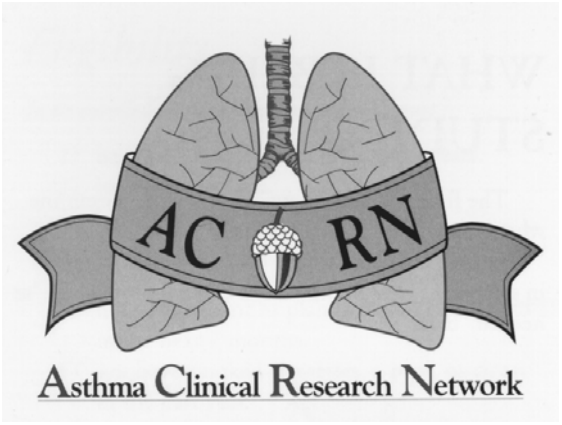

Asthma Clinical Research Network (II)
Best Adjustment Strategy for Asthma in Long Term (BASALT)

Mannitol Bronchoprovocation Challenge
Ancillary Study Protocol
Version 1.0

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ACRN (II) BASALT Protocol, Mannitol Bronchoprovocation Challenge Ancillary Study

I. HYPOTHESIS AND SPECIFIC AIMS

Mannitol bronchoprovocation appears to be a relatively simple, non-invasive way to assess the ability of mast cells in the airway to be activated. Since mast cell activation is thought to be important in the pathogenesis of asthma, decreases in the ability of these cells to be activated, as determined by mannitol bronchoprovocation, should reflect decreased airway inflammation and should be associated with improved measures of asthma control. Therefore, mannitol bronchoprovocation (as a representative "indirect airway challenge") might be very useful for guiding asthma therapy, determining response to asthma therapies, or defining asthma phenotypes. This ancillary study seeks to develop, implement, and evaluate a standardized mannitol bronchoprovocation procedure in an ongoing clinical trial conducted by the NHLBI-funded Asthma Clinical Research Network (ACRN) II. Furthermore, because one of the goals of ACRN II is the development of biomarkers predictive of treatment dependent changes in asthma symptoms and control, this ancillary study will analyze whether $P2RX_7$ pore function is linked to airway hyperresponsiveness induced by methacholine and/or mannitol challenges. The parent ACRN II trial is designated BASALT (Best Adjustment Strategy for Asthma over Long Term).

The overall hypotheses for this proposal are (1) an "indirect" challenge procedure using mannitol can safely characterize asthma phenotypes, predict asthma control and exacerbations, predict responses to interventions, and perform more specifically than a "direct" methacholine challenge; (2) $P2X_7$ pore function in whole blood correlates with measures of airway hyperresponsiveness induced by methacholine and/or mannitol challenges.

To address this hypothesis, the following specific aims are proposed:

(1) To assess the safety, tolerability, and effectiveness of mannitol bronchoprovocation procedures in a multi-center asthma clinical research trial, as compared to methacholine bronchoprovocation.

(2) To evaluate the applicability of mannitol bronchoprovocation to characterize the asthma phenotype, the baseline $PD_{15}FEV_1$, will be correlated with other baseline clinical and physiologic measurements collected in the BASALT trial. Comparisons will be made to methacholine bronchoprovocation performance.

(3) To determine if response to mannitol, serially performed during BASALT, predicts asthma exacerbations or predefined treatment failure, during the ICS treatment adjustments. Comparisons will be made to methacholine responses.

(4) To correlate changes in mannitol response to other secondary outcomes measured on a continuous scale in BASALT, such as FEV_1, morning PEF, ACQ, ASUI, exhaled nitric oxide, and EBC markers.

(5) To correlate $P2X_7$ pore function in whole blood with measures of airway hyperresponsiveness induced by methacholine and/or mannitol challenges.

II. BACKGROUND AND SIGNIFICANCE

Bronchial hyperresponsiveness (BHR) has been defined as an increase in the ease and degree of airflow limitation in response to a bronchoconstrictive stimulus. Although not entirely specific for asthma, BHR is considered one of its major pathophysiologic features.[1] Airway narrowing in asthma is the result of an interaction of multiple mechanisms, not necessarily and solely driven by airway inflammation. BHR has been called "non-specific", as most patients with active asthma will react to the bronchoconstrictive stimuli. However, these stimuli act by specific mechanisms, involving multiple pathophysiologic pathways. Therefore, the results of the different challenge tests available are only weakly correlated and not mutually interchangeable.

Bronchoconstrictor stimuli have been classified as "direct" or "indirect", based on the primary mechanism by which they cause airflow limitation.[2] Direct stimuli include: cholinergic agents such as methacholine; histamine; prostaglandin D_2; and the leukotrienes C_4, D_4, and E_4. Indirect stimuli include: pharmacologic agents such as adenosine, bradykinin, and propranolol; exercise; isocapnic hyperventilation with cold, dry air; and osmotic stimuli such as hypertonic saline, ultrasonically nebulized distilled water, and hypertonic mannitol dry powder. Combinations of mechanisms are possible.

Direct stimuli cause airflow limitation through direct action on the causative effector cells (primarily airway smooth muscle cells). Alternatively, indirect stimuli act on intermediary cells such as inflammatory cells, bronchial epithelial cells, or neuronal cells. The pro-inflammatory mediators and/or neurotransmitters, liberated by these cells, then interact with the effector cells to cause airflow limitation.[2]

Both clinical and research interest in indirect challenges has emerged. The ERS Task Force (2003) has proposed the following working definition of an indirect challenge:

"Indirect challenges act by causing the release of endogenous mediators that cause the airway smooth muscle to contract, with or without effect in inducing microvascular leakage. Because the responses to these challenges are modified or even completely inhibited by inhaled steroids, the airway response to these challenges may be a closer reflection of active airway inflammation."[3]

The hypertonic (4.5%) saline test, developed over a number of years by Anderson and colleagues in Australia, was one of the first indirect challenge procedures to be applied in research settings. [4-7] It has since been used safely in adults and children in a variety of studies, using a 15% reduction in FEV_1 to define the abnormality.[8-13] However, Anderson et al [7] reported technical difficulties with the hypertonic saline test, precluding it from adoption as a routine challenge procedure in clinical research.

To overcome the technical problems of the hypertonic saline test, Anderson and colleagues have developed a simpler osmotic challenge using dry powder mannitol.[14] Studies using mannitol challenge have demonstrated a very similar profile as other "indirect" tests, such as hypertonic saline, exercise, eucapnic voluntary hyperpnea, and adenosine monophosphate.[15-18] Further, the Aridol Study Group found the mannitol challenge to be generally safe and well tolerated in their large trial of 592 children and adults, with and without asthma.[18] No serious adverse events were reported. Mannitol PD_{15} had a sensitivity of 81% and specificity of 87% with respect to PD_{15} for 4.5% saline. A positive mannitol test cut off of a 15% fall in FEV_1 (PD_{15}) provided appropriate sensitivity and specificity with respect to clinical diagnosis of asthma even when the patient's baseline FEV_1 was within the normal range. Based on an analysis of patients with a clinical diagnosis of asthma, and excluding those with a negative test result and on current corticosteroid therapy, mannitol PD_{15} had a sensitivity of up to 89% to detect the presence of asthma and specificity of 95% for clinical diagnosis of asthma.

The research advantage of using provoking stimuli that act "indirectly" to cause airways to narrow is the high specificity for identifying the type of BHR that is altered by drugs used in the treatment of asthma,[19,20] and inhaled corticosteroids (ICS) in particular. Sensitivity to mannitol is reduced or even totally inhibited by chronic administration of ICS,[21] thereby allowing monitoring of therapy.[22] Furthermore, and of particular relevance to asthma clinical research networks, response to mannitol has been used to predict asthma exacerbations following back titration of ICS dose in subjects previously well controlled with ICS.[23]

The pharmaceutical company Pharmaxis Ltd. has received marketing approval for its inhaled dry powder mannitol (Aridol®) in Australia and Sweden, and is seeking approval in 27 other European Union member states. The use of Aridol® has been included in both the Australian and GINA guidelines. The U.S. Phase 3 studies are completed and under analysis. No serious or significant adverse events were reported in these trials. An NDA is planned for submission in 2007, with the potential of U.S. marketing in 2008.[24]

The dispassionate analysis of a new bronchoprovocation procedure by the ACRN is therefore timely, and consistent with the mission of this investigative group. The advantage of specificity of a safe and tolerable indirect challenge has high research appeal, especially in regards to phenotypic characterization of study populations and predictors of response to therapies such as ICS. The ACRN has vast experience with methacholine challenge, as a study entry criterion, a therapeutic outcome, and a predictor of drug response. This proposed ancillary study would provide the opportunity to make comparisons of "direct" versus "indirect" challenges in a clinical trial, and to share our experiences with the asthma research community.

This ancillary study also allows further investigation into $P2X_7$ pore function. The chromosomal location 12q24 has been linked to methacholine responsiveness in multiple asthma populations (25-30). Our group is testing the notion that a gene in this region, P2RX7, contributes to variability in the asthma phenotype. Surface expression of this gene by bronchial epithelial cells or leukocytes confers a homotrimeric nonselective cation channel/pore, $P2X_7$, that amplifies numerous cytokine and inflammatory responses including mast cell production of IL-13 when stimulated by extracellular ATP (31, 32). We have developed a screening assay for rapidly identifying subjects with loss-of-function P2RX7 alleles that is able to bridge disparate genomic, phenotypic and clinical results, while increasing statistical power in the setting of sample size limitations (33). In this regard, we have recently shown that $P2X_7$ pore function inversely correlates with the change in asthma symptoms during the course of an upper respiratory tract infection in 31 mild asthmatics (R_s = -0.486, p = 0.009, Denlinger et all ATS 07 poster, manuscript in preparation).

III. PRELIMINARY STUDIES

Mannitol has long been approved as an osmotic agent in both adults and children and has been given orally, in doses up to 200 grams to induce diarrhea for bowel preparation pre-surgery/procedures, and intraveneously to induce diuresis or to treat cerebral edema.

Inhaled mannitol acts as an indirect bronchial provocation test via an osmotic stimulus that induces the release of bronchoconstricting mediators (such as histamine, leukotrienes, and prostaglandins) resulting in constriction of airway smooth muscle. It has been hypothesized that indirect methods of monitoring airway hyperresponsiveness better reflect chronic inflammation than direct challenges. Indirect challenges might also be the preferred method of diagnosing asthma, monitoring disease activity, and responses to anti-inflammatory therapy, whereas, direct challenges may be the preferred method to exclude an asthma diagnosis, or monitor asthma control over the long term. Other indirect methods of measuring airway hyperresponsiveness include: hypertonic saline, adenosine monophosphate, exercise, cold air and voluntary hyperventilation. Cold air

hyperventilation has been used safely in clinical settings for more than 20 years. Airway hyperresponsiveness can also be identified by agents that have a direct constricting action on airway smooth muscle such as histamine or methacholine.

Previous clinical studies have shown that inhaled mannitol used as a bronchial provocation test for airway hyperresponsiveness is both safe and efficacious. At least 16 Phase 2 clinical trials have been performed involving over 1,100 subjects receiving inhaled mannitol bronchoprovocation to evaluate the safety and efficacy of this drug in healthy and asthmatic subjects (Pharmaxis Investigator's Brochure, 8th Edition 2007 – refer to Appendix I).

Reference	Objective	Study Population	Challenges per Subject	Results
Porsbjerg, 2007[34]	Subjects with asymptomatic AHR to methacholine would not have AHR to mannitol	16 healthy adult subjects	mannitol methaholine	Response to mannitol was within normal range in asymptomatic subjects with AHR to methacholine
Brannan 2005[18]	Phase 3 comparison study of mannitol with hypertonic saline	592 subjects	mannitol hypertonic saline	Mannitol is safe and effective
Brannan 2003[35]	To determine if mannitol inhalation causes mast cell activation and release of mediators	21 subjects; 12 asthmatic 9 healthy non-asthmatic	mannitol	Mannitol provoked airway narrowing in asthmatic subjects increased urinary excretion of metabolites related to mast cell activation
Currie 2003[17]	Determine the putative relationship between AMP and mannitol	15 adult asthmatics	Adenosine monophosphate (AMP) Mannitol	There is a significant relationship between the threshold concentrations of AMP and mannitol that cause a reduction in FEV_1
Koskela 2003[22]	Compare the sensitivity and validity of mannitol, histamine, and cold air bronchial challenges	17 adult asthmatics	Mannitol Histamine Cold air	Mannitol challenge is sensitive and valid to demonstrate the effectiveness of ICS in asthma

Bronchial provocation with mannitol has been proven safe and well tolerated in healthy subjects with and without asthma. Inhalation of mannitol dry powder generally provokes a decrease in FEV_1 in asthmatic subjects, reversible with the administration of short-acting $beta_2$-agonists.

Adverse Events Associated with Bronchial Provocation Tests

Adverse Event Reported	Mannitol (%)	Hypertonic saline (%)	Methacholine* (%)
Ophthalmic			
Eye pruritis	1.0	0.6	
Gastrointestinal			
Nausea	4.3	3.0	
Upper abdominal pain	1.9	1.1	
Diarrhea	1.3	0.6	
Vomiting	1.3	0.9	
Infections			
Nasopharyngitis	1.4	3.1	
Upper respiratory tract	1.3	1.7	
Musculosketal			
Back pain	1.0	0.6	
Neuronal			
Headache	17.2	19.0	2
Dizziness	1.1	1.3	6
Respiratory			
Pharyngolaryngeal pain	5.1	3.0	
Cough	2.2	2.4	25
Rhinorrhea	2.1	1.4	
Throat Irritation	1.3	0.2	
Asthma aggravated	1.1	1.3	
Chest tightness	1.0	0.6	transient
Dyspnea	1.0	0.8	21
Sneezing	0.8	1.1	
Nasal congestion	0.6	1.1	
Wheezing	0.5	1.1	10
General			
Fatigue	1.1	0.5	

*Product Information, Aridol™ Mannitol Powder for Inhalation, Pharmaxis Ltd., Australia *Limited data*

Pulmonary Response to Bronchial Challenge Testing

Reference	N	MC	CAC	HC	HS	AMP
Koskela[36] 2003	37	19 (51%)	9 (24%)	18 (49%)		
Koskela[36] 2003 * after treatement w/ICS	17	17 (100%) 7 (41%)	7 (41%) 2 (12%)	17 (100%) 9 (53%) ($PD_{15} \leq$ 1mg)		
Brannan[13] 2005	592	296 (50.0%)			322 (54.4%)	
Currie 2003[17]	15	15 (100%)				15 (100%)

MC = Mannitol challenge (fall in $FEV_1 \geq 15\%$); CAC = Cold Air challenge (fall in $FEV_1 \geq 9\%$); HC = Histamine challenge ($PD_{15} \leq 0.4mg$); HS = Hypertonic saline challenge (fall in $FEV_1 \geq 10\%$); AMP = adenosine monophosphate PC_{20} (fall in $FEV_1 > 20\%$)

Koskela and colleagues[36] have shown that mannitol is more sensitive than cold air, and as sensitive as histamine (PD_{15} 0.4mg) in demonstrating airway hyperresponsiveness. Subjects more responsive to mannitol tended to be male and dermatographic, those subjects that were cold air responsive were younger and also demonstrated dermatographism, and histamine responsiveness was related to old age. The Koskela group[36] has also demonstrated that a mannitol challenge is sensitive and valid in measuring the effects of inhaled corticosteroids in the treatment of asthma. Subjects were examined at baseline and 3 and 6 months after starting ICS therapy. Both mannitol and histamine challenges were shown to be sensitive tests demonstrating the effectiveness of ICS on airway hyperresponsiveness after 3 and 6 months of treatment Values in symptom scores and mean diurnal peak flow variation correlated with responses in the mannitol airway provocations.

The Brannan group[18] examined the safety and efficacy of dry powdered mannitol as an airway provocation challenge to hypertonic (4.5%) saline. Hypertonic saline challenges to measure airway hyperresponsiveness have been used safely and effectively for more than 20 years in both adults and children. The challenge procedures were well tolerated by subjects; withdrawal from the study due to severe adverse events occurred in four subjects (0.6%0 after mannitol and three subjects (0.5%) after the hypertonic saline challenge. Looking at adverse events in the 7 days following each challenge were not significantly different between the two procedures. The most common adverse events were: headache (mannitol 12.4%, HS 14.5%); infections and infestations (4.1% and 6.3%); gastrointestinal disorders (7.3% and 4.6% respectively). Cough is a recognized occurrence in conjunction with bronchial provocation challenges; however the majority of mannitol subjects (>85%) had either no cough due to the challenge or an occasional cough. Overall, Brannan and colleagues[18] concluded that the mannitol challenge was safe and well tolerated, with no serious adverse events reported. Mannitol PD_{15} was also shown to have a sensitivity of 81% and a specificity of 87% in comparison with hypertonic saline PD_{15}. They also demonstrated in patients with a clinical diagnosis of asthma currently using ICS the mannitol PD_{15} showed a sensitivity of up to 89% to detect asthma and a specificity of 95% for the clinical diagnosis of asthma.

Currie et.al.[17] compared the relationship of mannitol PD_{15} and adenosine monophosphate (AMP PC_{15}) bronchial challenges in asthmatic subjects. AMP has been widely used as an indirect bronchoconstricting stimulus to measure airway hyperresponsiveness. Fifteen subjects participated in the trial with good correlation ($p < 0.001$) between responses to mannitol PD_{15} and AMP PC_{15}. The authors concluded that mannitol PD_{15} is useful and practical, with a significant relationship to airway hyperresponsiveness measured using AMP PC_{15}.

IV. PROTOCOL

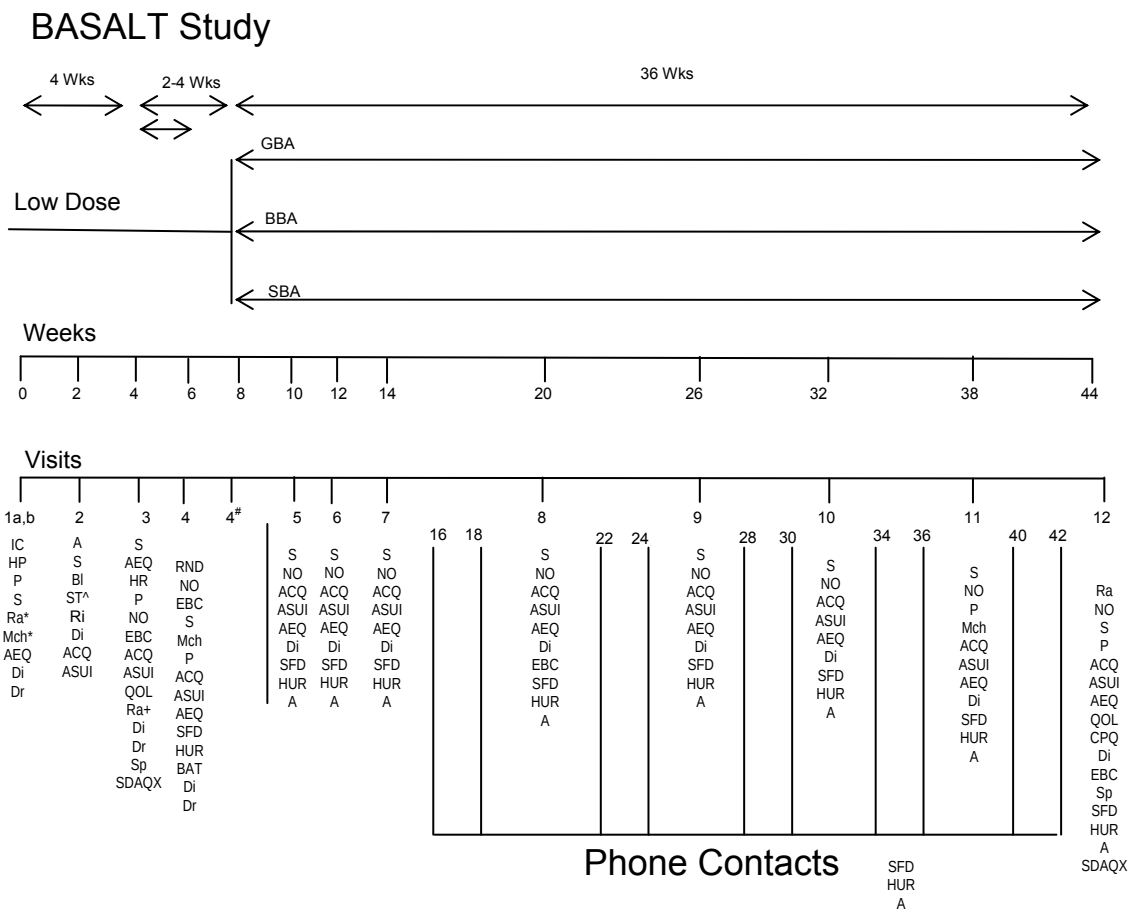
A. ACRN II BASALT RESEARCH DESIGN

The BASALT trial is being conducted at each of the ten designated ACRN II study centers. The primary hypothesis of BASALT is that in subjects with mild-moderate asthma initially well-controlled on daily low-dose ICS therapy, symptom-based adjustment (SBA) and/or biomarker-based adjustment (BBA) of ICS therapy will be superior to standard, guideline-based adjustment (GBA), in maintaining asthma control, as assessed by the time to pre-defined asthma treatment failure. Additionally, it is hypothesized that compared to GBA treatment, SBA and/or BBA treatment will result in: lower cumulative dose of ICS and oral corticosteroid treatment; improved asthma-related quality of life; fewer days lost from work or school; reduced estimated cost of care; a greater proportion of visit days with Asthma Control Questionnaire (ACQ) scores <1.25; greater reduction in markers of inflammation (FeNO, EBC pH and cytokines, PC_{20} FEV_1 methacholine, sputum eosinophils); reduced drop-out rate. Further, it is postulated that phenotypic subject features on BASALT entry correlate with the risk of treatment failure after treatment is shifted from low dose ICS taken twice daily to "adjusted dose" ICS. The phenotypic features to be evaluated in the parent BASALT study include: FEV_1% predicted; delta FEV_1 after 4 puffs of bronchodilator; change in pre-bronchodilator FEV_1 over 2 weeks after treatment is reduced; $PC_{20}FEV_1$ methacholine; FeNO, EBC pH and cytokines; sputum eosinophils. An exploratory hypothesis in BASALT is to compare the predictive value of FEV_1, FEV_1 bronchodilator response change in FEV_1 over two weeks after ICS reduction, $PC_{20}FEV_1$ methacholine, EBC pH and cytokines, sputum eosinophils, and FeNO for treatment failures when ICS treatment is reduced.

BASALT is a three-arm, parallel group randomized, double-blind, dual-dummy trial, consisting of a 4 week run-in period (common with TALC), a 2-4 week adherence monitoring period, and a 36 week intervention period. Subjects ≥ 18 years (n = 320) will be allocated to BASALT at Visit 3, if their pre-bronchodilator FEV_1 > 70% predicted and they meet pre-set asthma control criteria. BASALT participants must also demonstrate run-in period bronchodilator reversibility of ≥ 12% or PC_{20} FEV_1 methacholine ≤ 8 mg/ml if not on an ICS or ≤ 16 mg/ml if on an ICS. The BASALT protocol schema, visit schedule, and protocol activities are outlined below.

To achieve the specific aims of this proposal, a mannitol bronchoprovocation procedure will be added at visit 4a (within 1 week of visit 4), week 20, and week 32 noted in the parent BASALT Study. The blood sample for the $P2X_7$ pore function assay will be obtained at visit 4a.

PROTOCOL SCHEMA



*Ra-albuterol reversibility testing and/or *Mch-methacholine bronchoprovocation will be done at the investigator's discretion according to ACRN protocols. Subjects who are eligible for methacholine challenge will undergo this test at Visit 1a[1]. If asthma diagnosis is not confirmed, subjects may return for albuterol reversibility testing at Visit 1b at the investigator's discretion. Visits 1a and 1b will occur on different days. Subjects who are ineligible to perform methacholine challenge at Visit 1a will undergo albuterol reversibility testing at that visit. #V4 will be done 2 weeks after visit 3. If the subject is not compliant, V4 will be repeated in 2 more weeks (i.e., 4 weeks after visit 3). ^ If the subject cannot undergo skin testing at Visit 2 due to drug washouts or FEV1<60%, skin testing may be done at subsequent visits.

[1] Historical PC20 from an ACRN methacholine challenge performed within 6 months of the Visit 1 date by an ACRN-certified technician may be used to qualify the subject.

Abbreviations used in the protocol include the following: IC-informed consent; HP-medical history, brief physical examination; P-pregnancy test; NO-eNO measurement; EBC-exhaled breath condensate collection; Ra-reversibility testing with 4 puffs of albuterol; Mch-methacholine bronchoprovocation; AEQ-3 question ACRN Asthma Evaluation Questionnaire (Appendix 1); Di-diary dispensing, review; Dr-drug dispensing, adherence; A-specific adherence check and adherence encouragement; Bl-blood for IgE level, eosinophils, DNA, etc.; ST-skin tests; Ri-reversibility testing with 4 puffs of ipratropium bromide; ACQ-asthma control questionnaire; ASUI-asthma symptom utility index; HR-resting heart rate as measured by ECG; QOL-asthma-specific quality of life questionnaire (AQLQ); S-spirometry; SP-sputum induction; BAT – BASALT Adherence Testing; SFD – symptom free day instrument; HUR – healthcare utilization review instrument, CPQ – coordinator/patient questionnaire, SDAQX – sleep and day alertness questionnaire

Visit	**1a[1]**	**1b[2]**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Week	0	0	2	4	6-8	10	12	14	20	26	32	38	44
Window (reg/ext) (Days)			±3/ ±5	±3/ ±5	±3/ ±5	±3/ ±5	±3/ ±5	±3/ ±5	±5/ ±7	±5/ ±7	±5/ ±7	±5/ ±7	±5/ ±7
Study Phase													
Allocation to BASALT				X									
Informed Consent	X												
Randomization					X								
Medical History	X												
Long Physical Exam	X												X
Short Physical Exam				X	X	X	X	X	X	X	X	X	
Blood for IgE/Eosinophils			X										
Blood for genetic analysis (optional)			X										
Pregnancy Test	X			X	X							X	X
Skin Testing			X*										
Heart Rate Assessment (ECG)				X									
ENO				X	X	X	X	X	X	X	X	X	X
EBC				X	X				X				X
Spirometry	X	X	X	X	X	X	X	X	X	X	X	X	X
Albuterol Reversal (4 puffs)	(X)	X		X									X
Ipratropium Reversal (4 puffs)			X										
Methacholine Challenge	(X)				X							X	
Sputum Induction				X									X
Asthma Evaluation Questionnaire (AEQ)	X			X	X	X	X	X	X	X	X	X	X
ACQ/ASUI Questionnaires			X	X	X	X	X	X	X	X	X	X	X
AQL Questionnaire				X									X
SFD Questionnaire					X	X	X	X	X	X	X	X	X
Healthcare Utilization Review (HUR)					X	X	X	X	X	X	X	X	X
Sleep and Daytime Alertness Questionnaire				X									X
Coordinator/Patient Questionnaire(CPQ)													X

[1] Subjects who do not have historical PC20 and who have prebronchodilator $FEV_1 \geq 55\%$ of predicted and are eligible to perform a methacholine challenge will undergo the challenge at Visit 1a to determine eligibility; subjects who do not have acceptable historical PC20 and are ineligible to perform the methacholine challenge will undergo albuterol reversibility testing with 4 puffs of albuterol at Visit 1a.

[2] Subjects who do not meet methacholine PC_{20} criteria at Visit 1a may return for albuterol reversibility testing (4 puffs) for eligibility assessment at Visit 1b at the study investigator's discretion.

* Subjects who are not eligible for skin testing at V2 due to drug washout or FEV1<60%, may undergo skin testing at subsequent visits.

ACRN Satisfaction Questionnaire													X
Diary Card Dispensation	X	X	X	X	X	X	X	X	X	X	X	X	
Diary Card Review			X	X	X	X	X	X	X	X	X	X	X
Medication Dispensation	X	X	X	X	X	X	X	X	X	X	X	X	
Compliance review (Doser, AM1, etc.)			X	X	X	X	X	X	X	X	X	X	X

B. SUBJECTS, INCLUSION AND EXCLUSION CRITERIA

All subjects randomized to the BASALT protocol are eligible for the Mannitol Bronchoprovocation Challenge Ancillary Study and will be invited to participate, using an addendum consent form. A negative urine pregnancy test must be demonstrated, in women of childbearing potential. The only exclusion criteria would be: (1) positive urine pregnancy test and (2) requirement for more than two albuterol treatments for reversal of the methacholine bronchoprovocation challenge procedure at Visit 4 in the BASALT parent trial.

C. MANNITOL BRONCHOPROVOCATION METHODS

An ACRN II Steering Committee-approved Mannitol Challenge Manual of Procedures (MOP), developed by the Madison Center, will be used for testing the ACRN II BASALT study participants (Appendix II). These procedures are comparable to those utilized by the Aridol® Study Group[18], and consistent with the format and procedures of the ACRN Methacholine Challenge (used in all ACRN I and ACRN II). Components of this Mannitol Challenge MOP include: (1) purpose of mannitol challenge testing; (2) inhalation methods used in mannitol challenge procedures; (3) measurement of airway responsiveness; (4) safety procedures during mannitol challenge testing; (5) performance of mannitol challenge testing. A baseline FEV_1 of ≥ 70% predicted will be used for the ACRN II subjects (consistent with the published safety cut-offs). Spirometers will be programmed for the mannitol challenges, and distributed to the 10 ACRN II study centers (including Madison). All mannitol challenge procedures will be overread by Madison personnel, who will also be responsible for study coordinator training and certification. Standardized forms will be utilized to collect mannitol procedure performance information and safety/tolerability information. All data will be managed by the ACRN II Data Coordinating Center (DCC).

An investigator-initiated IND will be submitted, articulating the study procedures and scientific aims outlined in this ancillary proposal. Pharmaxis Ltd., the manufacturer of mannitol (Aridol®), has committed to provide the cross-referencing letter required for this IND, as well as necessary mannitol supplies and delivery devices. An addendum consent form for this procedure will be used, outlining the mannitol challenge procedure, potential risks of the procedure, and participant compensation. This consent form will state that the mannitol procedure is optional and will not prevent participation in the parent study. The consent language is that approved at UW-Madison for other protocols that have included mannitol challenges.

D. P2X7 PORE ASSAY SAMPLE COLLECTIOON AND ANALYSIS

A 5 ml citrate-anticoagulated tube of whole blood will be obtained at Visit 4a in consenting individuals who also agree to the mannitol bronchoprovocation challenge testing. These samples will be shipped overnight at room temperature to the Madison ACRN center for processing.

The staining procedure has been previously published.[33] Briefly, aliquots of citrated whole blood (blue top, 500 µL/liquot) are washed twice in HEPES-buffered saline (HBS; 130 mM NaCl, 5 mM KCl, 20 mM HEPES pH 7.4, 0.1% bovine serum albumin, 10 mM glucose; components purchased

at Sigma, St. Louis, MO) and labeled at room temperature with 125 ng of an anti-human CD14 antibody conjugated to phycoerythrin (BD Biosciences, San Diego, CA). After twenty minutes, the cells are washed twice in a potassium glutamate buffer (130 mM potassium glutamate, 5 mM KCl, 20 mM HEPES pH 7.4, 0.1% bovine serum albumin, 10 mM glucose; components from Sigma) to maximize the differences between high and low pore activities. In the absence of NaCl, cells were stimulated for twenty minutes with 0 or 250 μM 2'-3;'-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (Bz-ATP; Sigma) in the presence of 1 μM YO-PRO-1 (Molecular Probes, Eugene, OR). Samples were then adjusted to 10 mM magnesium chloride, washed in HEPES-buffered saline and diluted to a volume of 2.5 mL in HBS. Propidium iodide is added immediately prior to data collection, after $P2X_7$ pore closure by the magnesium addition.

Flow cytometry is performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), calibrated daily using standard fluorimetric beads in conjunction with the CellQuest and CellQuestPro acquisition and analysis software (v.3.3 and 4.0; Becton Dickinson). Instrument settings are calibrated to bead standards to minimize systematic variability. Finally, dead cells staining with propidium iodide are excluded from the analysis, further assisting feasibility of consistent results in a multi-center study with shipped samples (Korpi-Steiner et al. manuscript in preparation).

E. STATISTICAL METHODS AND POWER ANALYSES

All primary and secondary statistical analyses will follow the intent-to-treat paradigm, although there may be some exploratory analyses that do not.

Specific Aim 1. To assess the safety, tolerability, and effectiveness of mannitol bronchoprovocation procedures, as compared to methacholine bronchoprovocation. Standardized equipment, procedures, and quality control measures will be implemented at the 10 ACRN sites and incorporated into the BASALT trial. Standardized data collection forms (comparable to those for ACRN methacholine challenges) will be used to identify subject characteristics that modify safety and tolerability, as well as to collect effectiveness measures (ability to complete the procedure, need for interruption or rescue, time to completion, subject acceptability of the procedure). Data from all mannitol procedures performed during BASALT (baseline, week 20, week 32) will be summarized, separately and in aggregate. Assuming 75% accrual of the study's participants, it is estimated that 720 mannitol challenge procedures will be performed. The analysis plan for **Specific Aim 1** is largely descriptive. We will provide a descriptive summary of the distribution of the mannitol $PD_{15}FEV_1$ values, as well as summarize all information recorded regarding safety and tolerability of the procedure, ability to complete the procedure, frequency of need for interruption or rescue, time to completion, and subject acceptability of the procedure. Comparisons to the same information recorded regarding methacholine challenges will be made.

Specific Aim 2. To evaluate the applicability of mannitol bronchoprovocation to characterize the asthma phenotype. In BASALT, the baseline mannitol PD_{15} FEV_1 will be correlated with other baseline measurements collected in the parent trial by ACRN standardized procedures. These measurements include: methacholine PC_{20} FEV_1, bronchodilator reversibility with 4 puffs of albuterol, exhaled nitric oxide, EBC analytes, sputum eosinophils and ECP, ACQ, and other asthma control measures. To address **Specific Aim 2**, we will use baseline data collected during the BASALT run-in period. We will statistically analyze the relationship between baseline $PD_{15}FEV_1$ and the other baseline clinical and physiologic measurements. For measures that are normally distributed or normally distributed after transformation, we will evaluate Pearson correlation coefficients, and for measures that are not normally distributed Kendall's tau coefficient will be reported. We will also assess these relationships with adjustment for clinical center via partial correlations. Based on the proposed start date of the BASALT trial and the participation of these subjects in this ancillary trial, we assume that we will have data on approximately 75% of the 320

subjects enrolled (n=240 subjects), which will provide 95% confidence limits within 0.10 of the true correlation values.

Specific Aim 3. To determine if response to mannitol, serially performed at baseline, week 20, and week 32 in BASALT subjects, predicts asthma exacerbations or predefined treatment failure, during the ICS treatment adjustments. To address **Specific Aim 3**, we will utilize a Cox proportional hazards model to evaluate the time to treatment failure or asthma exacerbation, and include mannitol $PD_{15}FEV_1$ (or likely a transformed version) as a time-dependent covariate to determine if response to mannitol predicts treatment failure or exacerbations. In the model we will adjust for center and treatment, and evaluate the $PD_{15}FEV_1$ by treatment interaction to determine whether the relationship needs to be explored for each treatment group separately. We will then fit an additional Cox proportional hazards model to evaluate the effect of $PD_{15}FEV_1$ on exacerbation or treatment failure with adjustment for any additional baseline covariates that might be important (along with center and treatment effects). This will allow us to assess the importance of $PD_{15}FEV_1$ as a predictor of exacerbation or treatment failure in the presence of other important baseline clinical information and markers of severity. Since each subject may experience multiple treatment failures or exacerbations, as a secondary analysis we will also evaluate a repeated measures proportional hazards regression model to incorporate all events simultaneously, which is available in the SUDAAN (SUrvey DAta ANalysis) statistical package. The same analytic approach will be applied to the methacholine PC_{20} FEV_1, to compare performance of the "indirect" and "direct" challenge procedures.

Specific Aim 4. To correlate changes in mannitol response to other secondary outcomes measured on a continuous scale in BASALT, such as FEV_1, morning PEF, ACQ, ASUI, exhaled nitric oxide, and EBC markers. **Specific Aim 4** will be evaluated by assessing the correlation between changes in mannitol response to other secondary outcomes measured on a continuous scale in BASALT, such as FEV_1, am PEF, ACQ, ASUI, exhaled nitric oxide, and EBC markers. The change in mannitol will be evaluated based on the transformation that is found to be most informative in Specific Aim 3. For BASALT, the correlations will be evaluated within each treatment arm, however other methods including partial correlations, where adjustment for treatment group can be made, will be investigated which would allow for assessing the data as a whole. The precision of the 95% confidence limits will be within 0.20 of the true correlation values within each treatment group (n=80), and within 0.10 of the true correlation vales when the data is evaluated combined (n=240).

Specific Aim 5. To correlate $P2X_7$ pore function in whole blood with measures of airway hyperresponsiveness induced by methacholine and/or mannitol challenges. Exploratory Hypotheses: (1) The frequency of P2RX7 loss-of-function alleles will be enriched in individuals with a low methacholine PC_{20} (e.g. < 1 mg/mL) or low mannitol PD_{15}. (2) Attenuated pore activity and/or loss of function P2RX7 alleles will have a higher frequency in subjects having an exacerbation during the course of the BASALT protocol. (3) $P2X_7$ pore activity will correlate with other asthma biomarkers such as FeNO and/or sputum eosinophils. To address **Specific Aim 5**, $P2X_7$ pore activity will be treated as a continuous variable to calculate Spearman correlation coefficients with $PC_{20\text{-methacholine}}$, $PD_{15\text{-mannitol}}$, or other biomarkers after appropriate transformations as needed to achieve normal distribution of the data. Multivariate logistic regression analysis will be used to model the predictors of asthma exacerbations. Finally, genotyping for P2RX7 will be performed at the Wake-Forest Center. The distribution of alleles will be monitored for conformity to the Hardy-Weinberg equilibrium in order to detect genotyping errors and sampling bias. Haplotype analyses will be done using the score test implemented in the computer program Haplo.stats. The primary endpoints will be analyzed using the Fisher's exact test and logistic regression with adjustment for potential confounding variables such as age, gender, ethnicity and lung function where appropriate.

F. ADVERSE EVENT REPORTING AND DATA SAFETY MONITORING PLAN

Adverse event definitions and reporting for this Ancillary Study will be in accordance with the ACRN General Manual of Operations, Adverse Event Section, dated February 20, 2007 as applied to the ACRN II BASALT parent trial (see Appendix III).

This BASALT ancillary protocol has been approved by the Asthma Clinical Research Network Steering Committee, the NHLBI Protocol Review Committee and will be monitored by a NHLBI Data Safety Monitoring Board (DMSB). The Chairperson of the DSMB and all members are appointed by, and responsible to, the Director of NHLBI. The DSMB is made up of 6 external members and 3 representatives from the NHLBI. They are experts in statistics, genetics, pulmonary medicine, asthma management, ethics, clinical trial design, and basic science. One member is a layperson from the "Mothers of Asthmatics" group and provides a consumer perspective. The members act independently of the researchers, and report directly to the NIH.

The DSMB is responsible for review of study data in order to ensure quality and safety of study subjects and to provide NHLBI advice regarding the progress of studies in the ACRN. The DSMB reviews information on a monthly basis and schedules conference calls or meetings when needed during the course of the trial. The PI of the Data Coordinating Center attends DSMB meetings to present data. No clinical center PI attends the DSMB meetings. All data and deliberations of the DSMB are strictly confidential. The DSMB reviews interim reports of subject accrual and outcome measures provided by the DCC. Each report includes tabulations of study subject characteristics, major clinical events, and primary/secondary outcomes arranged by clinical center and by treatment group. After reviewing each interim report, the DSMB assesses the need to perform further in-depth evaluation of the benefits and risks of continuing the study. If necessary, the Chairperson of the Steering Committee is contacted (via mail or phone) to answer questions. The DSMB may recommend protocol modifications or early termination of studies based on concerns for the welfare of study subjects or scientific integrity. If it is determined that the study objectives have been satisfied based on data accrued to date; if patient safety would be compromised by continuation of the study; or if there are severe unanticipated problems with study conduct (that is, inadequate recruitment or problems with supplies of medications, etc.), the DSMB may recommend to the Director of NHLBI that the study be terminated or suspended temporarily. The NHLBI works with members of the Steering Committee to assure that appropriate steps are taken to implement the recommendations of the DSMB.

At the conclusion of individual studies, and at other times as may be deemed appropriate by the DSMB, the DSMB recommends release of unmasked study data summaries to the full Steering Committee for analysis interpretation and publication in scientific journals. No other release of unmasked study data to the Steering Committee or the protocol Review Committee will be authorized.

V. Literature Cited

1. Global Initiative for Asthma, Revised 2002. National Institutes of Health. National Heart, Lung, and Blood Institute. NIH publication 02-3659A, July 2002. www.ginasthma.com
2. Van Schoor J, Pauwels R, and Joos G. Indirect bronchial hyper-responsiveness: the coming of age of a specific group of bronchial challenges. Clin Exp Allergy 2005;35:250-261.
3. Joos GF, O'Connor B, ERS Task Force. Indirect airway challenges. Eur Respir J 2003; 21:1050-68.
4. Smith CM, Anderson CD. Inhalation provocation tests using non-isotonic aerosols. J Allergy Clin Immunol 1989, 84:781-790.
5. Smith CM, Anderson SD. Hyperosmolarity as the stimulus to asthma induced by hyperventilation? J Allergy Clin Immunol 1986, 77:729-736.

6. Smith CM, Anderson SD. Inhalational challenge using hypertonic saline in asthmatic subjects: a comparison with responses to hyperpnoea, methacholine and water. Eur Respir J 1990, 3:144-151.
7. Anderson SD, Smith CM, Rodwell LT, du Toit JI, Riedler J, Robertson CF: The use of non-isotonic aerosols for evaluating bronchial hyperresponsiveness. In Provocation Challenge Procedures Edited by: Spector S. New York, Marcel Dekker; 1995:249-278.
8. Riedler J, Read T, Dalton M, Hoist DI, Robertson CF: Hypertonic saline challenge in an epidemiological survey of asthma in children. Am J Respir Crit Care Med 1994, 150:1632-1639.
9. Rabone S, Phoon WO, Anderson SD, Wan KC, Seneviratne M, Gutierrez L, Brannan J: Hypertonic saline challenge in an adult epidemiological field survey. Occup Med 1996, 46:177-185.
10. deMeer G, Marks GB, deJongste JC, Brunekreef B: Airway responsiveness to hypertonic saline: dose-response slope or PD 15? Eur Respir J 2005, 25:153-158.
11. Riedler J, Gamper A, Eder W. Oberfeld G: Prevalence of bronchial hyperresponsiveness to 4.5% saline and its relation to asthma and allergy symptoms in Austrian children. Eur Respir J 1998, 11:355-360.
12. Barben J, Riedler J: Measurement of bronchial responsiveness in children. In Paediatric Pulmonary Function Testing Volume 33. Edited by: Hammer J and Eber E. Basel, Karger; 2005:125-136.
13. Anderson SD, Brannan JD: Methods for “indirect” challenge tests including exercise, eucapnic voluntary hyperpnea and hypertonic aerosols. Clin Rev Allergy Immunol 2003, 24:63-90.
14. Anderson SD, Brannan J, Spring J, Spalding N, Rodwell LT, Chan K, Gonda I, Walsh A, Clark AR: A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. Am J Respir Crit Care Med 1997, 156-758-765.
15. Leuppi JD, Brannan JD, Anderson SD: Bronchial provocations tests: The rationale for using inhaled mannitol as a test for airway hyperresponsiveness. Swiss Med Wkly 2002, 132:151-158.
16. Brannan JD, Koskela H, Anderson SD, Chew N: Responsiveness to mannitol in asthmatic subjects with exercise-and hyperventilation-induced asthma. Am J Respir Crit Care Med 1998, 158:1120-1126.
17. Currie GP, Haggart K, Brannan JD, Lee DK, Anderson SD, Lipworth BJ: Relationship between airway hyperresponsiveness to mannitol and adenosine monophosphate. Allergy 2003, 58:762-766.
18. Brannan JD, Anderson SD, Perry CP, Freed-Martens R, Lasseg AR, Charlton B and the Aridol Study Group. The safety and efficiency of inhaled drug powder mannitol as a bronchial provocation test for airway hyperresponsiveness: a phase 3 comparison study with hypertonic (4.5%) saline. Respiratory Research 2005; 6(144):1-12.
19. Brannan JD, Anderson SD, Freed R, Leuppi JD, Koskela H, Chan HK: Nedocromil sodium inhibits responsiveness to inhaled mannitol in asthmatic subjects. Am J Respir Crit Care Med 2000, 161:2096-2099.
20. Brannan JD, Anderson SD, Gomes K, King GG, Chan HK, Seale JP: Fexofenadine decreases sensitivity to and montelukast improves recovery from inhaled mannitol. Am J Respir Crit Care Med 2001, 163:1420-1425.
21. Brannon JD, Koskela H, Anderson SD, Chan HK: Budesonide reduces sensitivity and reactivity to inhaled mannitol in asthmatic subjects. Respirology 2002, 7:37-44.
22. Koskela H, Hyvärinen L, Brannan JD, Chan HK, Anderson SD: Sensitivity and validity of three bronchial provocation tests to demonstrate the effect of inhaled corticosteroids in asthma. Chest 2003;124:1341-1349.
23. Leuppi JD, Salome CM, Jenkins CR, Anderson SD, Xuan W, Marks GB, Koskela H, Brannan JD, Freed R, Andersson M, Chan HK, Woolcock AJ: Predictive markers of asthma exacerbations during stepwise dose-reduction of inhaled corticosteroids. Am J Respir Crit Care Med 2001; 163:406-412.

24. Personal communication with Gary Phillips, Commercial Director, Pharmaxis Ltd.
25. Xu J, Postma DS, Howard TD, Koppelman GH, Zheng SL, Stine OC, Bleecker ER, Meyers DA. Major genes regulating total serum immunoglobulin E levels in Families with asthma. Am J Hum Genet 2000;67(5):1163-73.
26. Malerba G. Lauciello MC, Scherpbier T, Trabetti E, Galavotti R, Cusin V, Pescollderungg L, Zanoni G, Martinati LC, Boner Al and others. Linkage analysis of chromosome 12 markers in Italian families with atopic asthmatic children. Am J Respir Crit Care Med 2000; 162(4 Pt 1):1587-90.
27. Raby BA, Silverman EK, Lazarus R, Lange C, Kwiatkowski DJ, Weiss ST. Chromosome 12q harbors multiple genetic loci related to asthma and asthma-related phenotypes. Hum Mol Genet 2003;12(16):1973-9.
28. Chi PB, Duggal P, Kao WH, Mathias RA, Grant AV, Stockton ML, Garcia JG, Ingersoll RG, Scott AF, Beaty TH and others. Comparison of SNP tagging methods using empirical data: association study of 713 SNPs on chromosome 12q14.3-12q24.21 for asthma and total serum IgE in an African Caribbean population. Genet Epidemiol 2006;30(7):609-19.
29. Brasch-Andersen C, Tan Q, Borglum AD, Haagerup A, Larsen TR, Vestbo J, Kruse TA. Significant linkage to chromosome 12q24.32-q24.33 and identification of SFRS8 as a possible asthma susceptibility gene. Thorax 2006;61(10):874-9.
30. Celedon JC, Soto-Quiros ME, Avila L, Lake SL, Liang C, Fournier E, Spesny M, Hersh CP, Sylvia JS, Hudson TJ and others. Significant linkage to airway responsiveness on chromosome 12q24 in families of children with asthma in Costa Rica. Hum Genet 2007;120(5):691-699.
31. Khakh BS, North RA. P2X receptors as cell-surface ATP sensors in health and disease. Nature 2006;442:527-32.
32. Bulanova E, Budagian V, Orinska Z, Hein M, Petersen F, Thon L, Adam D, Bulfone-Paus S. Estracellular ATP induces cytokine expression and apoptosis through P2X7 receptor in murine mast cells. J Immunol 2005;174(7):3880-90.
33. Denlinger LC, Coursin DB, Schell K, Angelini G, Green DN, Guadarrama AG, Halsey J, Prabhu U, Hogan KJ, Bertics PJ. Human P2X7 pore function predicts allele linkage disequilibrium. Clin Chem 2006;52(6):995-1004.
34. Porsbjerg C, Rasmussen L, Thomsen SF, Brannan JD, Anderson SD, Backer V. Response to mannitol in asymptomatic subjects with airway hyper-responsiveness to methacholine. Clinical and Experimental Allergy. 2007; 37:22-28.
35. Brannan JD, Gulliksson M, Anderson DS, Chew N, Kumlin M. Evidence of mast cell activation and leukotriene release after mannitol inhalation. Eur Respir J. 2003; 22:491-496.
36. Koskela HO, Hyvarinen L, Brannan JD, Chan HK, Anderson SD. Responsiveness to Three Bronchial Provocation Tests in Patients with Asthma. Chest. 2003; 124:2171-2177.