



# The leaky lung test: A pilot study using inhaled mannitol to measure airway barrier function in asthma

Steve Georas, Nicole Ransom, Sara Hillman, Sophia Eliseeva, Janelle Veazey, Timothy Smyth, Kim Le & Jon Meddings

To cite this article: Steve Georas, Nicole Ransom, Sara Hillman, Sophia Eliseeva, Janelle Veazey, Timothy Smyth, Kim Le & Jon Meddings (2018): The leaky lung test: A pilot study using inhaled mannitol to measure airway barrier function in asthma, Journal of Asthma, DOI: [10.1080/02770903.2018.1536145](https://doi.org/10.1080/02770903.2018.1536145)

To link to this article: <https://doi.org/10.1080/02770903.2018.1536145>

 View supplementary material 

 Published online: 16 Nov 2018.

 Submit your article to this journal 

 Article views: 8

 View Crossmark data 



# The leaky lung test: A pilot study using inhaled mannitol to measure airway barrier function in asthma

Steve Georas, MD<sup>a,b,c</sup>, Nicole Ransom, RRT<sup>a</sup>, Sara Hillman, RN<sup>a</sup>, Sophia Eliseeva, BS<sup>a</sup>, Janelle Veazey, BS<sup>b</sup>, Timothy Smyth, BS<sup>c</sup>, Kim Le, BS<sup>d</sup>, and Jon Meddings, MD<sup>d</sup>

<sup>a</sup>Department of Medicine, University of Rochester Medical Center, Rochester, NY, USA; <sup>b</sup>Department of Microbiology & Immunology, University of Rochester Medical Center, Rochester, NY, USA; <sup>c</sup>Department of Environmental Health Sciences, University of Rochester Medical Center, Rochester, NY, USA; <sup>d</sup>Cumming School of Medicine, University of Calgary, Calgary, Alberta

## ABSTRACT

**Objective:** Airway epithelial barrier dysfunction is emerging as an important feature of asthma pathogenesis, but this is difficult to measure in individual subjects. We aimed to develop a noninvasive way to measure airway permeability in asthma. **Methods:** Healthy controls and subjects with mild asthma inhaled dry powder mannitol in a dose-escalating manner on two separate occasions, stopping at 155 mg or 315 mg. Serum mannitol levels were measured at baseline and then 30, 90, and 150 min after mannitol inhalation. Mannitol absorption was compared with measurements of airflow obstruction (FEV1) and airway inflammation (FeNO). **Results:** Serum mannitol levels increased in a time- and dose-dependent manner in both healthy control and subjects with asthma. There were no significant differences in mannitol absorption when comparing healthy controls and subjects with asthma. Mannitol absorption did not correlate with markers of airway obstruction or inflammation. **Conclusions:** Measuring serum concentrations of mannitol after inhalation challenge can potentially provide insights into airway barrier function in asthma.

**Abbreviations:** FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; CCSP: club cell secretory protein (CCSP); FDA: Food and Drug Administration; HPLC: high-performance liquid chromatography; COPD: chronic obstructive pulmonary disease; ICS: inhaled corticosteroids; LABA: long-acting beta agonist; LTRA: leukotriene receptor antagonist; AUC: area under the curve; FeNO: Fractional exhaled nitric oxide

## ARTICLE HISTORY

Received 17 July 2018  
Revised 23 September 2018  
Accepted 10 October 2018

## KEYWORDS

Asthma; airway epithelial cell; epithelial barrier dysfunction; airway inflammation; tight junction

## Introduction

Airway epithelial cells form a barrier to the outside world, and are a key part of the mucosal immune system in the lung [1]. Epithelial cells can be divided into secretory and ciliated subsets. Secretory cells produce airway mucins and other protective substances, whereas ciliated cells propel airway mucus upwards and out of the lung [2]. Secretory and ciliated epithelial cells are joined together by cell surface junction proteins through homotypic and heterotypic interactions [3]. Junction proteins can be broadly categorized into the apical tight junctions (including members of the claudin and MARVEL family), and subapical adherens junctions (including cadherin family-members). There is growing evidence that dysfunction of junction proteins contributes to airway diseases

including asthma, cystic fibrosis, and COPD [4,5]. For example, airway cells and biopsies from patients with these diseases show reduced expression and function of junction proteins, which in the case of asthma is associated with more severe disease [6–8]. The mechanisms for junction protein dysfunction in disease states are not entirely clear, and both genetic predisposition as well environmental exposures have been implicated in this process [4].

Although obtaining lower airway cells and biopsies has proven useful for the analysis of junction protein expression and function in patients with airway diseases, this requires bronchoscopy and is not a practical approach for large scale studies. Non-invasive ways to measure epithelial barrier integrity would therefore be useful. One noninvasive way to analyze

**CONTACT** Steve N. Georas, MD  [steve\\_georas@urmc.rochester.edu](mailto:steve_georas@urmc.rochester.edu)  Department of Medicine, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, 14624 NY, USA.

Color versions of one or more of the figures in the article can be found online at [www.tandfonline.com/ijas](http://www.tandfonline.com/ijas).

 Supplemental data for this article can be accessed on the [publisher's website](#).

© 2018 Taylor & Francis Group, LLC

epithelial function is by measuring the concentration of molecules such as club cell secretory protein (CCSP) in systemic circulation [9]. CCSP is normally contained in the thin layer of fluids that coats the epithelial cell surface. In the presence of intact tight junctions and normal epithelial barrier function, little CCSP is thought to escape from the airway lumen into the circulation, whereas elevated serum CCSP concentrations are thought to indicate reduced airway epithelial barrier integrity. Supporting this possibility, elevated serum CCSP has been detected after respiratory virus infection or in association with air pollution exposure [10–13]. However, this approach assumes that airway epithelial CCSP production is constant over time and not affected by disease state or environmental exposures. In fact, epithelial CCSP production is reduced in asthma [14], which may help explain the observation that serum CCSP levels tend to be lower in asthmatics compared to controls [15,16].

We reasoned that a direct method to measure airway epithelial barrier function would be useful, and envisioned a test in which subjects inhale an inert compound followed by measurements of its absorption into the blood stream. We were inspired by studies of intestinal permeability, which measure the absorption of swallowed lactulose and mannitol [17,18]. To be a useful test agent, the inhaled compound would need to: (i) be safe and well tolerated, (ii) translocate across epithelial barriers via paracellular routes, and (iii) not undergo extensive metabolism once absorbed into the bloodstream. Mannitol fulfills each of these criteria. First, mannitol is a widely used food additive generally recognized as safe by the US Food and Drug Administration (FDA). Intravenous mannitol is used therapeutically to induce a hyperosmolar state and reduce cerebral edema, whereas inhaled mannitol is an alternative to methacholine for bronchoprovocation studies [19,20]. Second, mannitol is commonly used to assay epithelial monolayer integrity *in vitro*, since it diffuses paracellularly between cells when intercellular junctions are disrupted. Third, mannitol undergoes very little metabolism and is excreted almost entirely unchanged by the kidneys. Here we report preliminary studies comparing the absorption of inhaled mannitol in healthy subjects and mild asthmatics.

## Methods

### Control and asthmatic subjects

Healthy controls and mild asthmatics were recruited for study (subject demographics are indicated in Table 1). Healthy control subjects had no prior history of asthma

**Table 1** Subject demographics.

|                                   | Healthy     | Asthma                          | <i>p</i> value |
|-----------------------------------|-------------|---------------------------------|----------------|
| Number of subjects                | 10          | 11                              |                |
| Age (yrs)                         | 37 ± 11     | 35 ± 11                         |                |
| Sex (F:M)                         | 3:7         | 8:3                             |                |
| BMI                               | 27 ± 4      | 31 ± 7                          | 0.126          |
| Controller medications            | None        | ICS (2), ICS/LABA (5), LTRA (3) |                |
| Serum CCSP (ng mL <sup>-1</sup> ) | 9.5 ± 3.9   | 10.6 ± 3.3                      | 0.509          |
| <b>Visit 1</b>                    |             |                                 |                |
| FeNO (ppb)                        | 14 ± 7      | 20 ± 14                         | 0.279          |
| Baseline FEV1 (L)                 | 3.69 ± 0.86 | 2.99 ± 0.78                     | 0.015          |
| Baseline FEV1:FVC                 | 0.80 ± 0.05 | 0.75 ± 0.07                     | 0.032          |
| Nadir FEV1 (L)                    | 3.56 ± 0.85 | 2.79 ± 0.74                     | 0.007          |
| Average change FEV1 (L)           | 0.12 ± 0.09 | 0.20 ± 0.13                     | 0.179          |
| PD15 (# subjects)                 | 0           | 2                               |                |
| PD15 (mg mannitol)                | n/a         | 32, 33                          |                |
| <b>Visit 2</b>                    |             |                                 |                |
| FeNO (ppb)                        | 13 ± 5      | 18 ± 10                         | 0.231          |
| Baseline FEV1 (L)                 | 3.61 ± 0.78 | 3.06 ± 0.86                     | 0.062          |
| Baseline FEV1:FVC                 | 0.79 ± 0.04 | 0.70 ± 0.24                     | 0.234          |
| Nadir FEV1 (L)                    | 3.51 ± 0.76 | 2.72 ± 0.77                     | 0.010          |
| Average change FEV1 (L)           | 0.10 ± 0.13 | 0.33 ± 0.15                     | 0.003          |
| PD15 (# subjects)                 | 0           | 3                               |                |
| PD15 (mg mannitol)                | n/a         | 268, 295, 296                   |                |

or allergies and were on no medications. Asthmatic subjects had a doctor diagnosis of asthma, and had to demonstrate evidence of bronchodilator reversibility within the previous year (FEV1 or FVC increase by ≥12% or 200 mL after 400 mcg albuterol). Asthmatic subjects with severe disease (ongoing symptoms despite high-dose inhaled or oral glucocorticoids) or recent exacerbation within 6 months were excluded. Other exclusion criteria included: 5 pack year history of tobacco use or active smoking, other chronic or active lung diseases (e.g. COPD, pulmonary fibrosis, lung cancer), history of significant renal insufficiency of liver disease, asthma subjects with a baseline FEV1 < 65% predicted, asthma subjects unwilling or unable to withhold medications prior to testing, and pregnant women.

### Modified mannitol challenge protocol

We used prepackaged kits with capsules containing escalating doses of mannitol for inhalation (Aridol challenge kits, Pharmaxis). The capsules contain spray-dried mannitol powder consisting of particles that are 5 µm in diameter, and generate uniformly distributed particles under a range of inspiratory flow rates. We followed the FDA approved bronchoprovocation protocol with three modifications. First, based on pilot studies, we stopped Aridol challenges at the 4th and 5th test doses respectively (corresponding to a total of 155 and 315 mg of inhaled mannitol). The rationale for choosing two pre-determined stopping doses was to identify the lowest possible dose generating detectable serum levels. The goal of our study was not to induce bronchospasm, but this was monitored

carefully as during any bronchoprovocation test, and challenges were stopped if the FEV1 decreased by  $\geq 15\%$  between doses. Second, subjects were provided water and instructed to rinse and spit after each dose, and were also encouraged to expectorate (and not swallow) all sputum during and after testing. These measures helped ensure that serum mannitol levels reflected absorption from the respiratory tract, and not mannitol that was swallowed. Third, subjects were instructed to fast for 6 h prior to testing, and to avoid mannitol-rich foods for 24 h after testing, to minimize confounding effects of mannitol from dietary sources. We call this procedure The Leaky Lung Test, which was granted an IND-waiver from the US FDA, and approved by the University of Rochester Research Subjects Review Board. This study was registered on ClinicalTrials.gov (Study #NCT02306473).

Subjects were scheduled for two separate challenges, stopping at 155 mg (Visit 1) or 315 mg (Visit 2) of inhaled mannitol. Visits 1 and 2 were separated by at least 3 weeks. On the day of testing, subjects reported to the UPMC General Clinical Research Center and had an IV placed for serial blood draws. Baseline blood and urine samples were obtained, followed by measurements of exhaled nitric oxide (FeNO), FEV1 and FVC. Next, subjects proceeded with mannitol challenge protocol as above. Spirometry was performed after each dose, and a positive test was defined as  $\geq 15\%$  decrease in FEV1 between doses. Blood samples were obtained at 30, 90, and 150 min after the last mannitol dose. At the completion of testing, subjects were monitored for 3 h and were given water and a light snack. Subjects were given a urine jug and asked to collect all voided urine for 24 h, and discharged to home.

### Serum measurements

Serum samples were obtained on the day of testing, and aliquots were stored at  $-80^{\circ}\text{C}$  and analyzed in batches. Serum CCSP was measured by ELISA according to manufacturer's instructions (Abnova Inc.). Mannitol was measured using ion exchange high-performance liquid chromatography (HPLC). Briefly, cellobiose was used as an internal standard, samples were filtered and deionized, and then mannitol was detected using pulsed amperometric detection on a gold electrode [21].

### Statistical analysis

Means, standard deviations, and area under the curve analyses were performed using Prism Software (Graphpad). Differences between groups were

determined using ANOVA with Tukey's multiple comparisons test, or Mann Whitney non-parametric test (Figure 4). Correlation analyses were performed assuming non-Gaussian distribution of data, and the Spearman  $r$  correlation coefficient and two-tailed  $p$  values are reported. Data were analyzed with and without log transformation, without exclusion of outliers. A  $p < .05$  was considered statistically significant.

## Results

Ten healthy controls and eleven adult asthmatic subjects were recruited for study (see Table 1). Healthy controls had no prior history of asthma and were not taking any medications on a regular basis. All of the asthmatic subjects used an as needed short acting beta-agonist, and had not experienced an asthma exacerbation in the 6-month period prior to testing. Ten of the asthmatic subjects were using regular controller medications including inhaled corticosteroids (ICS,  $n = 2$ ), ICS plus long-acting beta agonist (ICS/LABA,  $n = 5$ ), or a leukotriene receptor antagonist (LTRA,  $n = 3$ ). Subjects were instructed to withhold these medications prior to testing according to established guidelines (12 h for ICS, 24 h for ICS/LABA, and 4 days for LTRA). When comparing baseline FeNO and FEV1 at both visits, asthmatic subjects tended to have higher FeNO ( $19 \pm 12$  vs.  $14 \pm 6$  ppb, mean  $\pm$  SD,  $p = 0.1$ ), and had significantly lower FEV1 ( $3.02 \pm 0.80$  vs.  $3.82 \pm 0.63$  liters,  $p = 0.0001$ ), than healthy controls. Baseline values for FeNO, FEV1, and FEV1:FVC ratios at each study visit are shown in Table 1.

The range of time between Visits 1 and 2 averaged 48 days (range 26–96 days) for healthy subjects, and 30 days (range 20–43 days) for asthmatics. There were no significant differences in FeNO or baseline FEV1 in either healthy or asthmatic subjects between Visits 1 and 2. In healthy controls, the average change in FeNO was  $-1$  ppb (range  $-10$  to  $5$  ppb), and the average change in FEV1 was  $-0.07$  L (range  $-0.23$  to  $0.16$  L), between visits. In asthmatic subjects, the average change in FeNO was  $1$  ppb (range  $-7$  to  $12$  ppb), and the average change in FEV1 was  $0.04$  L (range  $-0.20$  to  $0.52$  L), between visits.

Subjects were challenged with 155 mg mannitol on Visit 1. Two healthy subjects experienced mild chest tightness or cough during the challenge, but no healthy subject demonstrated a significant decline in FEV1. In healthy controls, the average decline in FEV1 was  $120 \pm 90$  mL at the end of the challenge. Three asthmatic subjects experienced wheezing or cough during Visit 1, and two subjects had a  $> 15\%$

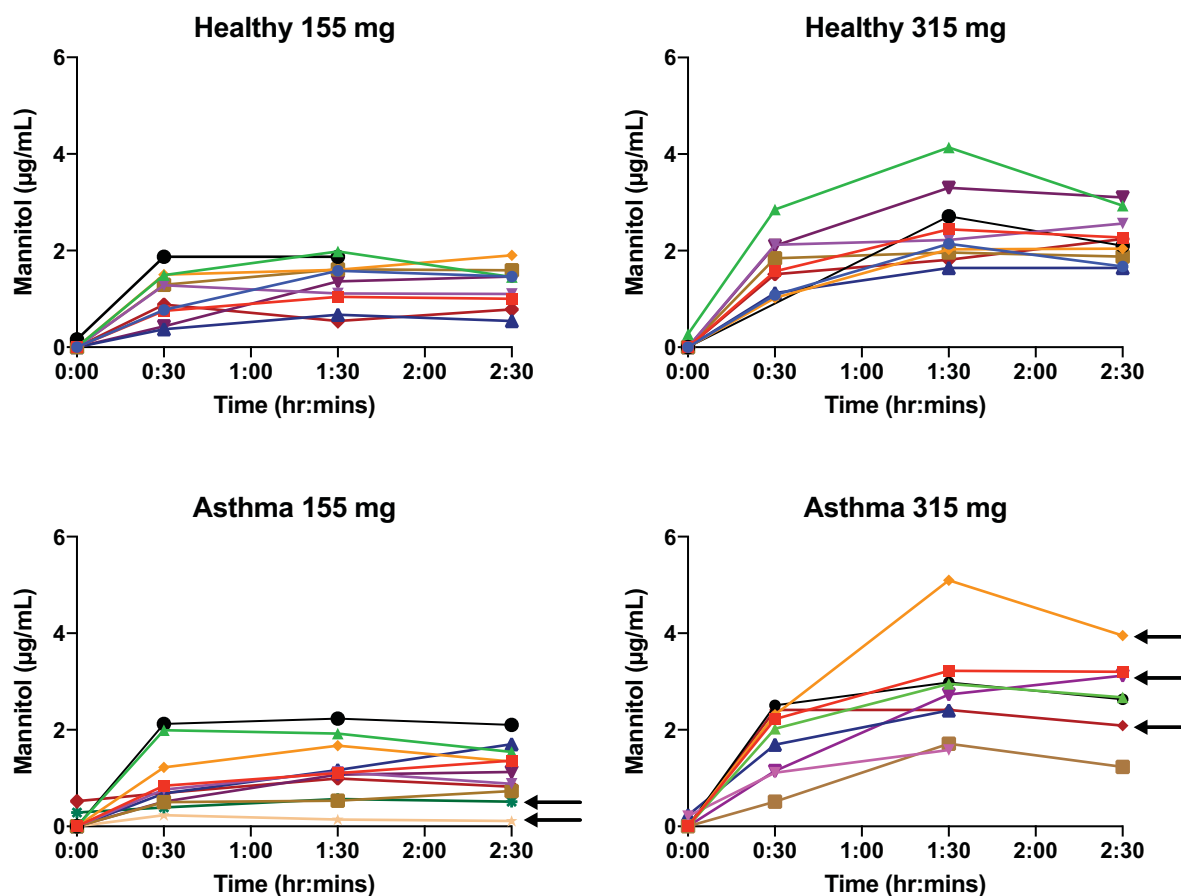


**Figure 1.** Individual serum mannitol levels in healthy controls (top row) or subjects with asthma (bottom row), after inhalation of either 155 mg mannitol (left graphs) or 315 mg mannitol (right graphs). Individual colors represent individual subjects matched for disease status. Arrows indicate asthmatic subjects that demonstrated bronchial reactivity to mannitol.

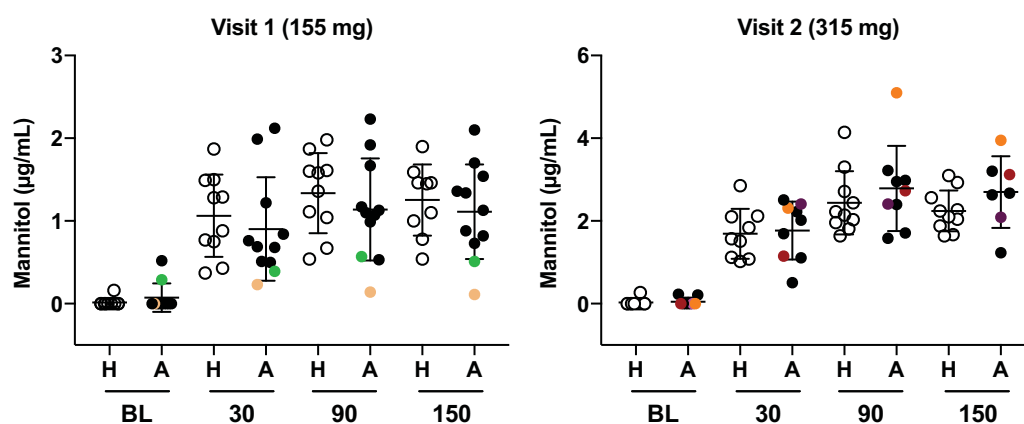


**Figure 2.** Average serum mannitol concentrations in healthy (H) or asthmatic (A) subjects at baseline (BL) or different time points after mannitol challenge. The colored dots correspond to subjects demonstrating bronchial reactivity in Figure 1.

decline in FEV1 at the target dose of mannitol (155 mg). These subjects were not scheduled for Visit 2. In asthmatic subjects, the average decline in FEV1 at the end of Visit 1 was  $200 \pm 130$  mL.

Ten healthy subjects and nine asthmatics were challenged with 315 mg mannitol on Visit 2. One

healthy subject experience mild chest tightness, but no healthy subject demonstrated a significant decline in FEV1. In healthy controls, the average maximal decline in FEV1 was  $100 \pm 130$  mL. Three asthmatic subjects experienced wheezing, chest tightness or cough during Visit 2, and also had a  $> 15\%$  decline in

FEV1 at the target dose of mannitol (315 mg). In asthmatic subjects, the maximal decline in FEV1 at Visit 2 was  $300 \pm 150$  mL. All of the reactions to mannitol were mild, with prompt resolution of symptoms and normalization of lung function within 30 min of receiving a short acting bronchodilator.

Serum samples were analyzed for mannitol using HPLC (see Methods). Samples were not available from all time points for each subject due to technical issues. Figures 1 and 2 shows changes over time of serum mannitol at Visit 1 (left graphs) and Visit 2 (right graphs). In Figure 1 individual colors represent individual subjects matched for disease state (Healthy vs. Asthma). Baseline serum mannitol values were below the detection limit of the assay in most subjects. Three subjects had detectable serum mannitol levels prior to challenge on both Visit 1 and Visit 2 (one healthy, two asthmatics), presumably reflecting mannitol absorption from dietary sources. Serum mannitol levels increased at 30, 90, and 150 min after challenge in both healthy and asthmatic subjects. Peak mannitol concentrations were observed by 1.5 h in most

subjects, and were lower at Visit 1 compared to Visit 2. There was greater variability in mannitol absorption in asthmatics compared to healthy subjects at both visits. At Visit 1, the lowest serum mannitol levels in asthmatics were in the two subjects who developed bronchoconstriction at the 155 mg dose (Figure 1, lower left graph, arrows). In contrast, the three subjects who developed bronchoconstriction at the 315 mg dose had high or average mannitol serum levels (Figure 2, lower right graph, arrows).

There were no significant differences when comparing average mannitol values between healthy controls and asthmatic subjects at any time point of either visit (Figure 2). Average serum mannitol values at Visit 2 were slightly higher in asthmatics compared to healthy controls at 90 min ( $2.8$  vs.  $2.4 \mu\text{g mL}^{-1}$ ) and 150 min ( $2.7$  vs.  $2.2 \mu\text{g mL}^{-1}$ ), but there was wide variability and these differences were not statistically significant. We next calculated the area under the curve (AUC) of serum mannitol values for subjects who had available data for all four time points. Figure 3 shows that the mannitol AUC was significantly higher at Visit 2 compared to Visit 1 for both healthy (H) and asthmatic (A) subjects, but there were no significant differences when comparing healthy versus asthmatics at either visit. Excluding subjects with detectable mannitol levels at baseline did not significantly influence results (not shown). Figure 4 shows that mannitol AUC increased on average 1.7-fold in healthy controls between Visit 1 and 2 (from  $2.3 \pm 0.9$  to  $4.8 \pm 1.4$ , mean  $\pm$  SD), and 2.4-fold in asthmatic subjects (from  $2.4 \pm 1.3$  to  $5.8 \pm 1.8$ ).

We explored correlations between peak mannitol levels and mannitol AUC with lung function (baseline FEV1, baseline FEV1/FVC, change in FEV1 after challenge), FeNO, serum CCSP levels, and body mass index. Correlation analyses were performed for

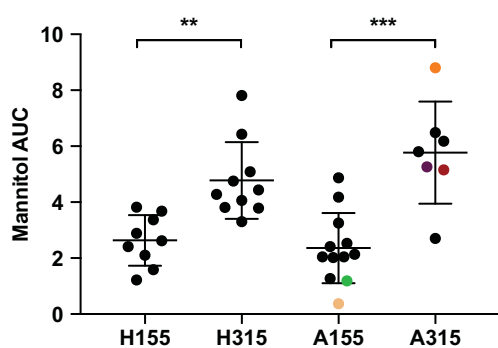


**Figure 3.** Mannitol area under the curve (AUC) in healthy subjects (H) or asthmatics (A) challenged with either 155 or 315 mg mannitol. The colored dots correspond to subjects demonstrating bronchial reactivity in Figure 1. \*\* $p < 0.01$ , \*\*\* $p < 0.005$ .

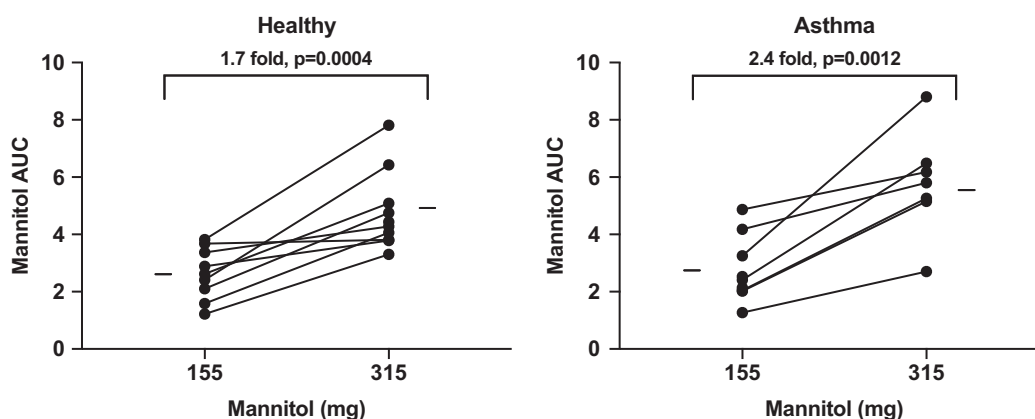


**Figure 4.** Changes in mannitol AUC in healthy controls or subjects with asthma challenged with either 155 or 315 mg mannitol. Not all subjects had matched samples available for analysis.  $p$  values determined using Mann Whitney test.

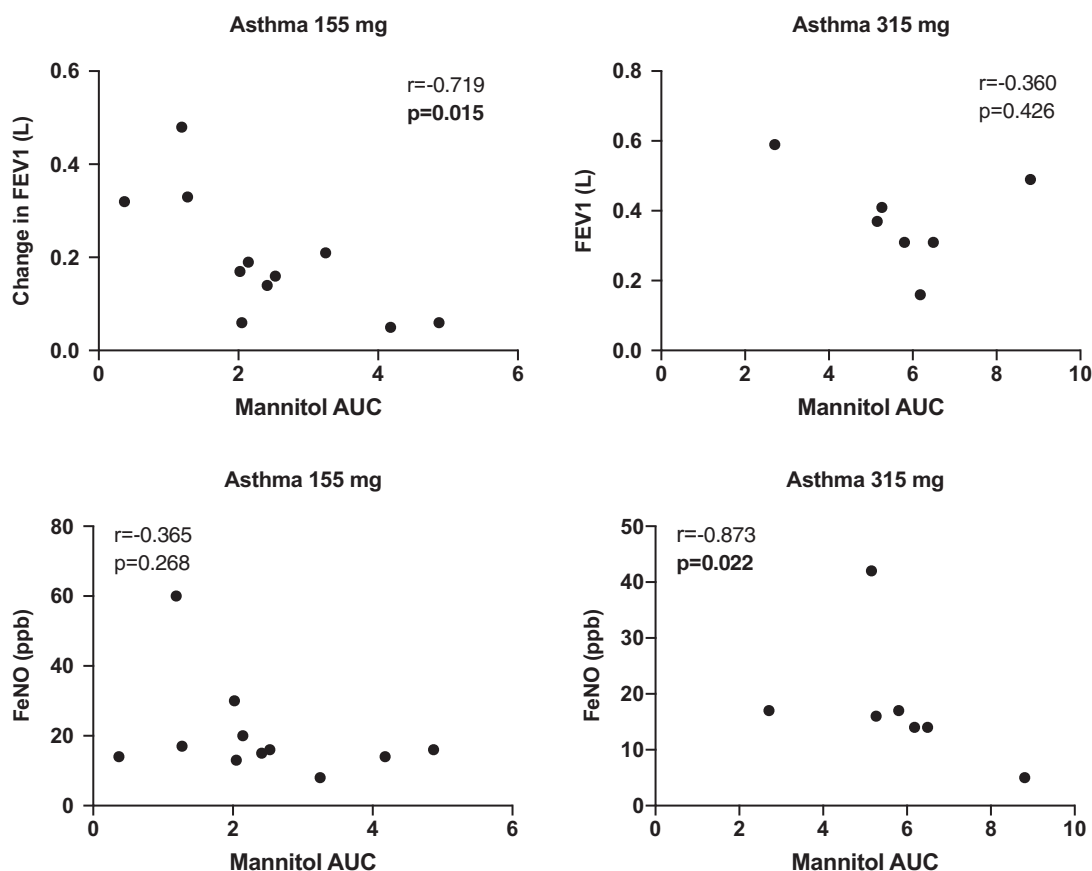


**Figure 5.** Correlation analyses between changes in FEV1 after mannitol challenge (top row) and baseline FeNO levels (bottom row) in asthmatic subjects challenged with 155 or 315 mg mannitol. Spearman  $r$  values and two-tailed  $p$  values are indicated (with  $p < 0.05$  indicated in bold text).

healthy controls (Supporting Information Figures 1 and 2) and asthmatic subjects (Supporting Information Figures 3 and 4) at each dose of mannitol administered. There were no significant associations between mannitol absorption and serum CCSP levels or body mass index (Supporting Information Figures 1–4). Baseline FEV1 was positively correlated with mannitol AUC in healthy subjects challenged with the 315 mg dose (Supporting Information Figure 2). In asthmatic subjects, the change in FEV1 after challenge was negatively correlated with mannitol AUC, with a stronger association observed at visit 1 (Figure 5, top row). Baseline FeNO in asthmatics was also negatively correlated with mannitol AUC, with a stronger association observed at visit 2 (Figure 5, bottom row).

Pre- and post-challenge urine samples were available from 10 healthy controls and nine asthmatic subjects. We analyzed urine mannitol concentrations, with and without normalization for urine creatinine, and also calculated total mannitol clearance by multiplying mannitol concentration  $\times$  total volume of collected urine. Although urine mannitol levels increased above baseline after both mannitol challenges, levels were

highly variable and did not increase in a predictable manner after Visit 2 compared to Visit 1 (Supporting Information Table S1, and data not shown).

## Discussion

Epithelial barrier dysfunction is emerging as an important feature of airway inflammation in asthma, but this is difficult to measure in individual subjects. Here we report our initial experience with the Leaky Lung Test, which uses the inert sugar alcohol mannitol as a probe of epithelial barrier integrity after inhalation challenge. Inhaled mannitol was well tolerated in all subjects, and as expected caused minor and reversible bronchoconstriction in a few asthmatics. Serum levels of mannitol increased in a time- and concentration-dependent manner after inhalation of 155 mg and 315 mg doses, with peak concentrations reached after about 90 min. We observed no significant differences when comparing mannitol absorption in healthy controls with mild asthmatics at either dose, but mannitol absorption was more variable in asthmatics. In future studies, it will be interesting to

determine if the asthmatics with more severe asthma or a history of asthma exacerbations demonstrate higher absorption of inhaled mannitol.

There are well-established techniques to monitor the integrity of airway epithelial monolayers grown *in vitro*. Typical approaches include measuring trans-epithelial electrical resistance, and paracellular flux of inert macromolecules [22]. However, there is no consensus about the best way to measure the integrity of the airway epithelial barrier in living organisms. It is important to distinguish between assays of “inside/out” versus “outside/in” barrier function when considering different approaches. Inside/out in this context refers to transudation of proteins from the circulation and interstitium into the airway lumen, whereas outside/in refers to translocation of inhaled particles and pathogens from the airway lumen into the sub-epithelial space. Inside/out barrier function is commonly investigated by measuring the concentration of macromolecules such as albumin in BAL fluids, which are increased in asthmatics compared to controls [23].

Outside/in barrier function in the lung has not been as well studied. Most research to-date has been conducted by measuring the movement of an inhaled test agent out of the airway or lung. Three general approaches have been used. One approach involves inhalation of technetium-labeled di-ethylenetriamine pentacetic acid (Tc-DTPA) and then measuring its clearance from the lung with a gamma counter [24]. It is difficult using this approach to distinguish between clearance via the mucociliary escalator versus translocation between epithelial cells, but one study concluded that epithelial permeability was higher in 10 asthmatic subjects compared to eight healthy controls [25]. Que et al. used an ozone challenge model and found that Tc-DTPA clearance was increased after ozone inhalation [26]. Interestingly, these authors found little overlap between subjects who demonstrated increased Tc-DTPA clearance times, reduced FEV1, or methacholine sensitivity, suggesting that airway permeability, airflow obstruction, and airway hyper-reactivity are regulated by distinct mechanisms [26].

A second approach involves inhaling a test agent and measuring its accumulation in serum over time. For example, Taylor et al. instilled intra-tracheal mannitol and measured its accumulation in plasma in a dog model of asthma [27]. Because peak mannitol concentrations increased in antigen-challenged animals, the authors concluded that epithelial permeability had increased. More recently, Chen et al. instilled intranasal FITC-dextran in mice and found that

plasma fluorescence intensity was significantly enhanced after challenge with inhaled lipopolysaccharide (LPS) [28]. This was associated with reduced expression of tight and adherens junctions in airway epithelial cells. Since the translocation of inhaled compounds from airway lumen to circulation is thought to occur passively, these techniques are direct measurements of epithelial integrity. A third approach to measuring outside/in barrier function involves inhalation of fluorescent molecules or beads, and measuring their active uptake by lung cell subsets using immunofluorescence and flow cytometry [29,30]. This approach has the advantage of identifying specific lung cell subsets that sample luminal contents, and whether or not they traffic migrate out of the lung. However, because the relationship between active uptake of a luminal antigens by airway cells and epithelial barrier integrity is not known, this approach provides an indirect estimate of barrier function.

Airway epithelial barrier dysfunction in asthma is caused by environmental exposures, especially respiratory viruses. Both rhinovirus (RV) and respiratory syncytial virus (RSV) cause tight junction dysfunction, probably as a mechanism to promote viral replication [31–33]. The contribution of virus-induced tight junction dysfunction to asthma symptoms or exacerbations is not known, and it will be interesting in future studies to analyze mannitol permeability before and after viral infections in selected subjects. These studies will need to use the lowest dose mannitol possible to avoid inducing bronchoconstriction. Based on the results reported here, we conclude that 155 mg mannitol should be sufficient, but it remains formally possible that even lower doses will generate detectable plasma mannitol levels.

Inhaled mannitol caused a significant drop in FEV1 in two out of 10 asthmatics at visit 1 (155 mg), and three out of nine asthmatics at visit 2 (315 mg). The two subjects with bronchial reactivity at visit 1 had the lowest mannitol plasma values, whereas we did not observe a similar pattern in the three subjects who reacted at visit 2 (Figure 1). Reasons for these differences are not clear, but one possibility is that bronchoconstriction limited the deposition of mannitol in the subjects who reacted at visit 1. There are several potential limitations in using the Leaky Lung Test to measure airway epithelial barrier integrity in asthma. First, studies of airway barrier integrity are potentially most useful in subjects with severe or exacerbation prone asthma, but these subjects will also be at greater risk of mannitol-induced bronchospasm. An ideal scenario for future studies would be to have

subjects inhale a single, very low-dose of mannitol that does not cause bronchoconstriction but is still readily detectable in serum: this will require additional optimization of methods. Second, inhaled mannitol (or other probes) might become trapped by airway mucus in some subjects, and might not be evenly distributed throughout the respiratory tract, depending on patterns of regional airflow. Third, although we encouraged all subjects to expectorate and not swallow mucus and oral secretions after challenge, we cannot exclude the possibility that small amounts of mannitol were absorbed from the GI tract in some subjects. Finally, although all asthmatic subjects withheld all asthma medications prior to study, it remains possible that medication use could have confounded our results. Along these lines, it should be noted that albuterol (10  $\mu$ M) was shown to increase the permeability of epithelial monolayers *in vitro* [34], but serum mannitol concentrations were not consistently enhanced in subjects who received albuterol in our study (and in fact were lower in subjects who received albuterol at Visit 1). One interesting possibility to explore in the future would be to prophylactically pre-medicate subjects with a short-acting beta-agonist prior to mannitol challenge, which should result in more uniform patterns of aerosol deposition in the lung and might improve the tolerability of mannitol inhalation.

Neither peak mannitol concentrations nor the mannitol AUC correlated with indices of asthma severity including baseline FEV1 or FeNO, or with serum CCSP levels. In fact, mannitol AUC was negatively correlated with both the change in FEV1 after challenge as well as baseline FeNO levels (Figure 5). This suggests that airflow obstruction, airway inflammation, and barrier integrity are regulated by distinct mechanisms, although we acknowledge our small sample size and need for future studies in larger cohorts. We were hoping to use of urinary mannitol clearance as a surrogate of airway barrier function, but wide variability in urine mannitol levels interfered with these analyses. We suspect that dietary indiscretion was one cause of variability (e.g. eating mannitol rich foods), or possibly that some mannitol was swallowed and excreted in stool, but this will need to be followed up in future studies.

Finally, although we were interested in studying a cohort of mild asthmatics in our pilot study, the approach reported here will be applicable to other lung diseases characterized by dysfunction of the epithelial barrier (e.g. interstitial lung diseases). Inhaled mannitol should be better tolerated in these subjects, who do not have underlying airway hyper-reactivity.

## Conclusion

In summary, we conclude that measuring the absorption of inhaled mannitol has the potential to provide new insights into airway barrier dysfunction in asthma and other lung diseases. Future studies in additional cohorts of more severe asthmatics may provide insights into the magnitude and duration of epithelial barrier dysfunction at baseline or after specific environmental exposures, but the risks of severe bronchospasm will need to be carefully considered in these subjects.

## Acknowledgements

Aridol<sup>®</sup> kits were provided by Pharmaxis, Inc. None of the funding sources or agencies had a role in the study design, data collection, data analysis, data interpretation, or writing of the manuscript.

## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

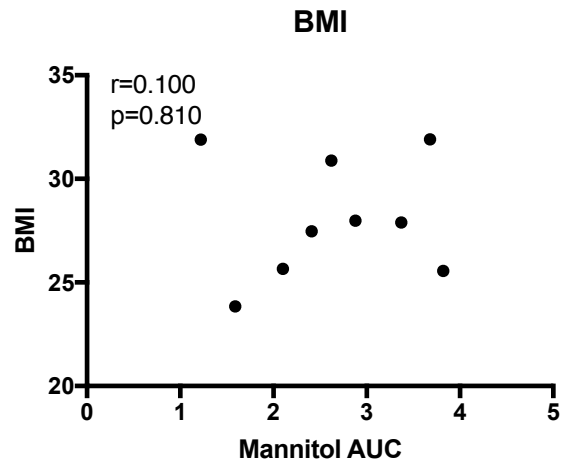
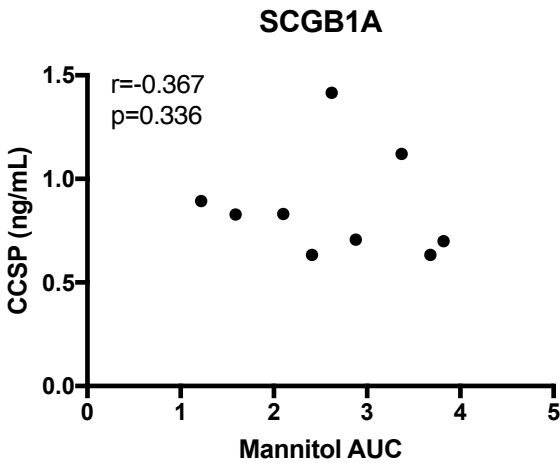
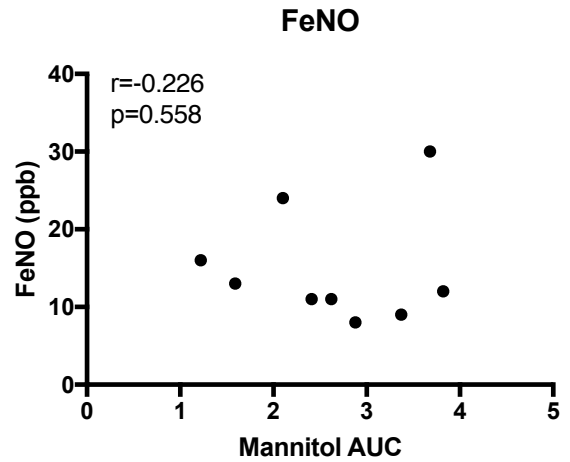
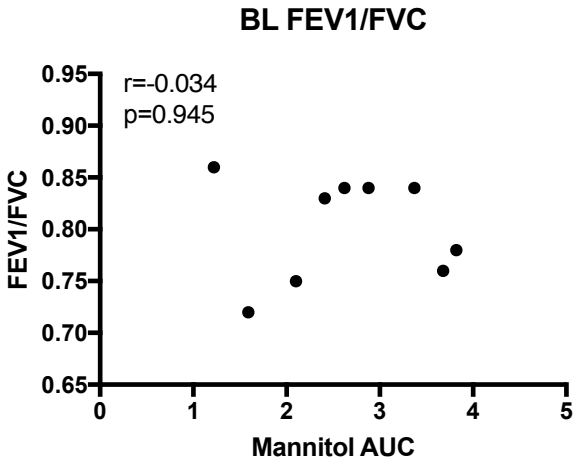
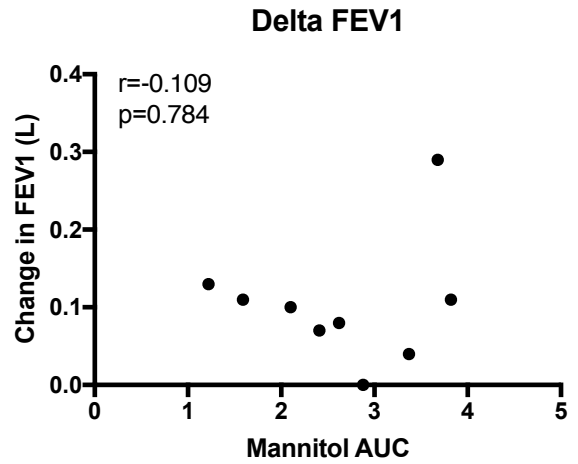
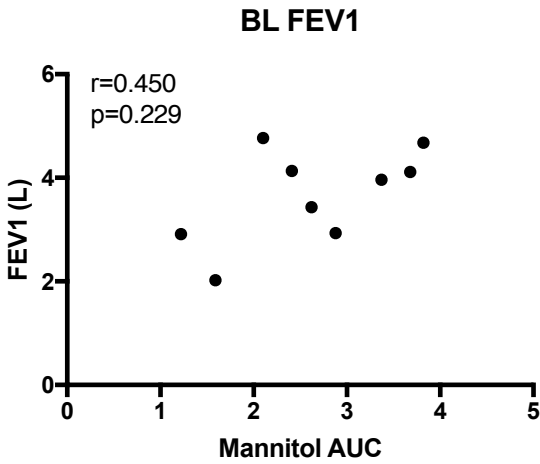
## Funding

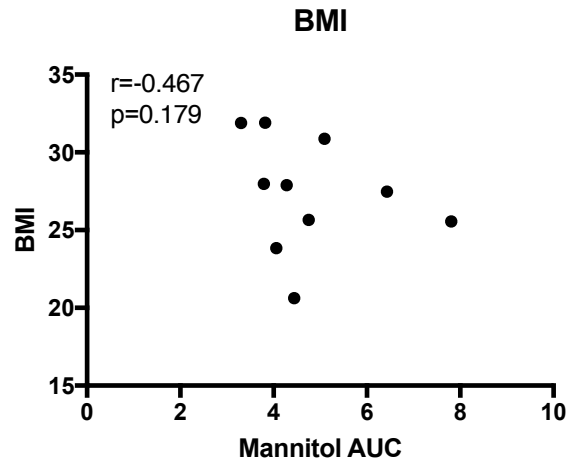
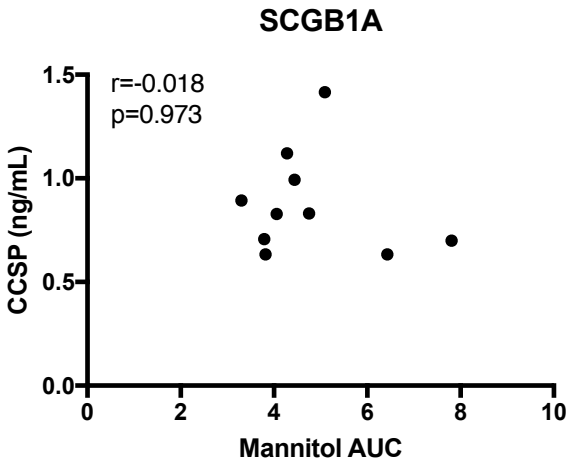
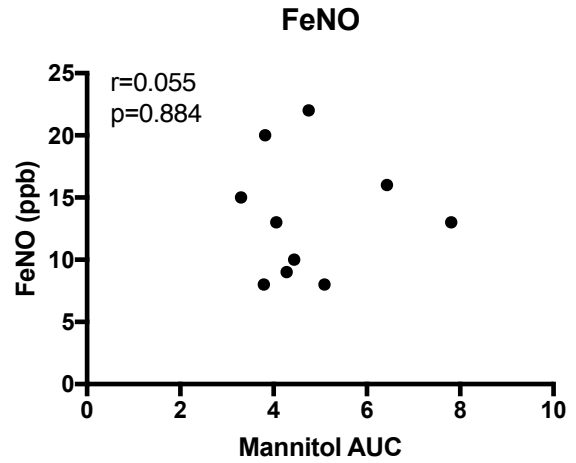
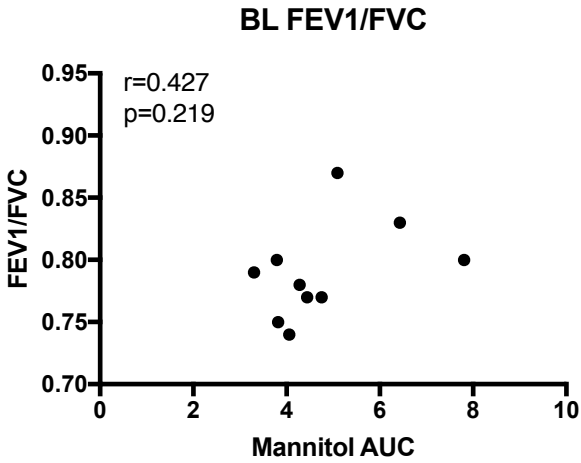
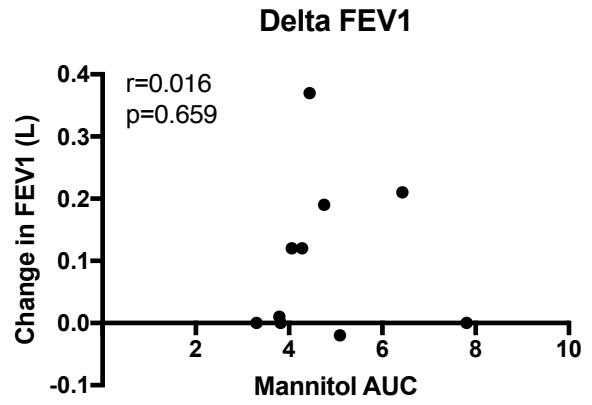
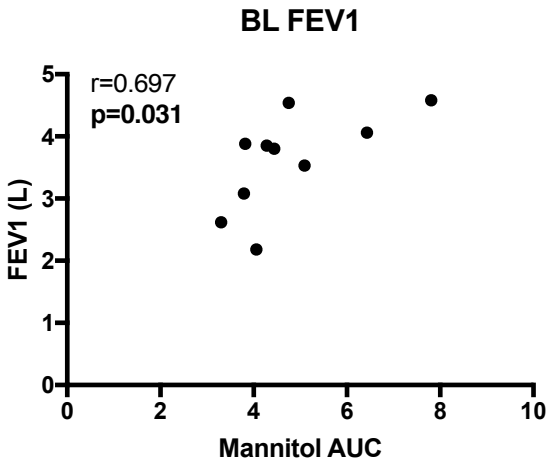
Supported in part by The Parkes Family Foundation, and NIH grants R01 HL014224, F31 HL140795, T32 HL140795.

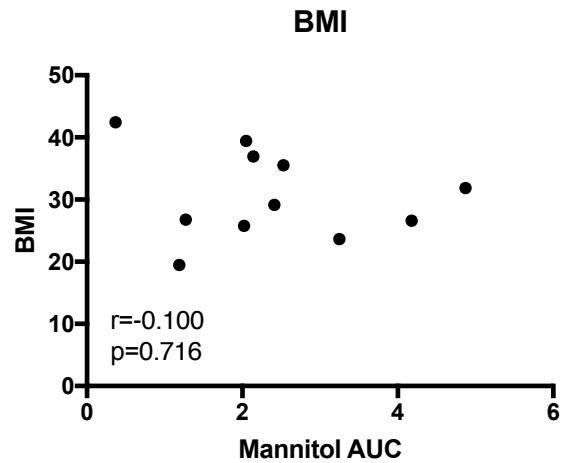
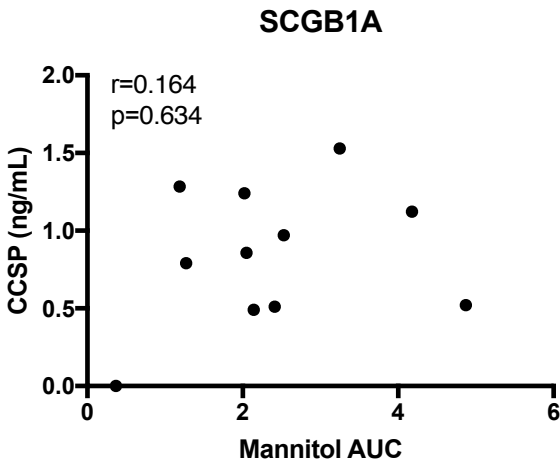
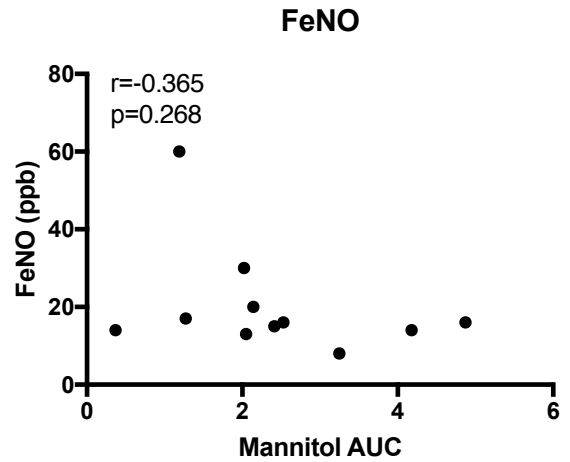
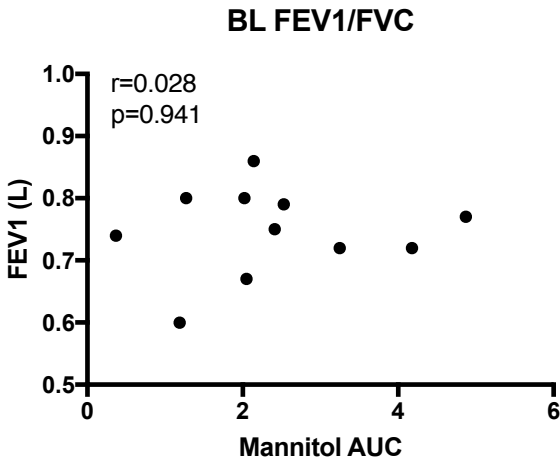
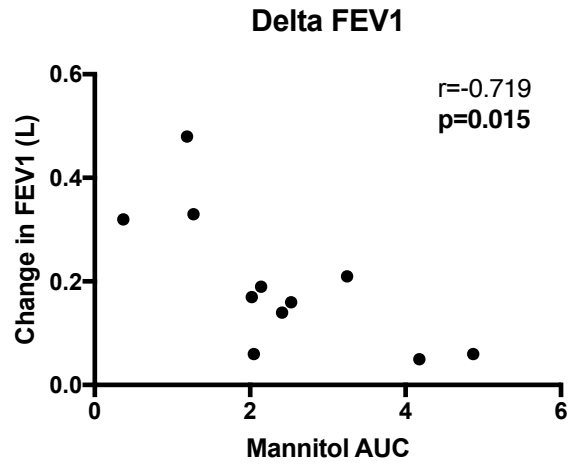
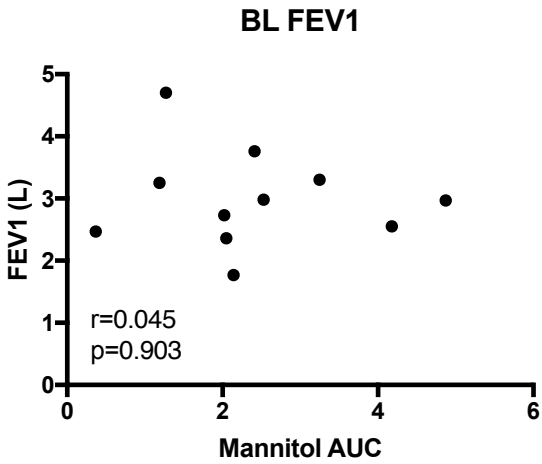
## References

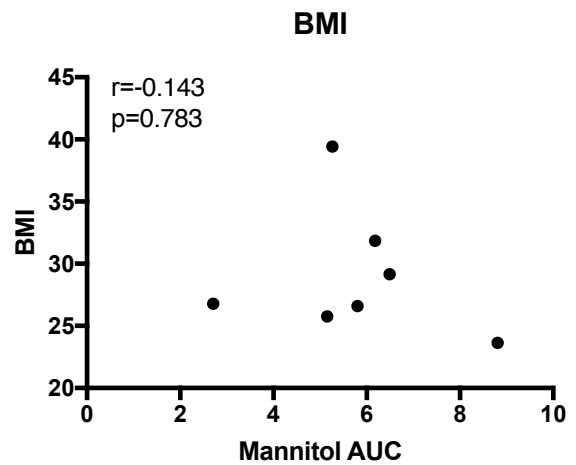
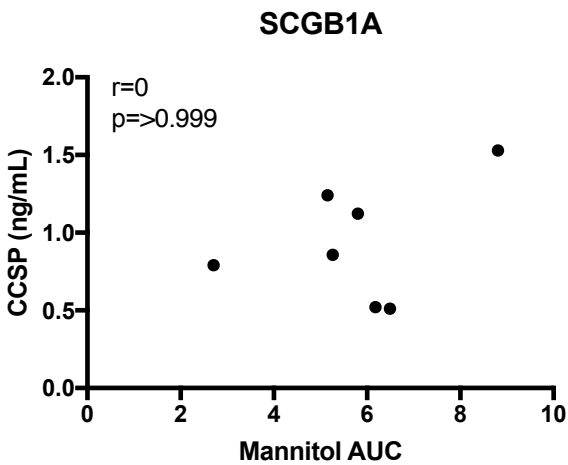
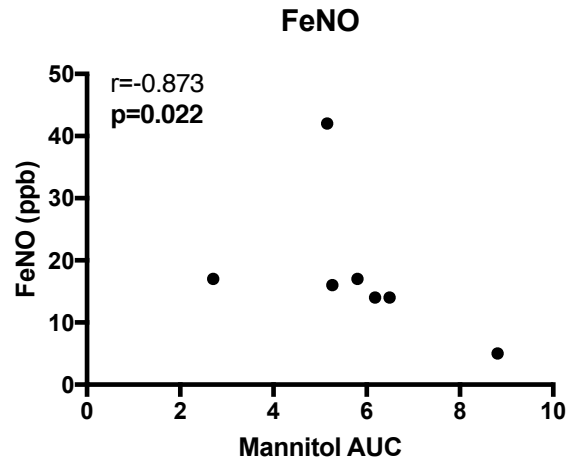
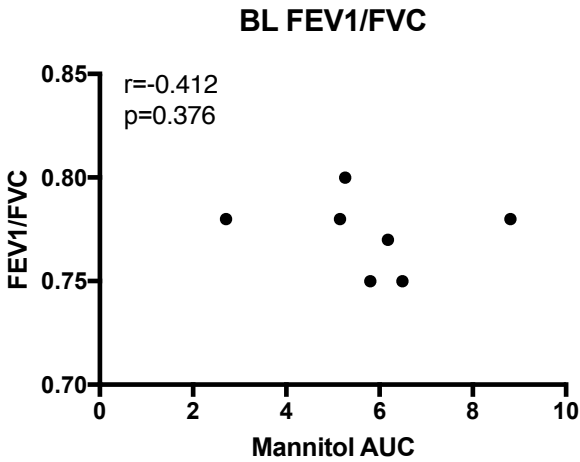
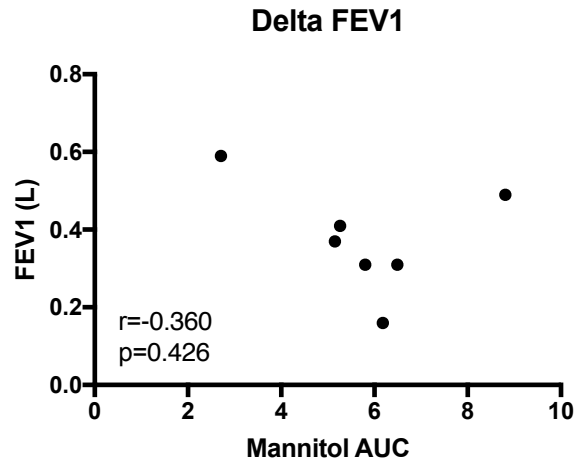
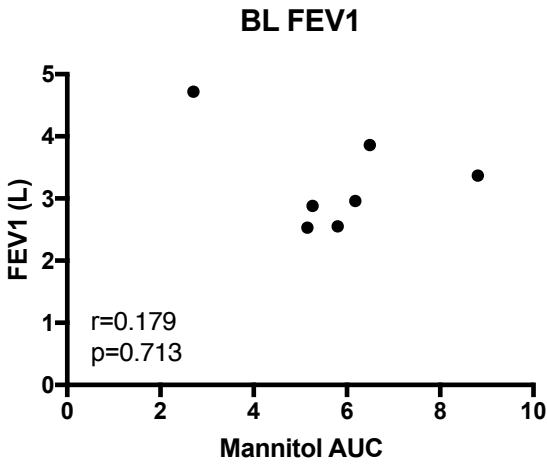
1. Schleimer RP, Kato A, Kern R, Kuperman D, Avila PC. Epithelium: at the interface of innate and adaptive immune responses. *J Allergy Clin Immunol* 2007; 120:1279–1284.
2. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010;363:2233–2247.
3. Shen L, Weber CR, Raleigh DR, Yu D, Turner JR. Tight junction pore and leak pathways: a dynamic duo. *Annu Rev Physiol* 2011;73:283–309.
4. Georas SN, Rezaee F. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. *J Allergy Clin Immunol* 2014;134: 509–520.
5. Rezaee F, Georas SN. Breaking barriers. New insights into airway epithelial barrier function in health and disease. *Am J Respir Cell Mol Biol* 2014;50:857–869.
6. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can J Physiol Pharmacol* 2008;86:105–112.
7. Hackett T-L, Singhera GK, Shaheen F, Hayden P, Jackson GR, Hegele RG, Van Eeden S, et al. Intrinsic phenotypic differences of asthmatic epithelium and its

- inflammatory responses to respiratory syncytial virus and air pollution. *Am J Respir Cell Mol Biol* 2011;45:1090–1100.
8. Xiao C, Puddicombe SM, Field S, et al. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol* 2011;128(3):549–556.
  9. Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. *Am J Respir Crit Care Med* 1999;159:646–678.
  10. Broeckaert F, Arsalane K, Hermans C, Bergamaschi E, Brustolin A, Mutti A, Bernard A, et al. Serum clara cell protein: a sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. *Environ Health Perspect* 2000;108:533–537.
  11. Bolger C, Tufvesson E, Anderson SD, Devereux G, Ayres JG, Bjermer L, Sue-Chu M, et al. Effect of inspired air conditions on exercise-induced bronchoconstriction and urinary CC16 levels in athletes. *J Appl Physiol* 2011;111:1059–1065.
  12. Johansson S, Kristjansson S, Bjarnarson SP, Wennergren G, Rudin A. Clara cell protein 16 (CC16) serum levels in infants during respiratory syncytial virus infection. *Acta Paediatr* 2009;98:579–581.
  13. Timonen KL, Hoek G, Heinrich J, Bernard A, Brunekreef B, de Hartog J, Hämeri K, et al. Daily variation in fine and ultrafine particulate air pollution and urinary concentrations of lung Clara cell protein CC16. *Occup Environ Med* 2004;61:908–914.
  14. Shijubo N, Itoh Y, Yamaguchi T, Imada A, Hirasawa M, Yamada T, Kawai T, et al. Clara cell protein-positive epithelial cells are reduced in small airways of asthmatics. *Am J Respir Crit Care Med* 1999;160:930–933.
  15. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, Kawai T, et al. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. *Lung* 1999;177:45–52.
  16. Rava M, Tares L, Lavi I, Barreiro E, Zock J-P, Ferrer A, Muniozguren N, et al. Serum levels of Clara cell secretory protein, asthma, and lung function in the adult general population. *J Allergy Clin Immunol* 2013;132:230–232.
  17. Doig CJ, Sutherland LR, Sandham JD, Fick GH, Verhoef M, Meddings JB. Increased intestinal permeability is associated with the development of multiple organ dysfunction syndrome in critically ill ICU patients. *Am J Respir Crit Care Med* 1998;158:444–451.
  18. Breslin NP, Nash C, Hilsden RJ, Hershfield NB, Price LM, Meddings JB, Sutherland LR, et al. Intestinal permeability is increased in a proportion of spouses of patients with Crohn's disease. *Am J Gastroenterol* 2001;96:2934–2938.
  19. Porsbjerg C, Brannan JD, Anderson SD, Backer V. Relationship between airway responsiveness to mannitol and to methacholine and markers of airway inflammation, peak flow variability and quality of life in asthma patients. *Clin Exp Allergy* 2008;38:43–50.
  20. Sverrild A, Porsbjerg C, Thomsen SF, Backer V. Diagnostic properties of inhaled mannitol in the diagnosis of asthma: a population study. *J Allergy Clin Immunol* 2009;124:928–932.
  21. Meddings JB, Gibbons I. Discrimination of site-specific alterations in gastrointestinal permeability in the rat. *Gastroenterology* 1998;114:83–92.
  22. Hubatsch I, Ragnarsson EG, Artursson P. Determination of drug permeability and prediction of drug absorption in CaCO-2 monolayers. *Nat Protoc* 2007;2:2111–2119.
  23. Van de Graaf EA, Out TA, Roos CM, Jansen HM. Respiratory membrane permeability and bronchial hyperreactivity in patients with stable asthma. Effects of therapy with inhaled steroids. *Am Rev Respir Dis* 1991;143:362–368.
  24. Oberdorster G, Utell MJ, Weber DA, Ivanovich M, Hyde RW, Morrow PE. Lung clearance of inhaled 99mTc-DTPA in the dog. *J Appl Physiol Respir Environ Exerc Physiol* 1984;57:589–595.
  25. Ilowite JS, Bennett WD, Sheetz MS, Groth ML, Nierman DM. Permeability of the bronchial mucosa to 99mTc-DTPA in asthma. *Am Rev Respir Dis* 1989;139:1139–1143.
  26. Que LG, Stiles JV, Sundry JS, Foster WM. Pulmonary function, bronchial reactivity, and epithelial permeability are response phenotypes to ozone and develop differentially in healthy humans. *J Appl Physiol* 2011;111:679–687.
  27. Taylor SM, Downes H, Hirshman CA, Peters JE, Leon D. Pulmonary uptake of mannitol as an index of changes in lung epithelial permeability. *J Appl Physiol Respir Environ Exerc Physiol* 1983;55:614–618.
  28. Chen H, Wu S, Lu R, Zhang YG, Zheng Y, Sun J. Pulmonary permeability assessed by fluorescent-labeled dextran instilled intranasally into mice with LPS-induced acute lung injury. *PLoS One* 2014;9:e101925.
  29. Jakubzick C, Helft J, Kaplan TJ, Randolph GJ. Optimization of methods to study pulmonary dendritic cell migration reveals distinct capacities of DC subsets to acquire soluble versus particulate antigen. *J Immunol Methods* 2008;337(2):121–131.
  30. Jakubzick C, Randolph GJ. Methods to study pulmonary dendritic cell migration. *Methods Mol Biol* 2010;595:371–382.
  31. Sajjan U, Wang Q, Zhao Y, Gruenert DC, Hershenson MB. Rhinovirus disrupts the barrier function of polarized airway epithelial cells. *Am J Respir Crit Care Med* 2008;178:1271–1281.
  32. Comstock AT, Ganesan S, Chattoraj A, Faris AN, Margolis BL, Hershenson MB, Sajjan US, et al. Rhinovirus-induced barrier dysfunction in polarized airway epithelial cells is mediated by NADPH oxidase 1. *J Virol* 2011;85:6795–6808.
  33. Rezaee F, DeSando SA, Ivanov AI, Chapman TJ, Knowlden SA, Beck LA, Georas SN, et al. Sustained protein kinase D activation mediates respiratory syncytial virus-induced airway barrier disruption. *J Virol* 2013;87:11088–11095.
  34. Unwalla HJ, Horvath G, Roth FD, Conner GE, Salathe M. Albuterol modulates its own transepithelial flux via changes in paracellular permeability. *Am J Respir Cell Mol Biol* 2012;46:551–558.









## Supplementary Figure Legends

**Figure S1.** Correlation analyses for healthy subjects at visit 1 (155 mg mannitol). Correlations between mannitol area under the curve (AUC) and baseline FEV1 (L), change in FEV1 after challenge (L), baseline FEV1/FVC, FeNO (ppb), serum CCSP (also known as SCGB1A), and body mass index (BMI). Spearman r vales and two-tailed p values are indicated (with  $p < 0.05$  indicated in bold text).

**Figure S2.** Correlation analyses for healthy subjects at visit 2 (315 mg mannitol). Correlations between mannitol area under the curve (AUC) and baseline FEV1 (L), change in FEV1 after challenge (L), baseline FEV1/FVC, FeNO (ppb), serum CCSP (also known as SCGB1A), and body mass index (BMI). Spearman r vales and two-tailed p values are indicated (with  $p < 0.05$  indicated in bold text).

**Figure S3.** Correlation analyses for asthmatic subjects at visit 1 (155 mg mannitol). Correlations between mannitol area under the curve (AUC) and baseline FEV1 (L), change in FEV1 after challenge (L), baseline FEV1/FVC, FeNO (ppb), serum CCSP (also known as SCGB1A), and body mass index (BMI). Spearman r vales and two-tailed p values are indicated (with  $p < 0.05$  indicated in bold text).

**Figure S4.** Correlation analyses for asthmatic subjects at visit 2 (315 mg mannitol). Correlations between mannitol area under the curve (AUC) and baseline FEV1 (L), change in FEV1 after challenge (L), baseline FEV1/FVC, FeNO (ppb), serum CCSP (also known as

SCGB1A), and body mass index (BMI). Spearman r vales and two-tailed p values are indicated (with  $p < 0.05$  indicated in bold text).

**Table S1.** Urine mannitol values

|                                    | <b>Healthy</b> | <b>Asthma</b> |
|------------------------------------|----------------|---------------|
| <b>Visit 1</b>                     |                |               |
| Baseline mannitol (mg/ml)          | 0.038±0.012    | 0.033±0.014   |
| Post-challenge mannitol (mg/ml)    | 0.128±0.141    | 0.046±0.041   |
| Post-challenge mannitol/creatinine | 2.12±2.19      | 1.00±0.79     |
| Mannitol clearance                 | 187±216        | 84±65         |
|                                    |                |               |
| <b>Visit 2</b>                     |                |               |
| Baseline mannitol (mg/ml)          | 0.025±0.005    | 0.033±0.012   |
| Post-challenge mannitol (mg/ml)    | 0.156±0.097    | 0.059±0.037   |
| Post-challenge mannitol/creatinine | 2.07±1.85      | 1.27±0.85     |
| Mannitol clearance                 | 195±180        | 97±60         |

