

ORIGINAL ARTICLE

Mannitol challenge for assessment of airway responsiveness, airway inflammation and inflammatory phenotype in asthma

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Summary

Background Assessment of airway inflammation in asthma is becoming increasingly important, as the inflammatory phenotype underpins the treatment response.

Objective This study aimed to evaluate mannitol as a tool for assessing airway responsiveness and airway inflammation in asthma, compared with hypertonic saline.

Methods Fifty-five subjects with stable asthma completed a hypertonic (4.5%) saline challenge and a mannitol challenge at two separate visits, performed 48–72 h apart, in random order.

Results Induced sputum was obtained from 49 (89%) subjects during the saline challenge and 42 (76%) subjects during the mannitol challenge ($P > 0.05$). There was a significant correlation between the greatest percentage fall in forced expiratory volume in 1 s (FEV₁) ($r = 0.6$, $P < 0.0001$), the dose–response slope ($r = 0.73$), cumulative dose ($r = 0.55$) and PD₁₅ ($r = 0.46$) for mannitol and hypertonic saline. The greatest percentage fall in FEV₁ to mannitol was less in non-eosinophilic asthma. There was a lower total cell count in mannitol vs. hypertonic-saline-induced sputum. However, sputum eosinophils and neutrophils were not significantly different. Using mannitol, a higher proportion of subjects were classified as having eosinophilic asthma. There were no differences in IL-8, neutrophil elastase or matrix-metalloproteinase 9 concentrations in sputum samples induced with mannitol or hypertonic saline.

Conclusion We conclude that mannitol can be used to induce good-quality sputum, useful for analysis of inflammatory mediators and for predicting the inflammatory phenotype in asthma.

Keywords airway inflammation, airway responsiveness, asthma, hypertonic saline, inflammatory phenotypes, mannitol

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Introduction

Asthma is now recognized as a complex disease, being heterogeneous on many different levels, including pathology, natural history and treatment responsiveness. Of particular importance is the recognition of the heterogeneity of airway inflammation in asthma. The inflammatory pattern underpins the treatment response, with non-eosinophilic forms of the disease responding poorly to inhaled corticosteroid (ICS) therapy. Recent examples of this are the lack of response to corticosteroids in smokers with asthma [1] and the lack of efficacy of corticosteroids in viral exacerbations of wheeze in chil-

dren [2]. Corticosteroids were recommended for these groups based on an extension of the T helper type 2 (Th₂)-eosinophilic inflammation paradigm to all forms of asthma. We now understand that the non-response to ICS in smokers and viral infections is due to non-eosinophilic inflammation [3]. As newer therapies for asthma focus on increasingly specific mechanistic pathways, identification of different forms of inflammation in asthma will assume greater importance. Current approaches using symptoms and lung function are inferior to an approach based on objective measurement of airway hyperresponsiveness (AHR) [4] or airways inflammation [5]. Green et al. [5] demonstrated that asthma treatment based on levels of

sputum eosinophils led to a 50% reduction in asthma exacerbations. This highlights the need for reliable methods of collecting airway samples and assessing airway inflammation and AHR in asthma, in order to optimize and individualize treatment approaches.

Hypertonic saline and mannitol are two different bronchial challenge agents used to assess AHR and induce expectoration of airway lining fluid for assessment of inflammation. Both act by increasing osmolarity at the airway surface, thereby inducing the release of mediators from mast cells [6]. Hypertonic saline has successfully been used to measure both AHR and induce sputum for analysis of inflammatory cells and mediators in asthma [7]. In our laboratory, we have previously shown that various mediators of airway inflammation, including IL-8, matrix-metalloproteinase 9 (MMP-9) and neutrophil elastase, can be reliably measured using assays that we have validated for use in saline-induced sputum samples [8]. Mannitol, given by inhalation as a dry powder, has been developed to overcome the practical limitations of hypertonic saline, which involves delivery of a wet aerosol via an ultrasonic nebulizer. It is possible that mannitol could also be used to induce sputum for cellular and mediator analysis in asthma. This study evaluates the relevance of using mannitol as a tool for assessing AHR and airway inflammation across the various inflammatory phenotypes of asthma, in comparison with hypertonic saline.

The aims of the study were to determine whether in subjects with asthma:

1. Intrasubject airway responsiveness and airway inflammatory markers vary according to the challenge agent.
2. Airway responsiveness to mannitol and levels of inflammatory markers in mannitol-induced sputum vary across inflammatory phenotype.

Methods

Study subjects

Subjects aged over 18 years with stable asthma were recruited. Asthma was diagnosed based on current (past 12 months) episodic respiratory symptoms, a doctor's diagnosis of asthma and AHR to hypertonic saline (ever). Asthma stability was confirmed, defined as no exacerbation, change in asthma medications, respiratory tract infection or oral corticosteroids in the past 4 weeks. Subjects completed 2 visits. Before each visit, subjects fasted for 12 h and withheld short-acting β -agonist medications for 6 h and long-acting β -agonist medications for 24 h. Subjects were excluded from the study if they were current smokers or had any respiratory-related illness other than asthma on presentation. Informed consent was

obtained, and the study was approved by the Hunter New England Human Research Ethics Committee.

Assessing airway responsiveness

Subjects underwent two separate airway responsiveness challenges. One challenge used mannitol and the other challenge used hypertonic (4.5%) saline. The challenges were performed 48 to 72 h apart, in random order. During each bronchial provocation challenge, sputum was induced for assessment of airway inflammation.

Hypertonic saline challenge. Spirometry (Minato Auto-spiro AS-600; Minato Medical Science, Osaka, Japan) and combined bronchial provocation and sputum induction with hypertonic saline (4.5%) were performed as described [7]. Following baseline spirometry and expectoration instruction, hypertonic saline was administered for increasing time periods (30 s, 1, 2, 4, 4, and 4 min) via an ultrasonic nebulizer (ULTRA-NEBTM Model 2000, DeVilbiss Healthcare Inc., Somerset, PA, USA) with a Hans Rudolph two-way valve (Hans Rudolph Inc., Shawnee, KS, USA). Spirometry was measured 1 min after each dose and participants were asked to expectorate after each dose of hypertonic saline. If the forced expiratory volume in 1 s (FEV₁) declined 15% below baseline, salbutamol 200 μ g was administered via a pressurized inhaler and valved holding chamber. Sputum induction was ceased once the maximum nebulization time (15.5 min) was reached or when subjects experienced a 15% decline from baseline FEV₁.

Mannitol challenge. At the alternate visit, a mannitol challenge was performed. The mannitol challenge involved inhalation of incremental doses of mannitol (0, 5, 10, 20, 40, 80, 160, 160 and 160 mg mannitol). After each inhalation, the subject was instructed to cough and expectorate sputum if present. Spirometry was measured 60 s after each dose. The challenge was completed when the subject experienced a 15% decline from baseline FEV₁ or when a cumulative dose totalling 635 mg had been given.

Sputum processing

Sputum was selected from saliva [7, 9], dispersed with dithiothreitol (DTT), and a total cell count of leucocytes and viability was performed. Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count was obtained from 400 non-squamous cells. The remaining solution was centrifuged (400 g, 10 min, 4 °C) and the cell-free supernatant was aspirated and stored at -70 °C.

Sputum supernatant measurements

IL-8 concentrations were determined by ELISA (R&D Systems, Minneapolis, MN, USA) with standard curves

based on dilutions of purified recombinant IL-8. Neutrophil elastase activity was detected using the Human Neutrophil Elastase Immunocapture Activity Assay Kit (Calbiochem, Darmstadt, Germany). MMP-9 was determined by ELISA (R&D Systems) with standard curves based on dilution of purified MMP-9. All fluid-phase mediators have been validated previously for assessment in induced sputum [8].

Statistical analysis

Phenotype characterization was performed from both hypertonic saline- and mannitol-induced sputum using previously defined criteria [10]. Subjects were classified as eosinophilic (eosinophils $\geq 3\%$), neutrophilic (neutrophils $\geq 61\%$), paucigranulocytic (eosinophils $< 3\%$ and neutrophils $< 61\%$) or mixed (eosinophils $\geq 3\%$ and neutrophils $\geq 61\%$). Phenotype classifications between saline and mannitol-induced sputum were compared using Fischer's exact test. Paired analyses were conducted between the saline and the mannitol challenge results for sputum cell counts and lung function. Paired analyses for AHR were not performed due to the measurements for saline and mannitol being in different units (mLs and mgs, respectively). Correlations and Bland-Altman plots were performed for sputum cell counts, lung function and AHR. Dose response and PD15 were log transformed. To allow for log transformation of values < 1 , 2 units were added to all DRS values. Non-parametric tests were performed for data that were not normally distributed. Significance was accepted when $P < 0.05$.

Results

Fifty-five subjects (31% male) with stable asthma completed both a hypertonic saline challenge and a mannitol challenge at two separate visits (Table 1). Of these, an

induced sputum sample was obtained from 49 (89%) subjects during the saline challenge and 42 (76%) subjects during the mannitol challenge ($P > 0.05$). An induced sputum sample was obtained during both challenges from 39 (71%) subjects.

Airway responsiveness

There were no significant differences in lung function before the two challenges (Table 2), $n = 55$. There was a moderate correlation for the greatest percentage fall in FEV₁ during both challenges ($r = 0.6$, $P < 0.0001$; Fig. 1). There were moderate correlations for the dose-response slope ($r = 0.73$, $P < 0.0001$), cumulative dose ($r = 0.55$, $P < 0.0001$) and PD15 for mannitol (mg) and saline (mLs) ($r = 0.46$, $P = 0.031$) (Table 2 and Fig. 2). Note that PD15 data are only available for subjects who demonstrated AHR on the day of testing ($n = 30$ and 25 for hypertonic saline and mannitol, respectively).

Table 1. Subject characteristics

N	55
Age (years)	53.2 (13.4)
Sex, M/F (n)	17/38
Height (cm)	166.5 (7.9)
Weight (kg)	86.2 (19.8)
FEV ₁ % predicted	85.7 (20.7)
FVC % predicted	99.5 (16.2)
FEV ₁ /FVC	0.70 (0.09)
Atopy, n (%)	39 (70.9)
No. subjects using ICS, n (%)	48 (87)
ICS dose, mcg/day BDP equivalent	1484 (837)

Results are mean (SD) unless otherwise stated.

BDP, beclomethasone dipropionate; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid.

Table 2. Airway calibre and hyperresponsiveness for hypertonic (4.5%) saline vs. mannitol

	4.5% saline	Mannitol	P-value
N	55	55	
FEV ₁ baseline, L*	2.47 (0.82)	2.44 (0.81)	0.319
FEV ₁ /FVC baseline*	0.70 (0.09)	0.69 (0.09)	0.560
% fall FEV ₁ *	13.19 (10.36)	12.19 (9.26)	0.406
			Spearman's ρ (P-value)
Dose-response slope [†]	1.11 (0.29, 4.81)	0.03 (0.01, 0.12)	0.73 ($P < 0.0001$)
PD15 [‡]	2.88 (1.5, 11.26) mL ($n = 30$)	98.5 (42.5, 220.3) mg ($n = 25$)	0.46 ($P < 0.031$)
Cumulative dose [‡]	16.3 (5.5, 23.0) mL	635 (155, 635) mg	0.55 ($P < 0.0001$)

*Results are mean (SD), paired *t*-test.

[†]Results are median (IQR), Mann-Whitney test.

[‡]Results are median (IQR).

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PD15, provocation dose causing a 15% decline in FEV₁ from baseline; IQR, interquartile range.

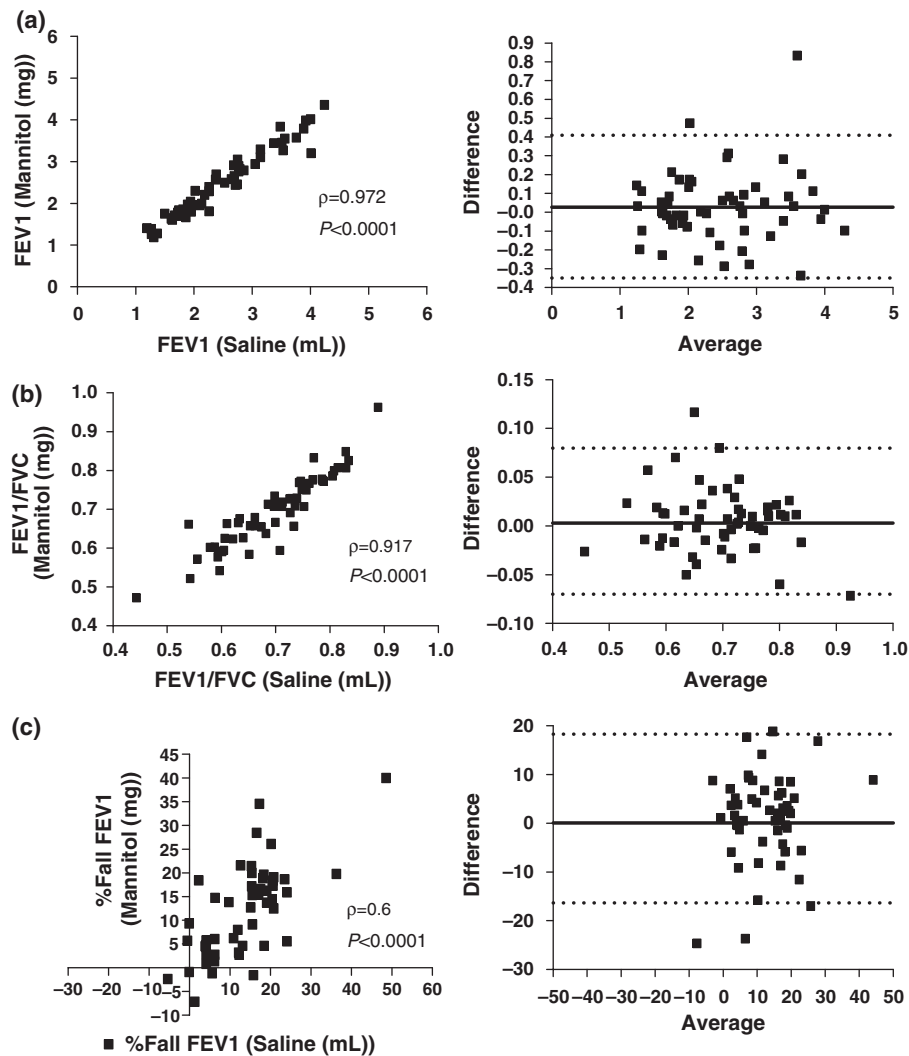


Fig. 1. Pearson's correlation and Bland-Altman plot of (a) forced expiratory volume in 1 s (FEV₁) at baseline, (b) FEV₁/FVC at baseline and (c) percentage fall in FEV₁ following challenge, using mannitol vs. 4.5% saline as the challenge agent ($n=55$). FVC, forced vital capacity.

Mannitol responsiveness by inflammatory phenotype

No significant differences were found for baseline lung function and AHR to mannitol when compared by the inflammatory phenotype (assigned using hypertonic saline-induced sputum cell counts; Table 5), $n=49$. Those with paucigranulocytic asthma had a higher baseline FEV₁, FEV₁/FVC and a lower percentage fall in FEV₁; however, this was not significant. Subjects with a mixed granulocytic phenotype had a lower baseline FEV₁ and a lower percentage fall in FEV₁; however, these results are difficult to interpret because there were only two subjects in this group.

When subjects were classified as having eosinophilic or non-eosinophilic asthma, the percentage fall in FEV₁ to mannitol was significantly less for those with non-eosinophilic asthma. Non-eosinophilic subjects tended to have a lower dose-response slope, a higher

PD₁₅ and a higher cumulative dose of mannitol although these were not significant (Table 6), $n=49$.

Sputum cell counts

Sputum cell counts in samples collected using each challenge agent are presented in Table 3, $n=39$. Sputum quality was good for both induction procedures. There was less squamous cell contamination with hypertonic saline-induced sputum; however, squamous contamination was acceptable for both induction procedures (Table 3). Sputum cell counts were compared between the saline- and the mannitol-induced sputum. Induced sputum eosinophils and neutrophils were similar for samples obtained from mannitol and hypertonic saline-induced sputum. There was a significantly lower median total cell count for the mannitol-induced sputum compared with saline

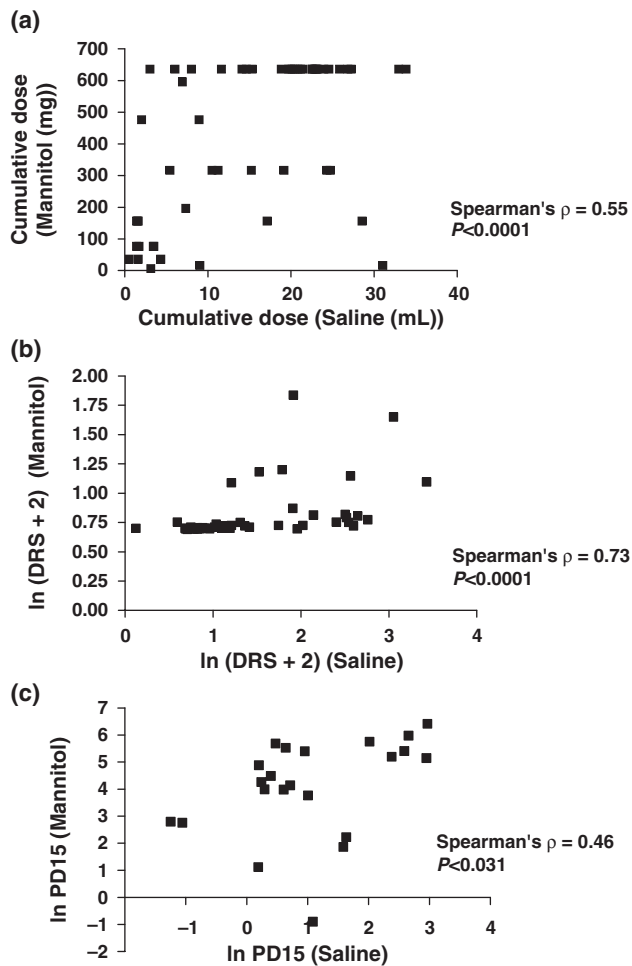


Fig. 2. Pearson's correlation of (a) cumulative dose ($n = 55$), (b) ln (dose response + 2) ($n = 55$) and (c) ln PD15 ($n = 22$) for challenge using mannitol vs. saline as the challenge agent.

(Table 3). Moderate and significant correlations were present between the two methods, except for sputum quality, which was poorly correlated (Figs 3 and 4). The agreement between both induction procedures for sputum eosinophil counts was good in the range $< 10\%$, but poor for higher levels of sputum eosinophilia. The agreement between sputum neutrophil proportions was poor between the two procedures.

There was a significant difference in the proportion of subjects with different phenotypes between saline and mannitol classifications and for eosinophilic or non-eosinophilic asthma, with three subjects (8%) misclassified by mannitol-induced sputum analysis (Table 4), $n = 39$. More subjects were classified as eosinophilic asthma and fewer as paucigranulocytic asthma following mannitol-induced sputum analysis than from hypertonic saline sputum analysis. There was no difference in the frequency of neutrophilic asthma between the two challenge procedures.

Induced sputum inflammatory mediators

There was no difference in induced sputum concentrations of IL-8, neutrophil elastase or MMP-9, in samples induced with mannitol or saline (Table 3), $n = 39$. In mannitol-induced samples, on examining inflammatory mediators according to phenotype, neutrophil elastase was increased in neutrophilic asthma compared with both eosinophilic and paucigranulocytic asthma, $n = 39$. IL-8 showed a similar trend, which did not quite reach statistical significance (Table 5). When grouped as eosinophilic vs. non-eosinophilic, there was no significant difference between groups in any mediator (Table 6), $n = 39$.

Discussion

This study establishes that mannitol inhalation can be used to both assess AHR and induce sputum in the same test. The data confirm the close association between airway responsiveness to the indirectly acting osmotic stimuli, hypertonic saline and mannitol. The study is also the first to demonstrate that mannitol challenge can be used to successfully induce sputum of good quality for analysis of inflammatory cells and inflammatory mediators, with levels comparable with those found in hypertonic saline-induced samples. We have demonstrated that the challenge agent used does not affect intra-subject airway responsiveness, the quality of sputum cells collected or the concentration of inflammatory mediators in sputum. Parameters of airway responsiveness to mannitol were different across inflammatory phenotypes. Subjects with eosinophilic asthma tended to have increased airway responsiveness to mannitol, although this was not likely to be clinically significant. There was also a significant difference in the proportion of subjects with different inflammatory phenotypes, depending on the challenge agent used, with the mannitol challenge resulting in more subjects classified as eosinophilic and less classified as paucigranulocytic asthma. However, this difference is likely to be within the limits of reproducibility for the techniques, and for clinical purposes, the techniques can be considered similar for the assessment of asthma phenotypes.

Various agents are used for bronchial provocation testing to assess AHR and induce expectoration of airway lining fluid for assessment of inflammation. Hypertonic saline has been used as a challenge agent for more than two decades [11]. It is believed to act by increasing osmolarity at the airway surface, thereby inducing the release of mediators from mast cells [6]. Mannitol, a newly developed challenge agent that involves dry powder inhalation, also transiently increases osmolarity, leading to the activation of mast cells and release of mediators such as leukotriene E_4 and prostaglandin D_2 , thereby indirectly causing contraction of airway smooth muscle [12].

Table 3. Sputum cell counts and fluid phase mediators in sputum supernatant collected during saline vs. mannitol challenge

	4.5% saline	Mannitol	<i>P</i> -value
<i>N</i>	39	39	
Total cell count $\times 10^6$ mL	3.8 (2.0, 5.4)	2.1 (1.2, 4.1)	0.003
Eosinophils (%)	1.0 (0.3, 3.0)	1.3 (0.3, 4.5)	0.446
Neutrophils (%)	49.0 (19.3, 66.8)	34.0 (18.3, 66.8)	0.507
Squamous (%)	2.7 (1.2, 9.5)	4.3 (2.4, 15.3)	0.010
Columnar epithelial cells (%)	1.8 (0.8, 3.0)	2.5 (1.0, 8.3)	0.123
Quality	17 (15, 19)	16 (14, 18)	0.153
viability (%)	76.6 (57.1, 87.5)	76.7 (64.8, 88.6)	0.748
IL-8 (ng/mL)	8.5 (4.1, 13.5)	7.5 (3.3, 23.8)	0.746
Neutrophil elastase (ng/mL)	1137 (569, 2949)	1175 (366, 5445)	0.784
MMP-9 (ng/mL)	5642 (2018, 9901)	4543 (2228, 11 108)	0.837
MMP-9 activity (U)	1367 (417, 2883)	1409 (640, 3431)	0.709

Results are median (IQR), Wilcoxon's sign-rank test.

IQR, interquartile range; MMP-9, matrix-metalloproteinase 9.

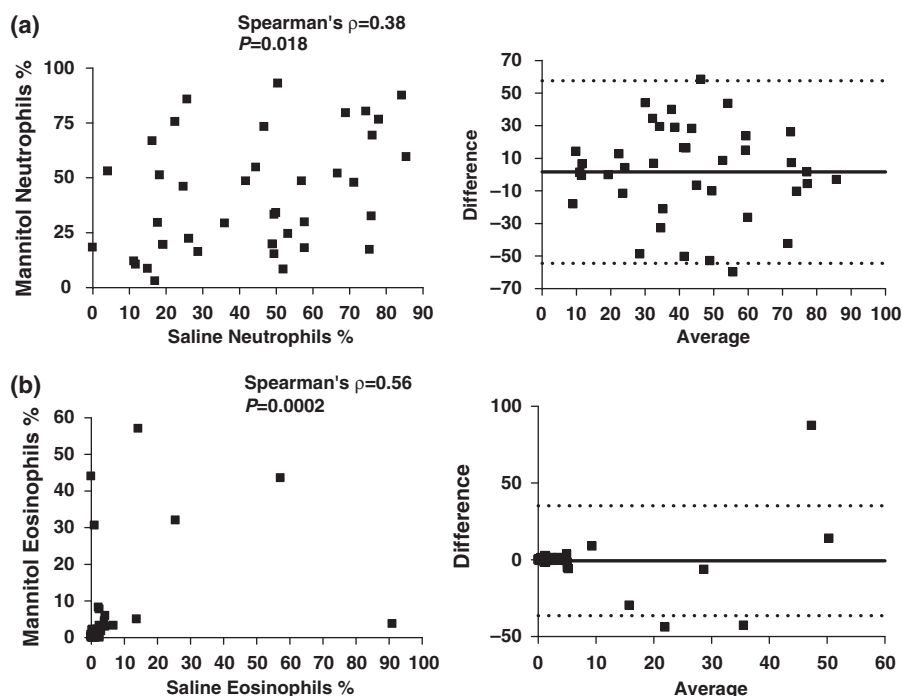


Fig. 3. Pearson's correlation and Bland–Altman plot of (a) percentage neutrophils and (b) percentage eosinophils, in induced sputum collected during a mannitol vs. a saline challenge ($n = 39$).

There were no differences in intra-subject airway responsiveness according to the challenge agent. The numbers of subjects who demonstrated AHR on the day of testing were $n = 30$ and 25 for hypertonic saline and mannitol, respectively. This reflects the fact that the majority of subjects were using corticosteroids and were thus relatively well controlled. Our data reveal that all parameters assessed, including the mean maximum fall in FEV₁, dose–response slope, cumulative dose and PD15, were correlated for the mannitol and saline challenges. Furthermore, significant correlations were observed for each of these variables when compared for the mannitol

vs. saline challenges. This is in agreement with previous reports of a correlation between PD15 for mannitol and saline [13]. A multi-centre trial of 592 subjects also demonstrated the strength of the association between mannitol and saline challenge data, as mannitol PD15 was shown to have a sensitivity of 81% and a specificity of 87% with respect to PD15 for hypertonic saline [14].

Our study extended the investigation of airway responsiveness to mannitol by examining AHR across inflammatory phenotype. We observed that when subjects were classified as eosinophilic vs. non-eosinophilic asthma, the eosinophilic group showed an increased fall in FEV₁ in

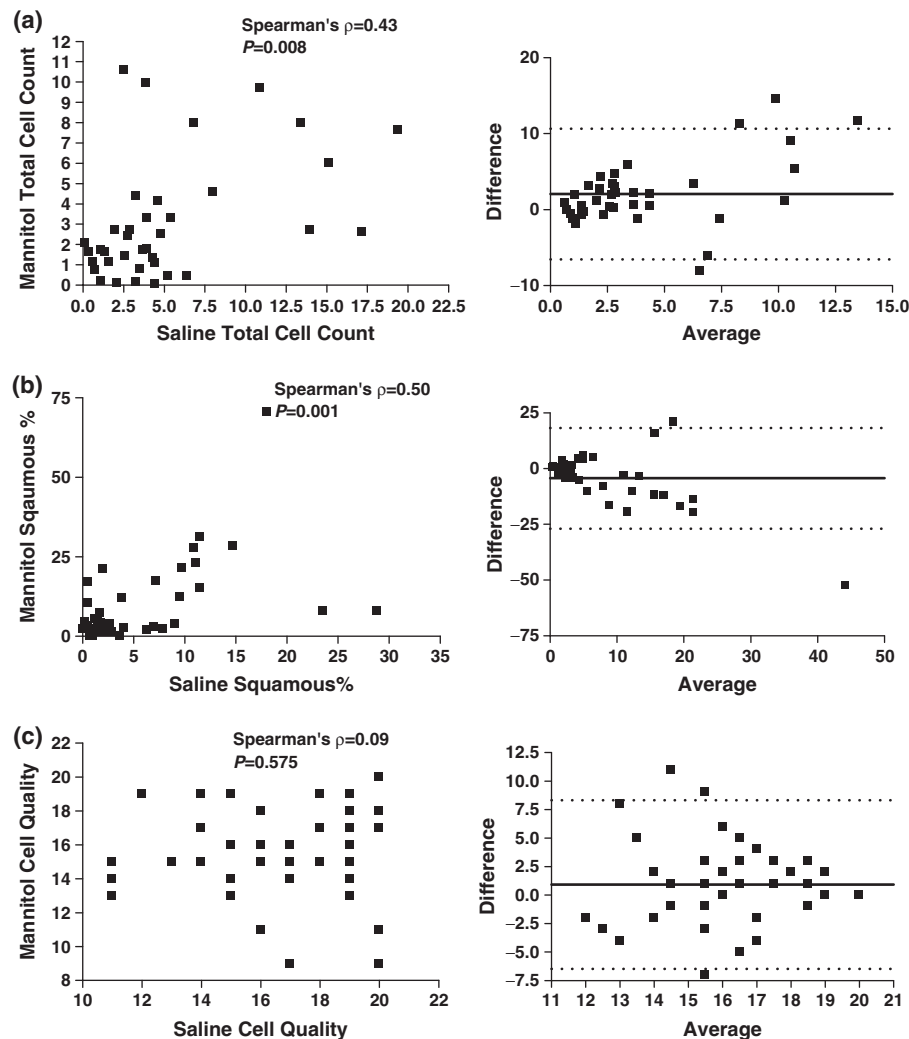


Fig. 4. Pearson's correlation and Bland-Altman plot of (a) total cell count, (b) percentage squamous cells and (c) cell quality in induced sputum collected during a mannitol vs. a saline challenge ($n = 39$).

Table 4. Phenotype proportions for hypertonic (4.5%) saline vs. mannitol ($n = 39$)

	4.5% saline <i>n</i> (%)	Mannitol <i>n</i> (%)	<i>P</i> -value
<i>Phenotype</i>			<0.0001
Eosinophilic	8 (20.5)	11 (28.2)	
Neutrophilic	8 (20.5)	7 (18.0)	
Paucigranulocytic	21 (53.9)	18 (46.2)	
Mixed	2 (5.1)	3 (7.7)	
<i>EA/NEA</i>			0.003
Eosinophilic	8 (20.5)	11 (28.2)	
Non-eosinophilic	31 (79.5)	28 (71.8)	

Results are *n* (%), Fisher's exact test.

EA, eosinophilic asthma; NEA: non-eosinophilic asthma.

response to mannitol, with a tendency towards increased AHR. The likelihood that eosinophilic inflammation worsens the degree of hyperreactivity to mannitol is supported by the observation that ICS therapy signifi-

cantly attenuates airway sensitivity to inhaled mannitol [15]. Furthermore, airway reactivity to mannitol has been shown to predict the likelihood of asthma exacerbations in an ICS down titration study [16]. However, the differences between the groups in our study are small and thus not likely to be of clinical significance.

The production of sputum samples was good using both challenge agents, with >70% of subjects producing adequate samples for laboratory analysis of sputum cells and inflammatory mediators, using both techniques. The quality of the cells collected using mannitol was similar to cells collected using saline. Although there was more squamous cell contamination using mannitol vs. saline, the difference was very small, the values were still within the acceptable range, and thus this difference is unlikely to be clinically important. Mannitol-induced samples had a lower total cell count than those induced by saline. The possible reasons for these effects include differential cell death between the two agents, sampling of different

Table 5. Mannitol airway responsiveness and mannitol-induced sputum inflammatory mediators by inflammatory phenotype (classified using cell counts from hypertonic saline-induced samples)

	Eosinophilic	Neutrophilic	Paucigranulocytic	Mixed	<i>P</i> -value
<i>N</i>	11	9	27	2	
FEV ₁ baseline (L)*	2.12 (0.72)	2.18 (0.76)	2.77 (0.82)	1.87 (0.98)	0.051
FEV ₁ /FVC baseline*	0.65 (0.08)	0.67 (0.1)	0.73 (0.09)	0.68 (0.01)	0.067
% fall FEV ₁ *†	17.16 (7.92)	14.16 (11.28)	10.09 (9.08)	1.5 (4.5)	0.066
Dose-response slope*	0.06 (0.02, 0.26)	0.03 (0.01, 0.96)	0.01 (0, 0.08)	0.01 (0, 0.01)	0.128
PD15 mannitol (mg)*	74.55 (34.35, 243.15) (<i>n</i> = 8)	16.1 (6.3, 98.5) (<i>n</i> = 3)	167.0 (42.5, 220.3) (<i>n</i> = 11)	NA	0.476
Cumulative dose (mg)*	231.13 (74.87, 426.5)	302.88 (25.01, 508.83)	326.88 (135.67, 576.5)	469.0	0.736
<i>N</i>	8	8	21	2	
IL-8 (ng/mL)†	4.9 (3.2, 12.1)	22.1 (5.1, 76.9)	5.6 (2.3, 15.4)	74.1	0.051
Neutrophil elastase (ng/mL)†	898 (467, 2886)	15 509 (5034, 42 084)†	752 (126, 2213)	15 009	0.010
MMP-9 (ng/mL)†	3501 (2045, 7365)	3853 (1858, 19 777)	3436 (1596, 10 447)	32 953	0.180
MMP-9 activity (U)†	1445 (615, 2697)	1721 (930, 2943)	782 (545, 3143)	4101	0.589

Spirometry and challenge data are presented for all subjects who produced a saline-induced sputum sample for phenotyping (*n* = 49). Inflammatory mediators are presented for the subset of subjects who also produced a mannitol-induced sputum sample (*n* = 39).

*Results are mean (SD), one-way ANOVA.

†Results are median (IQR), Kruskal–Wallis test.

‡Significantly different from eosinophilic and paucigranulocytic asthma. Inflammatory phenotype was assigned based on induced sputum cell counts obtained using hypertonic saline.

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PD15, provocation dose causing a 15% fall in FEV₁ from baseline; IQR, interquartile range; MMP-9, matrix-metalloproteinase 9.

Table 6. Mannitol airway responsiveness and induced sputum inflammatory mediators by eosinophilic/non-eosinophilic phenotype (classified using cell counts from hypertonic saline-induced samples)

	Eosinophilic	Non-eosinophilic	<i>P</i> -value
<i>N</i>	11	38	
FEV ₁ baseline (L)*	2.12 (0.72)	2.58 (0.84)	0.107
FEV ₁ /FVC baseline*	0.65 (0.08)	0.71 (0.09)	0.055
% fall FEV ₁ *†	17.16 (7.92)	10.60 (9.68)	0.046
Dose-response slope†	0.06 (0.02, 0.26)	0.01 (0, 0.11)	0.079
PD15 mannitol (mg)†	74.6 (34.4, 243.2)	84.2 (16.1, 217.1)	0.891
Cumulative dose (mg)†	231.1 (74.9, 426.5)	329.0 (76.2, 559.0)	0.459
<i>N</i>	8	31	
IL-8 (ng/mL)†	4.9 (3.2, 12.1)	8.2 (3.1, 27.2)	0.397
Neutrophil elastase (ng/mL)†	898 (467, 2886)	1340 (340, 6950)	0.417
MMP-9 (ng/mL)†	3501 (2045, 7365)	4689 (2090, 11 838)	0.778
MMP-9 activity (U)†	1445 (615, 2697)	1238 (601, 3143)	0.888

Spirometry and mannitol challenge data are presented for all subjects who produced a saline-induced sputum sample for phenotyping (*n* = 49).

Inflammatory mediators are presented for the subset of subjects who also produced a mannitol-induced sputum sample (*n* = 39).

*Results are mean (SD), Student's *t*-test.

†Results are median (IQR), Wilcoxon's rank-sum test.

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; PD15, provocation dose causing a 15% decline in FEV₁ from baseline; IQR, interquartile range; MMP-9, matrix-metalloproteinase 9.

airway regions and differences in the duration of the challenge. To address the possibility of increased cell death with mannitol, we examined cell viability and found no difference in the viability of cells collected using each challenge agent. Thus, we can exclude cell death as an explanation. The cellularity of airway regions differs, such that more proximal airway samples have a higher neutrophil count and a higher total cell count [17]. We found that mannitol-induced sputum had lower total cell

counts and tended to have lower neutrophil counts, suggesting that it may sample more peripheral airways than hypertonic saline delivered by ultrasonic nebulization. This is plausible because the particle size of mannitol dry powder by inhalation is <3.6 µm. In contrast, however, the inhalation technique with hypertonic saline that used tidal respiration and wet aerosol delivery should favour a more peripheral distribution. It is interesting that the direction of the difference was to detect more

eosinophilic inflammation using mannitol-induced sputum. If sampling of more peripheral airways by mannitol were correct, then this concurs with the known predominance of small airway eosinophilic inflammation in asthma. It would be interesting to further reevaluate this using other tests of peripheral eosinophilic inflammation such as alveolar nitric oxide. Importantly, the clinical significance of the difference in cell counts is likely to be minor, as only 8% of subjects were classified as a different phenotype when using mannitol compared with saline-induced sputum.

The proportion of subjects classified as eosinophilic, neutrophilic, paucigranulocytic and mixed inflammatory phenotypes using both saline- and mannitol-induced samples was comparable with the proportions described by our group previously [10]. The majority of subjects in this study (87%) were using maintenance ICS, and had not changed their medications for at least 4 weeks. Thus, the phenotypes reported in this paper describe these subjects when they are clinically stable. It would be expected that if steroids were withdrawn, the proportion of eosinophilic subjects, classified using either mannitol- or saline-induced samples, would increase. The extent by which the cellular proportions would change cannot be determined from this study, but is an interesting point for further investigation.

Our study assessed IL-8, MMP-9 and neutrophil elastase concentrations in induced sputum samples as markers of airway inflammation. These mediators were chosen as they can be reliably measured using assays that have been validated for use in saline-induced sputum samples [8]. Analysis of induced sputum supernatant demonstrated that the concentrations of inflammatory mediators in samples collected using mannitol and hypertonic saline were similar. Further analysis of the mannitol-induced samples indicated that neutrophil elastase was significantly elevated in subjects with neutrophilic asthma. This confirms previously reported observations in samples collected with hypertonic saline [8].

In summary, this study has evaluated various aspects of bronchial challenge combined with sputum induction, using hypertonic saline vs. mannitol as the challenge agent. The study has identified that mannitol challenge can be used to successfully induce sputum of good quality for analysis of inflammatory cells and inflammatory mediators. There are minor differences in the proportion of subjects with different inflammatory phenotypes, depending on the challenge agent used. However, these differences are likely to be within the limits of reproducibility for the techniques, and for clinical purposes, the techniques can be considered similar for the assessment of asthma phenotypes. Analysis by inflammatory phenotype indicates only minor differences in airway responses to challenge and inflammatory mediator concentrations. Thus, from these results, we conclude that mannitol

challenge can be used to induce sputum for analysis and for predicting the inflammatory phenotype in individual patients with asthma.

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