible pulmonary function tests.

Informed written consent was obtained from each child and at least one parent, and the study was approved by the Hospital Ethics Committee for Human Studies.

The first step of the study was to instruct each child in self-measurement of FEV<sub>1</sub> using a "hand held" spirometer (MicroLoop II; Micro Medical Ltd. Rochester, UK) with the aim of performing three consecutive FEV<sub>1</sub> readings

with a variability of <5%. The children were asked to avoid strenuous exercise during the study days which could imitate an LAR when carried out on the challenge day or mask an LAR when performed on a control day.

#### Control days

On days 1 and 2, children measured FEV<sub>1</sub> at home hourly between 10:00 h and 22:00 h. Three measurements were obtained on each occasion with variability <5%, and recorded in a protocol. The highest FEV<sub>1</sub> reading was chosen. Medication - if used - and respiratory symptoms were noted. These 2 days served as "control days" to assess diurnal changes in lung function for each child.

#### Intervention day

On day 3, all children performed an HS challenge in the Respiratory Laboratory according to a previously published standardization [2]. In brief 4.5% saline was nebulized *via* a De Vilbiss Ultraneb 2000 (De Vilbiss, Somerset, PA, USA) connected to 65 cm of corrugated aerosol tubing and a two-way non-rebreathing valve (Cat No. 560200; with diverter, Cat. No. 850500; Laerdel, Stavanger, Norway). During the challenge, the nebulizer output was kept constant and the dose of saline increased successively by doubling the inhalation time starting with 0.5 then 1, 2, 4 and 8 min with subjects inhaling at tidal volumes. FEV<sub>1</sub> was measured in duplicate 60 s after each challenge step. Lung function was measured with the same "hand held" spirometer as the child had used on control days. The challenge ended when the FEV<sub>1</sub> had fallen by  $\geq 15\%$ , or a cumulative inhalation time of 15.5 min had been achieved. The nebulizer canister plus tubing was weighed prior to the challenge and after the final step on an electronic balance to assess the total amount of aerosol nebulized and the nebulizer output-min<sup>-1</sup>. Individual dose-response curves were constructed by plotting the percentage fall in FEV<sub>1</sub> from baseline on a linear scale against the cumulative dose of aerosol delivered on a logarithmic scale. The value of the provocative dose causing a 15% fall in FEV<sub>1</sub> (PD<sub>15</sub>) was obtained by linear interpolation of the last two inhalation steps.

Children with a positive response to HS challenge subsequently inhaled a  $\beta$ -agonist (400 mcg salbutamol *via* a spacer). Comparisons of FEV<sub>1</sub> on the day of intervention with FEV<sub>1</sub> on control days started 4 h after inhalation of salbutamol.

All challenges were performed between 08:30 h and 10:00 h and FEV<sub>1</sub> measurements at home were carried out from 10:00 h to 22:00 h as on control days.

#### Statistical analysis

Data entry and analysis were made using SPSS for Windows (Release 6.0; SPSS Inc., Chicago, IL, USA). The possibility of an LAR was tested by comparing FEV<sub>1</sub> levels up to 12 h after HS challenge on intervention day to FEV<sub>1</sub> levels on control days without any intervention.

The aim of the statistical analysis was to establish whether the FEV<sub>1</sub> levels following the challenge fell considerably in comparison to the levels on normal days. This question was posed for each subject individually as well as

for the population as a whole. On the individual level, both an analysis based on the normal distribution (repeated t-tests with the significance level adjusted for multiple testing) as well as a nonparametric analysis (sign tests) were carried out. For the question generalized to the population as a whole, the sign test was carried out. (For further details see "Appendix".)

## Results

Of the 25 children initially enrolled, 22 completed the three consecutive study days. One child withdrew from the study because of time commitments, another was unable to perform reproducible FEV<sub>1</sub> measurements and one child had unstable asthma. Twenty-one of the 22 children responded positively to HS and were available for statistical analysis. A paediatric respiratory physician had diagnosed mild asthma in 18/21 children and asymptomatic BHR to 4.5% saline in 3/21 children based on respiratory symptoms, physical examination and lung function tests. Twelve children showed mild (PD<sub>15</sub>; >6 mL), and 9 children moderate (PD<sub>15</sub>; 2.01–6 mL) BHR. Further characteristics of these 21 children including atopic status, baseline FEV<sub>1</sub> % predicted, and PD<sub>15</sub> values are presented in table 1.

No child reported any deterioration of symptoms up to 12 h after the challenge test. Erroneously, two children (Subjects 2 and 19) inhaled one puff of a bronchodilator at 14:00 h and 21:00 h, respectively, on intervention day. Therefore valid comparisons are not available from 14:00 h–18:00 h in Child 2 and 22:00 h for Child 19.

Characteristics of the 21 subjects that responded positively to hypertonic saline challenge and performed forced expiratory volume in one second (FEV<sub>1</sub>) measurements on all three study days

Child	Sex	Age yrs	Atopic status	Baseline FEV <sub>1</sub> % predicted	PD <sub>15</sub>
1	M	14	Yes	89	4.1
2	F	14	Yes	87	2.1
3	M	14	Yes	93	26.0
4	M	14	Yes	94	29.0
5	M	14	Yes	92	42.5
6	M	14	Yes	77	4.6
7	M	15	Yes	75	5.4
8	M	15	Yes	84	5.5
9	M	15	Yes	83	4.8
10	M	14	Yes	89	8.8
11	F	15	Yes	90	7.0
12	M	15	Yes	94	25.7
13	F	15	Yes	92	5.4
14	M	15	ND	91	22.3
15	M	15	Yes	82	2.8
16	M	15	No	72	14.2
17	M	14	ND	92	7.2
18	M	14	Yes	88	12.4
19	M	15	No	101	23.1
20	F	15	Yes	75	3.9
21	F	15	Yes	96	8.0

\*: atopic status defined as a positive allergy test (radio-allergosorbent test RAST) or skin-prick test; PD<sub>15</sub>: provocative dose of 4.5% saline that causes a 15% fall in FEV<sub>1</sub> from baseline; M: male; F: female; ND: not done, because the child refused to undergo an allergy test.

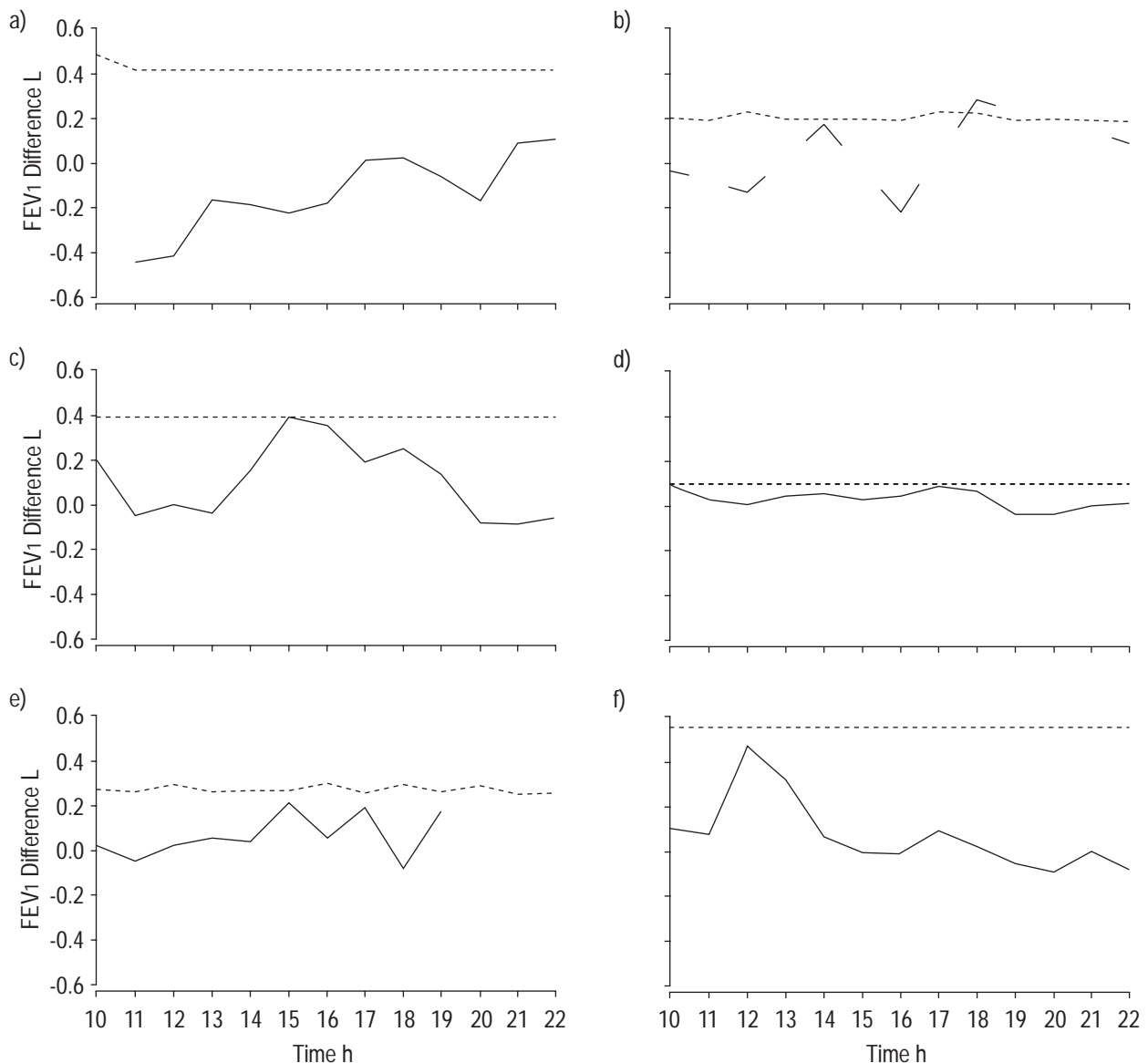


Fig. 1. – Difference in forced expiratory volume in one second (FEV<sub>1</sub>) against time for a) Child 1, b) Child 2, c) Child 8, d) Child 12, e) Child 14, f) Child 16. —: the difference between the sample mean (Control-FEV<sub>1</sub>) and the sample intervention-FEV<sub>1</sub> measurement; - - -: the critical value ( $\alpha=5\%$ ) for this difference, determined by the t-test. A late asthmatic response is indicated if the lower line crosses the upper line at two consecutive time points. The broken lower line (—) in b) and the early end of the lower line (—) in e) indicate missing values at these time points. Time is given in the 24-h clock.

#### t-tests

No subject had two consecutive significant drops in FEV<sub>1</sub> on intervention day and consequently no subject had an LAR. The results are best presented graphically and for 6 of the 21 subjects this is done in figure 1. The null hypothesis is rejected at some time point if the lower line (difference between the sample mean and the sample intervention-FEV<sub>1</sub> measurement) crosses the upper line (critical value for this difference determined by the t-test). This happened for Child 8 at 15:00 h and for Child 2 at 18:00 h. The results for Child 2 are somewhat problematical since there are no two consecutive time points 1 h apart at which both an intervention measurement and at least one control measurement were made. The other 4 children whose line diagrams are shown in figure 1 include two subjects (Child 12 and Child 14) who dem-

onstrated "almost" significant results in the sign-test (see below) and two subjects (Child 1 and Child 16) chosen randomly out of the remaining 17 who showed absolutely no evidence of any possible LAR.

SDs, degrees of freedom and number of valid comparisons for each child are presented in table 2.

#### Sign-tests

Applying the sign-test to each of the 21 subjects individually shows a trend towards a significant result in two cases: Child 12 and Child 14 ( $p=0.0547$  and  $p=0.0625$  respectively, see table 1). For Child 12, who has the smallest estimated SD, the FEV<sub>1</sub> measurement on the day of intervention is most consistently below the mean of the control measurements (fig. 1) although the drop is never

Table 2. – sds: degrees of freedom (df) and number of valid comparisons (T) (t-test), and p-values of the sign-test for all 21 subjects

Child No.	t-test			p-value of the sign test
	s	df	T	
1	0.111	12	10	0.828
2	0.063	9	4	0.500
3	0.071	8	7	0.773
4	0.256	11	9	0.746
5	0.031	13	10	0.828
6	0.255	13	9	0.746
7	0.095	9	7	1.000
8	0.104	13	10	0.377
9	0.076	13	9	0.910
10	0.040	13	10	0.989
11	0.050	8	6	0.109
12	0.026	13	10	0.055
13	0.140	11	5	0.813
14	0.071	9	7	0.063
15	0.065	11	7	0.773
16	0.150	13	10	0.377
17	0.087	11	10	1.000
18	0.116	13	10	0.377
19	0.198	12	12	0.387
20	0.053	13	10	0.172
21	0.075	11	7	0.992

s: pooled sd.

more than 2.1%. For Child 14, the FEV<sub>1</sub> on the day of intervention is also fairly consistently below the control measurements (fig. 1), with a maximum drop of 5.3%.

In the population as a whole, of 180 comparisons 84 are pluses (46.7%) giving a far from significant result ( $p=0.82$ ) (see "Appendix", paragraph 2).

### Discussion

The results of this study show no statistically significant and no clinically relevant fall in FEV<sub>1</sub> within 4–12 h after an EAR to a HS challenge in children with mild to moderate BHR, indicating that there is lack of an LAR after HS challenge. To the best of the authors' knowledge, this is the first study looking into a possible LAR after HS in children.

Verifying the existence of an LAR poses methodological problems because there is still a lack of a definition of an LAR. It is commonly described as a secondary deterioration in lung function several hours after a bronchial provocation but the amount of this decrease is not standardized. Therefore, comparisons of lung function after the challenge to diurnal variation on a control day are suggested, instead of an arbitrary chosen decrease in lung function. This method reduces the risk of false positive results due to high diurnal variations and of a false negative result in the case of very low diurnal variation. In the statistical analysis the authors applied different methods. Following STENTON *et al.* [21], FEV<sub>1</sub> measurements were compared on control and intervention days using the t-test for each child individually. This requires normally distributed values, an assumption which cannot be validated in small samples. Therefore, applying a nonparametric approach, the authors replaced the requirement of normal distributions and equal variances by the assumption that the

FEV<sub>1</sub> values on control and challenge days were symmetrically distributed about their respective means. A further approach was to look into the population as a whole using the sign-test. With these three different statistical methods, the authors were unable to detect any LAR after HS challenge.

BUSSIERES *et al.* [20] described a possible LAR after HS challenge in 2 of 12 adult subjects. In one of the two subjects, BUSSIERES *et al.* [20] proposed high diurnal fluctuation of expiratory flows to be responsible for the late fall in FEV<sub>1</sub> and possibly restriction from current medication might have contributed to some extent. The second subject with a significant difference between the maximal fall in FEV<sub>1</sub> 2–8 h after HS challenge and a control day also showed a high increase in methacholine responsiveness after HS challenge. It has been shown that the increase in nonspecific BHR to methacholine has a close relationship to the occurrence of an LAR after allergen challenge and the link between both is thought to be a complex inflammatory process in the airways [22–27]. However, there are some differences between the methods of BUSSIERES *et al.* [20] and the current study that may explain these findings. In their study, a fixed percentage of a fall in FEV<sub>1</sub> within 2–8 h after an EAR was chosen to define an LAR and both subjects with a late fall in FEV<sub>1</sub> inhaled 7.5% saline, in contrast to 4.5% saline in the present study. Besides a different statistical analysis, in the current study the FEV<sub>1</sub> comparisons were performed from 4–12 h after the HS challenge with respect to the inhalation of 400 mcg salbutamol immediately after the challenge. In the standardized protocol for the HS challenge in children, it is suggested to reverse the EAR by a  $\beta$ -agonist. This is particularly critical for field studies where children are not supervised for hours after the bronchial challenge as is the case in most laboratory studies. Theoretically, this medication could have influenced the course of an LAR and a secondary deterioration in lung function within 4 h after provocation would possibly not have been registered.

It also needs to be addressed that the current study population was fairly homogenous and did not include children with severe asthma or children with severe BHR to 4.5% saline. None of the children tested had used any anti-inflammatory medications in the previous 2 months.

Self-measurement of lung function could be criticised as being unreliable. However, in the present study each child was instructed individually by the same member of the study team in self-measuring lung function using the hand held spirometer. During the study an assessment of the technique of self-measurement of lung function was performed on the third study day. Only one child was not able to satisfy the criteria of correct self-measurement and was therefore excluded. The advantage of self-measurement was to avoid the need for an admission to hospital and to create conditions that reflect "real life".

Similar to HS challenge, hyperventilation during exercise seems to lead to hyperosmolarity in the airways [28]. Therefore, it is not unexpected that studies looking into a possible LAR after exercise challenges will be in line with the current results [14–18]. Delayed responses following recovery from EAR after exercise have been found in a minority of patients and have been suspected either to be not specifically related to the performance of exercise [14], to be a nonspecific epiphenomenon that is

neither dependent upon the existence of an early response nor is unique to exercise [15], to be an artefact due to pre-exercise bronchodilation [16] or to be related to withdrawal from bronchodilator therapy [14]. Secondary deterioration in lung function after exercise was not reproducible on two occasions one week apart [17] and was also seen on control days [14–18].

Some common mechanisms of airway narrowing have been postulated for the bronchial response to cold air and distilled water. Comparisons with the response to distilled water are somewhat difficult to interpret due to differing methodology and statistical analysis [11, 12]. In the only study looking into LAR after cold air challenge in children, VARGA *et al.* [19] found no LAR in 22 subjects as assessed by measurement of peak expiratory flow rate values.

In conclusion, the authors have shown the absence of a late asthmatic response 4–12 h after hypertonic saline challenge in children with mild to moderate bronchial hyperresponsiveness. These results further increase the safety of the bronchial provocation test with 4.5% saline, especially with regard to epidemiological studies in children.

## Appendix

### *Analysis based on the normal distribution (t-tests)*

Following STENTON *et al.* [21] the data was used to test the hypotheses by means of one sided t-tests:

$$H_{0t} : \mu_{it}(\text{Control}) - \mu_{it}(\text{Intervention}) = 0 \quad (1)$$

versus

$$H_{1t} : \mu_{it}(\text{Control}) - \mu_{it}(\text{Intervention}) > 0 \quad (2)$$

whereby  $H_{0t}$  is the null hypothesis at time  $t$  ( $t=10:00\text{h}$ ,  $11:00\text{h}$ , ...  $22:00\text{h}$ );  $H_{1t}$  is the alternative hypothesis at time  $t$ ;  $\mu_{it}(\text{Control})$  is the theoretical (population) mean of all possible measurements on control days for subject  $i$  ( $i=1, \dots, 21$ ) at time  $t$ , and  $\mu_{it}(\text{Intervention})$  is the theoretical (population) mean of all possible measurements on intervention days for subject  $i$  ( $i=1, \dots, 21$ ) at time  $t$  ( $t=10:00\text{h}$ ,  $11:00\text{h}$ , ...  $22:00\text{h}$ ).

Means, variances and sds of the FEV<sub>1</sub> measurements on control days were calculated for each subject at each time point where sufficient data was available. For each subject the sds at different time points were pooled to give a single estimate of the SD which was assumed to remain constant over time. (This approach was taken after a repeated measures ANOVA on the sds revealed that pooling over time was justified, pooling over subjects not.) For each subject, the pooled variance estimate was calculated by averaging the estimates calculated at the separate time points. The pooled SD is then the square root of this average variance and the number of degrees of freedom associated with this pooled SD equals the number of time-points at which two control measurements were made

The number of  $t$ -tests carried out for each subject ( $T_i$ ), equals the number of time points at which valid comparisons between control and intervention measurements can be made. A valid comparison can be made at time  $t$  if at least one control measurement is available, an intervention measurement is available and no medication has been taken during the previous 4 h. For each subject the significance levels for the  $T_i$  separate  $t$ -tests are adjusted for

multiple testing so that for each subject an overall 5% significance level is maintained. A subject is said to have a late asthmatic response if there are two consecutive time points  $t$  and  $t-1$  at which the null hypotheses  $H_{0t}$  and  $H_{0t+1}$  are rejected in favour of the alternative hypotheses  $H_{1t}$  and  $H_{1,t+1}$ .

### *A nonparametric approach*

Replacing the assumptions of normal distributions with equal variances by the much weaker assumption that the control and intervention forced expiratory volume in one second measurements were symmetrically distributed about their respective means the authors tested:

$$H_0 : \mu_{\text{Intervention}} = \mu_{\text{Control}} \quad (3)$$

versus

$$H_1 : \mu_{\text{Intervention}} < \mu_{\text{Control}} \quad (4)$$

for each subject individually by the sign-test. Where,  $H_0$  is the null hypothesis;  $H_1$  is the alternative hypothesis;  $\mu_{\text{Intervention}}$  is the population mean of FEV<sub>1</sub> levels on the intervention day; and  $\mu_{\text{Control}}$  is the population mean FEV<sub>1</sub> on control days. The authors compared the measured mean FEV<sub>1</sub> level on control days with the level on intervention days at all time points, recording a plus if the control level was higher, a minus if the control level was lower. Then the authors tested whether the probability of obtaining a positive comparison was equal to or higher than 0.5. In order to answer the question whether in the population as a whole the mean FEV<sub>1</sub> level following an intervention dropped below the mean "normal" level, the sign-test procedure was extended by accumulating the counts of plus and minus over all subjects.

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