Asthma Clinical Research Network
Long Acting Beta Agonist Response by Genotype (LARGE)

Study Protocol
Version 22.0
February 2, 2006

A study to compare the effects of a long acting beta agonist in patients with asthma receiving inhaled corticosteroids who express two distinct polymorphisms of the β_2 -adrenergic receptor.

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I. PROPOSED TRIAL

We propose a 62-week randomized, double-blind, crossover trial assessing the effects of regularly scheduled use of a long-acting beta-agonist in two groups of patients with asthma receiving inhaled corticosteroids who differ by their genotype at the codon for the 16th amino acid of the β_2-adrenergic receptor.

II. HYPOTHESES TO BE TESTED BY THIS TRIAL

A. PRIMARY RESEARCH HYPOTHESIS

The regularly scheduled administration of an inhaled long-acting beta-agonist will have a detrimental effect on asthma control, as defined by AM peak expiratory flow (PEF), in subjects with asthma receiving inhaled corticosteroids who bear the B16-Arg/Arg[1] genotype of the β_2-adrenergic receptor gene, compared to subjects with asthma of similar severity who bear the B16-Gly/Gly genotype. This primary research hypothesis leads to the development of the following:

B. NULL HYPOTHESIS

The regularly scheduled administration of inhaled long-acting beta-agonist will have similar effects on asthma control, as defined by AM PEF, in subjects with asthma receiving inhaled corticosteroids who bear the B16-Arg/Arg genotype, as in subjects with asthma receiving inhaled corticosteroids who bear the B16-Gly/Gly genotype of the β_2-adrenergic receptor.

C. ADDITIONAL RESEARCH HYPOTHESES TO BE TESTED

1. The regularly scheduled administration of inhaled long-acting beta-agonist will have a detrimental effect on asthma control in subjects receiving inhaled corticosteroids who harbor the B16-Arg/Arg genotype, compared to patients with asthma of similar severity who harbor the B16-Gly/Gly genotype, as defined by the secondary outcome variables listed below.

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[1] Throughout this protocol, we use the terminology B16-Arg/Arg or B16-Gly/Gly to indicate the genotype at the codon for the 16th amino acid of the β_2-adrenergic receptor.

a) Physiologic Variables:

   (i) FEV_1
   (ii) PM PEF
   (iii) peak flow variability (PM-AM PEF difference divided by PM PEF)
   (iv) level of FEV_1 achieved in response to 4 puffs ipratropium
   (v) level of FEV_1 achieved in response to 4 puffs albuterol
   (vi) methacholine PC_{20}

b) Indices of Asthma Control and Asthma-Related Quality of Life:

   (i) Asthma symptoms as assessed by the Asthma Symptom Utility Index (ASUI)
   (ii) Asthma symptom free days
   (iii) Asthma control as assessed by the Asthma Control Questionnaire (ACQ)
   (iv) Use of "as needed" rescue medication
   (v) Asthma-specific quality of life (QOL)

c) Biomarkers of Inflammation:

   (i) Exhaled nitric oxide (eNO)
   (ii) Markers of oxidative stress (e.g. pH) in exhaled breath condensates (EBC)
   (iii) Levels of biological mediators (e.g. cysteinyl leukotrienes) in EBC

2. The regularly scheduled administration of inhaled long-acting beta-agonist will have a detrimental effect on asthma control, as defined by AM PEF, physiologic variables, indices of asthma control, and biomarkers of inflammation in subjects with asthma receiving inhaled corticosteroids who bear the B16-Arg/Arg genotype.

A detrimental effect of regularly scheduled long-acting beta-agonist **will not be observed** in subjects with asthma of a similar severity who bear the B16-Gly/Gly genotype at the β_2-adrenergic receptor.

D. EXPLORATORY HYPOTHESES

1. The regularly scheduled administration of inhaled long-acting beta-agonist will have a detrimental effect on asthma control as defined by asthma exacerbations, in subjects with asthma who bear the B16-Arg/Arg genotype at the β_2-adrenergic receptor gene, compared to subjects with asthma of a similar severity who bear the B16-Gly/Gly genotype.

2. Combinations of alleles aligned on the same chromosome (haplotypes) at different positions along the β_2-adrenergic receptor gene and its 5' leader and 3' terminal sequences will be associated with differential effects on asthma control, as defined by AM PEF, in the setting of regularly scheduled administration of an inhaled long-acting beta-agonist.

III. BACKGROUND AND RATIONALE

(a) β_2-adrenergic Receptor Gene Variants and the Response to Short-Acting β_2-agonists

BAGS trial genetics

Within the United States alone, there are thought to be 15 million people with asthma (National Heart Lung and Blood Institute, 1997). All patients with asthma are advised to use inhaled β_2-adrenergic receptor agonists for the treatment of episodic bronchospasm; for the patients with relatively mild asthma, such treatment may be the only form of asthma therapy (National Heart Lung and Blood Institute, 1995). In the Beta Agonist Study (BAGS) trial conducted by the ACRN, we demonstrated that the regularly scheduled use of inhaled albuterol had neither a detrimental nor beneficial effect in patients with mild asthma (Drazen et al., 1996). A subsequent analysis of the BAGS data was performed in which most of the BAGS patients were genotyped at the β_2-adrenergic receptor locus and their data stratified by genotype at this locus (Israel et al., 2000). We found that patients with a specific genotype (those with B16-Arg/Arg genotype, which comprised 15% of patients in this trial) experienced an adverse asthma outcome while on regularly scheduled albuterol while other genotype-stratified subgroups did not. (Figure 1).

Figure 1. BAGS trial. Compared to GLY/GLY subjects, a decline in AM PEF was observed in ARG/ARG subjects receiving albuterol.

BARGE trial genetics

Our retrospective finding that Arg/Arg patients experienced a detrimental response to β_2-agonist use was corroborated by a recent prospective study by the ACRN, the Beta Adrenergic Response by Genotype (BARGE) trial. In this study, we demonstrated that patients homozygous for arginine at B16 (B16-Arg/Arg) showed improvements in peak expiratory flow (PEF) rate, (Figure 2), FEV_1, and symptoms when they minimized their use of albuterol, even as an "as needed' medication for relief of symptoms of airflow obstruction (Israel

BARGE AM PEF

Difference between Regular Albuterol vs. Placebo Treatment-induced Changes over 16 Wks

Figure 2. BARGE trial. Compared to GLY/GLY subjects, a decline in AM PEF was observed in ARG/ARG subjects receiving albuterol.

et al., 2004). Taken together, these data suggest that patients with the B16-Arg/Arg genotype may experience adverse effects from use of short-acting β-agonists. These data naturally raise the question of whether such effects might occur with long-acting ß-agonists.

(b) β_2-adrenergic Receptor Gene Variants and the Response to Long-Acting β_2-agonists

Accordingly, to assess whether β_2-adrenergic receptor gene variants had an effect on the response to long-acting β_2-agonists, we undertook a retrospective analysis of the subjects who had received the long-acting β_2-agonist, salmeterol, while participating in two of our earlier studies, the Salmeterol Off CorticoSteroids (SOCS) Study (Lazarus et al., 2001) and the SaLmeterol +/- Inhaled Corticosteroid (SLIC) trial (Lemanske et al., 2001).

SOCS trial

In the SOCS trial, we examined the arm in which mild-moderate patients with asthma discontinued inhaled corticosteroids and started regular salmeterol treatment for 16 weeks followed by a 6 week run-out period during which salmeterol was discontinued. Compared to subjects with the B16-Gly/Gly genotype, subjects with the B16-Arg/Arg genotype had a slow and steady decline in AM PEF (Figure 3) as well as an increase in

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peak expiratory flow variability throughout the trial, both of which were accentuated during the run-out period of that trial.

SOCS Trial
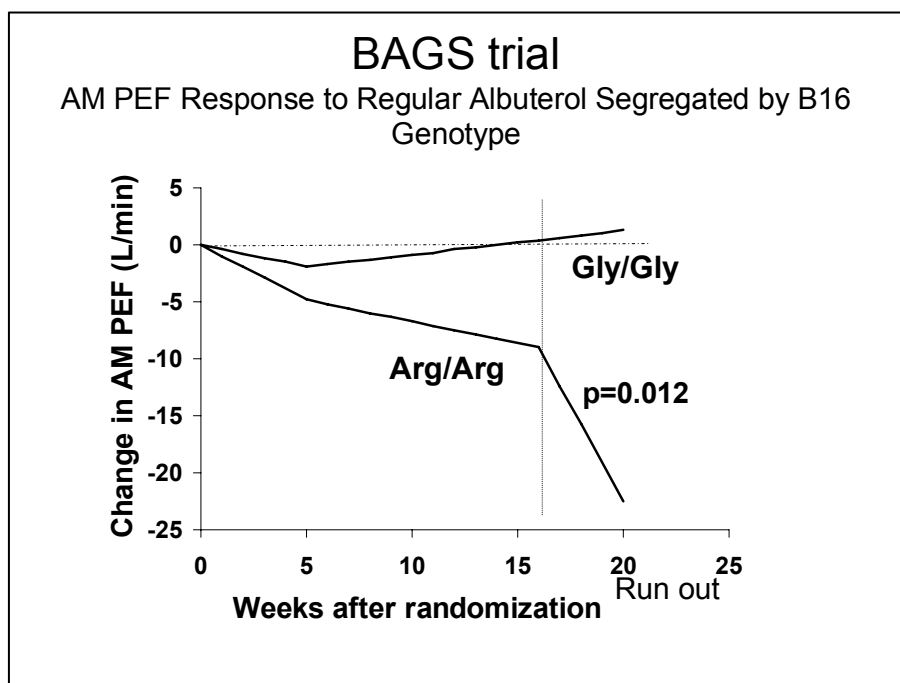### AM PEF Response to Salmeterol Segregated by B16 Genotype

Figure 3. SOCS trial. Compared to GLY/GLY subjects, a decline in AM PEF was observed in ARG/ARG subjects receiving salmeterol.
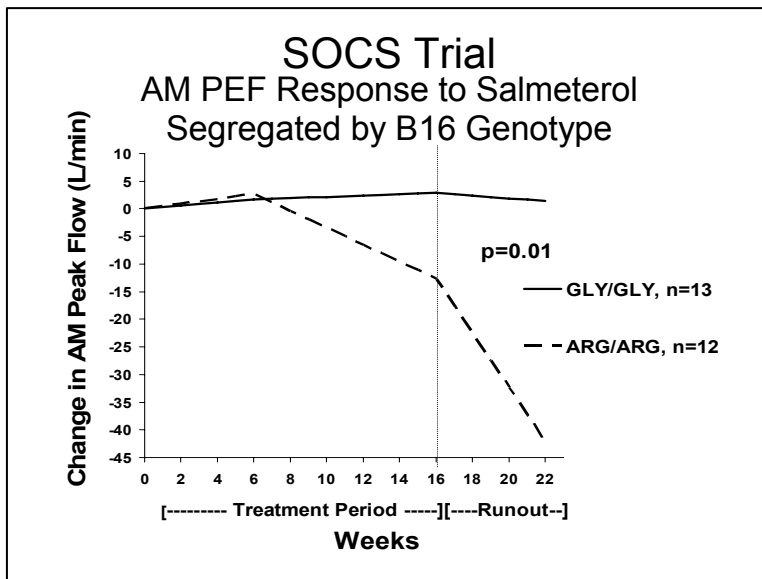
SLIC trial

Our retrospective analysis of the SLIC trial allowed us to explore the question of whether a genotype-specific response to salmeterol occurs in the setting of concurrent inhaled corticosteroid use. In the SLIC trial, we examined the arm in which patients, with greater asthma severity than in the SOCS trial, were randomized to continue inhaled corticosteroids and add salmeterol. In that study, despite the use of concurrent inhaled corticosteroids, compared to B16-Gly/Gly subjects, B16-Arg/Arg subjects receiving salmeterol demonstrated worse AM peak expiratory flow, FEV_1 (Figure 4), asthma symptom scores, and increased albuterol rescue use.

SLIC Trial
FEV1 Response to Salmeterol and Inhaled Corticosteroid Segregated by B16 Genotype
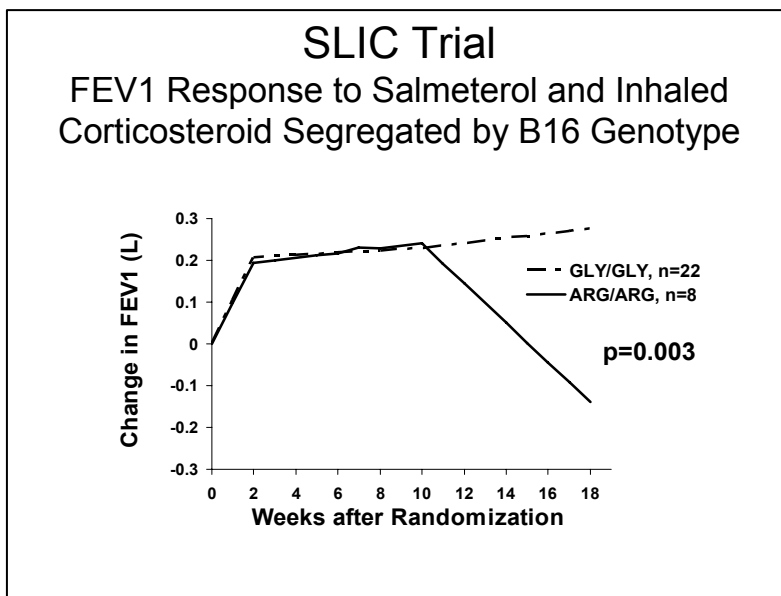
Figure 4. SLIC trial. Compared to GLY/GLY subjects, a decline in FEV1 was observed in ARG/ARG subjects receiving salmeterol despite concurrent inhaled corticosteroid use.

Both of these retrospective analyses suggest that B16-Arg/Arg patients may experience adverse outcomes when using long-acting β-agonists.

(c) <u>Significance</u>

Combination therapy with long-acting beta-agonists and inhaled corticosteroids is currently the most commonly used controller medication for asthma. Over the last several years, a number of case reports have suggested that there may be an association between regular use of a long-acting beta-agonist and serious asthma exacerbations or death (Castle et al., 1993;Clark et al., 1993;Mann et al., 2003). Further, another very recent, and so far unpublished, placebo-controlled study of the effects of adding salmeterol to usual treatment for asthma was halted prematurely due to an increase in asthma-related deaths or life-threatening experiences in patients receiving salmeterol (Food and Drug Administration, 2003). That study suggested that increased risk may occur in African Americans. Taken together with our pharmacogenetic findings, and considering the fact that Arg/Arg patients represent at least 1 out of 6 Caucasian patients with asthma and up to 1 out of 5 African Americans (Ellsworth et al., 2002), these studies suggest that there may be a subset of patients who do not benefit from, or who experience adverse effects with, the use of long-acting beta-agonists.

We therefore propose a trial to confirm our pharmacogenetic findings prospectively. The proposed trial is termed the **<u>L</u>ong-<u>A</u>cting Beta-Agonist <u>R</u>esponse by <u>G</u>enotype Trial,** or the **LARGE Trial**.

IV. PROTOCOL OVERVIEW

A. Trial Design/Schema

This randomized, double-blind, crossover, placebo-controlled trial will examine the effects of regularly scheduled long-acting beta-agonist in a group of asthmatic patients harboring the B16-Arg/Arg genotype and in a separate group of FEV_1- and race- (Caucasian versus non-Caucasian) matched patients harboring the B16-Gly/Gly genotype at the β_2-adrenergic receptor. Both groups will receive concurrent inhaled corticosteroids. To minimize confounding by use of β_2-agonists during the treatment periods, subjects will use an anticholinergic as a rescue reliever. See protocol schema below.

STAGE 1

STAGE 2

B16 -Arg/Arg ;
B-16 -Gly /Gly

ICS 240 bid +
LAB +
PRN Atrovent**

ICS 240 bid +
Pbo LAB +
PRN Atrovent**

Screen &
Genotype

ICS 240 bid
+ PRN Albuterol

ICS 240 BID +
PRN Albuterol

ICS 240 BID+
PRN albuterol

ICS 240 bid +
Pbo LAB +
PRN Atrovent**

ICS 240 bid +
LAB +
PRN Atrovent**

Matched subjects

RANDOMIZE

CROSSOVER

Screen &
Genotype

Prematch
phase:
Visits q 3 4
weeks

8 wks. run in

18 wks. active treatment

8 wks. run-out/run-in

18 wks. active treatment

10 wks. run-out

Double Blind, Placebo Controlled, Crossover Design

Hx &P
DR
QOL

Consent
Hx
S
G

Hx &P
S
MW
Disp

M Ch
or
BD
TA
Disp

DR
S
Disp

DR
S
Disp

ACQ
NO/S
EBC
ST
TA
Disp

DR
S
Disp

DR
NO/S
BD4
Disp

DR
QOL
ACQ
NO/S
EBC
M Ch
TA
Disp

DR
NO/S
Disp

DR
NO/S
Disp

DR
NO/S
EBC
AtRev
Disp

DR
NO/S
Disp

DR
QOL
ACQ
NO/S
EBC
M ch
Disp

DR
NO/S
Disp

DR
NO/S
BD4
Disp

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NO/S
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DR
NO/S
Disp

DR
NO/S
EBC
Atrev
Disp

DR
NO/S
BD4
Disp

DR
QOL
ACQ
NO/S
EBC
M ch
Disp

DR
NO/S
EBC
AtRev
Disp

DR
NO/S
BD4
Disp

DR
QOL
ACQ
NO/S
EBC
M Ch
Disp

DR
QOL
ACQ
NO/S
P

Visit	Sa	Sb	Sc*/Sd	Pa	Ph..Px	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

Screening

Week						0	3	6	8	10	14	18	22	26	29	32	34	36	40	44	48	52	55	58	60	62

Sa = screen & genotype, Sb = for genotype eligible subjects, if necessary to withdraw from meds, Sc = to assess study eligibility by M Ch/BD, Sd - If necessary, if failed Sc,
* Visit Sc may take place at same time as visit Sb if no withhold

AtRev: Atroven reversibility	**ACQ:** Asthma control questionnaire	**BD** Bronchodilator reversibility w/ 2 puffs albuterol	**BD4** Bronchodilator response to 4 puffs abuterol
DR: Diary card review	**Disp** Dispensing of medications, PF device and/or diaries	**EBC**: Exhaled breath condensate	**G:** Genotype Phlebotomy
Hx & P: History /Phys	**M Ch:** Mechacholine challenge (+ pregnancy test if female)	**MW** Medication withhold instructions	
NO/S: Exhaled nitric oxide and Spirometry	**QOL:** Asthma quality of life/ ASUI	**ST:** Skin test	**TA:** MDI or Diskus technique assessment

** Abuterol to be used as superceding rescue medication when ipratropium fails to control symptoms

B. Trial Overview

1. Screening

After signing the study consent form, which includes permission to draw blood for genetic testing, potential participants will have their history reviewed (e.g. medication use and asthma exacerbation history) and will be screened for eligibility based on baseline lung function (visit Sa). If they qualify, blood will be obtained for the ascertainment of B16 genotype. We estimate that one in 3 subjects screened will have the B16-Gly/Gly genotype, and 1 out of 6 subjects screened will have the B16-Arg/Arg genotype.

Genotype-eligible subjects will return for visit Sb for spirometry, history, physical exam and safety evaluation to assess capacity to withhold for 48 hours from current medications e.g. salmeterol (if necessary) prior to returning for visit Sc. Following the appropriate drug withhold period, if necessary, the third screening visit, visit Sc, will include methacholine challenge to assess for airway responsiveness (or bronchodilator reversibility if FEV_1 is too low) for study entry eligibility. If no medication withhold is necessary, then visit Sc may take place on same day as visit Sb. A 4th screening visit, visit Sd, will include a bronchodilator reversibility assessment with 2 puffs albuterol that will only be administered to individuals who failed methacholine challenge at visit Sc. Subjects who qualify for methacholine challenge must attempt to meet the PC_{20} eligibility criterion first; bronchodilator reversibility will only be used as a secondary means of proving eligibility in these subjects.

2. Pre-Matching/Matching

Individuals who have met study entry criteria (see Section VIII) and who are either Arg/Arg or Gly/Gly will enter a pool of entry-criteria eligible patients awaiting to be matched against their opposite genotype, stratified by FEV_1 and race (Caucasian vs. non-Caucasian). Match-eligible subjects will begin treatment with open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and PRN albuterol use and will return after 3 weeks of treatment for Visit Pa for spirometry to establish baseline FEV_1 for matching with individuals of the alternate genotype. They will return every 4 weeks for diary review, compliance review, spirometry and safety checks until a match is found. Matched subjects (within 10 percentage points of percent predicted FEV_1 at Visit Pa and in the same race category (Caucasian vs. non-Caucasian)) will be contacted to return for Visit 1.

3. 8-Week Run-In Period

Matched subjects will enter an 8-week run-in period to establish a baseline. During this period, they will continue to be treated with an open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and given inhaled albuterol (RESCUE) for use as a "rescue medication." Asthma control will be characterized by AM peak flow, spirometric values, AM/PM peak flow variability index, asthma symptoms, quality of life, asthma control questionnaires, use of rescue medications, and occurrence of events of adverse asthma control. In addition, we will obtain baseline data on airway responsiveness, bronchodilator response to ipratropium, methacholine PC_{20}, the bronchodilator response to albuterol, exhaled nitric oxide and inflammatory indices in exhaled breath condensates.

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4.   18-Week Double-Blind Treatment Period 1

Subjects then will be randomized to an 18-week double-blind treatment phase in which they will receive open-label regularly scheduled inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and either inhaled long-acting beta-agonist (salmeterol 50 mcg BID) or placebo. During this period, asthma control will be monitored by the above indicators and ipratropium (RESCUE 1) will be used as the primary rescue therapy.

5.   8-Week Run-Out Period

At the end of the blinded treatment period, all subjects will be returned to regular use open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) with PRN albuterol (RESCUE) for an 8-week run-out period. This 8-week run-out period also will serve as the run-in (or washout) period for the second stage of the study.

6.   18-Week Double-Blind Treatment Period 2

At the end of the first run-out period, subjects will be crossed over to the alternate double-blinded treatment regimen with either a long-acting beta-agonist or placebo. During this stage, asthma control will be monitored by the same indicators as in the first stage.   Ipratropium (RESCUE 1) will be used as the primary rescue therapy during this study period.

7.   10-Week Study Run-Out

At the end of the second blinded treatment period, all subjects will be returned to regular use open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) with PRN albuterol (RESCUE) for a 10-week run-out period. During this period, asthma control will be monitored by the same indicators as in the first run-out period

During the two treatment periods, participants will use inhaled ipratropium bromide as rescue medication (RESCUE 1) to avoid the confounding effects of β_2-adrenergic stimulation on the outcome variables to be monitored[2]. However, in the event that an episode of adverse asthma control responds incompletely to ipratropium, albuterol will be used as a superceding rescue medication (RESCUE 2).

Due to prohibitive costs associated with the acquisition of additional placebo salmeterol, treatment during the run-in and run-out periods will consist of open-label inhaled corticosteroid without any placebo long-acting beta-agonist.

We will use the same measures for assessment of asthma throughout the entire study.

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[2] Ipratropium bromide has been used in a number of asthma clinical trials (GlaxoSmithkline, unpublished data), including the ACRN's BARGE trial, as the primary rescue therapy. No adverse outcomes have been reported as a result of this use of ipratropium. The experiences in clinical trials with this drug indicate that it can be used safely in the LARGE trial, as proposed.

C. Outcome Variables

The *primary question* to be addressed by this study is:

Does the regularly scheduled administration of an inhaled long-acting beta-agonist have a detrimental effect on asthma control, as defined by AM PEF, in patients with asthma receiving inhaled corticosteroids who bear the B16-Arg/Arg genotype of the β_2-adrenergic receptor gene, compared to patients with asthma of a similar severity who bear the B16-Gly/Gly genotype?

Primary outcome variable: The primary outcome variable will be morning peak expiratory flow (AM PEF). The difference in AM PEF between the ends of the two 18-week treatment periods will be assessed between both B16 genotype groups (B16-Arg/Arg, B16-Gly/Gly) as well as separately within each genotype. Change in AM PEF in both B16 genotype groups will be assessed at the beginning and at the end of each 18-week treatment period as well as after 8 weeks of run-out period. See Statistical Analysis, Section XVIII.E, for further details of the analytic plan.

Secondary comparisons will be conducted, regarding:
a) **Physiologic indices** (FEV$_1$; PM PEF; PEF variability; ipratropium responsiveness; bronchodilator effect of albuterol; methacholine PC$_{20}$);

b) **Symptom-based indices of asthma control and asthma-related quality of life** (asthma symptoms (ASUI); asthma symptom-free days; the duration of effect of long-acting beta-agonist inhaled on a regularly scheduled basis; use of "as needed" medication; quality of life and asthma control).

Exploratory analyses will include:
a) Analysis of **Biomarkers of inflammation** (exhaled nitric oxide (eNO)[3] and markers of oxidative stress (e.g. pH) and levels of biological mediators (e.g. cysteinyl leukotrienes) in exhaled breath condensates);

b) Assessment of whether the regularly scheduled administration of inhaled long-acting beta-agonist will have a detrimental effect on asthma control as defined by **asthma exacerbations** in subjects with asthma who bear the B16-Arg/Arg genotype at the β_2-adrenergic receptor gene, compared to subjects with asthma of a similar severity who bear the B16-Gly/Gly genotype;

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[3] **Exhaled nitric oxide** has been advocated as a non-invasive marker of airway inflammation (Deykin et al., 1998;Dupont et al., 1998;Jatakanon et al., 1998) . It is appropriate to make this measurement because one of the adverse effects attributed to the regular use of β_2-agonists is airway inflammation , and this effect of β_2-agonists may vary by β_2-adrenergic receptor genotype, as noted in SOCS genetics analysis.

c) Assessment of whether combinations of alleles aligned on the same chromosome (**haplotypes**) at different positions along the β_2-adrenergic receptor gene and its 5' leader and 3' terminal sequences will be associated with differential effects on asthma control, as defined by AM PEF and other outcomes, in the setting of regularly scheduled administration of an inhaled long-acting beta-agonist. (See Section XVIII.H for further details regarding haplotype analysis.)

V.   RATIONALE FOR OUR DECISIONS WITH RESPECT TO TRIAL DESIGN

1. Choice of Genotypes

We have decided to compare the treatment response to regularly scheduled inhaled long-acting beta-agonist in two groups defined by genotype at the β_2-adrenergic receptor locus. The two genotypes are B16-Arg/Arg and B16-Gly/Gly. We chose these genotypes for the reasons detailed below.

Our preliminary data from the BAGS and BARGE trials, and the findings of Hancox and colleagues (Hancox et al., 1998), suggest that patients with the B16-Arg/Arg genotype have a detrimental response to regularly scheduled use of inhaled beta-agonists, while patients with the B16-Gly/Gly genotype do not. Our preliminary retrospective data from the SOCS and SLIC trials suggest a similar effect of the long-acting beta-agonist salmeterol in individuals who are B16-Arg/Arg, even in the presence of inhaled corticosteroids. Based on these observations, it would seem reasonable to stratify our patient groups based on the genotype at the B16 locus alone. We have chosen not to study the effects of genotype at the B27 position or other beta-receptor polymorphisms on salmeterol response as these loci have not been shown to have any significant effect on outcomes of beta-agonist therapy. Similarly, we have chosen not to study B16-Arg/Gly heterozygotes nor individuals with different β_2-adrenergic receptor gene haplotypes as these individuals have not had a predictable response in any of our previous studies. Thus, because of the widespread use of salmeterol in combination with inhaled corticosteroids, we propose to evaluate the effect of regular salmeterol treatment in individuals bearing the B16-Arg/Arg genotype and in those bearing the B16-Gly/Gly genotype who are receiving inhaled corticosteroid therapy.

2. Rationale for Study Design

We have chosen a double-blinded, randomized, placebo-controlled, crossover design for this trial. Randomized allocation to treatment groups within each genotype will help control for potential confounding variables. The crossover design reduces the sample size while maintaining adequate statistical power for testing the primary hypothesis of this study. Without such reduction in sample size, the conduct of this randomized trial would be cost-prohibitive. Because random allocation to each genotypic group is not feasible, we are matching by FEV_1 percent of predicted (within 10 percentage points) and race (Caucasian versus non-Caucasian) across genotypes in an attempt to minimize confounding caused by potential differences between these two genotypes in the severity of airways obstruction at baseline or in racial makeup.

Because the first run-out period serves as the run-in period for the second phase of the study, the first run-out period is identical to the run-in period with respect to treatment modalities so as not to bias second phase treatment period baseline characteristics or treatment effects and outcomes.

While a statistical carryover effect did not occur in the BARGE trial, which included a 6-week washout period, we extended the washout further in LARGE to 8 weeks. Additionally, our statistical analysis plan can accommodate even a lengthier carryover effect. The primary outcome of the LARGE trial is AM PEF achieved at the end of the treatment period, not a change from baseline AM PEF. The planned analysis includes a segmented linear model within a longitudinal data analysis. The first segment within a treatment period is during weeks 1-6, whereas the second segment is during weeks 7-18 (the primary segment to be analyzed). Thus, there will be at least 14 weeks (8 weeks of the washout period + 6 weeks of the first segment of the treatment period) between the end of the previous treatment period and the onset of the second (and primary) segment of the next treatment period. We think it highly unlikely that the carryover effect will last more than 14 weeks.

This amount of time approaches the entire time of the intervention (18 weeks) and we think it highly unlikely that such an extended (> 14 weeks) run-out effect would occur. While we think that the run-out period is adequate, we had already chosen to add an extra two weeks to the second run-out period (10 instead of 8 weeks) to better observe an "unadulterated" longer run-out.

During the two treatment periods, participants will use inhaled ipratropium bromide as rescue medication to avoid the confounding effects of β_2-adrenergic stimulation on the outcome variables to be monitored[2]. However, in the event that an episode of adverse asthma control responds incompletely to ipratropium, albuterol will be used as a superceding rescue medication. Ipratropium bromide has been used in a number of asthma clinical trials (GlaxoSmithkline, unpublished data), including the ACRN's BARGE trial, as the primary rescue therapy and was well tolerated. No adverse outcomes have been reported as a result of this use of ipratropium. The experiences in clinical trials with this drug indicate that it can be used safely in the LARGE trial, as proposed.

In order to ensure that subjects harboring different genotypes are of similar severity at baseline and that the effects observed are genotype-specific, and not due to potential differences in B16 allele distribution in people of different races, subjects will be matched by FEV_1 (after they have received 3 weeks of identical therapy) and by race

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[2] Ipratropium bromide has been used in a number of asthma clinical trials (GlaxoSmithkline, unpublished data), including the ACRN's BARGE trial, as the primary rescue therapy. No adverse outcomes have been reported as a result of this use of ipratropium. The experiences in clinical trials with this drug indicate that it can be used safely in the LARGE trial, as proposed.

(Caucasian vs. non-Caucasian) so that a sub-analysis in Caucasians alone can be undertaken. See Section XVIII.C.

3. Choice of Outcome indicators

a) Primary Outcome Indicator:

The primary outcome indicator will be AM PEF measured by the subject on a recurrent basis. This primary outcome indicator was chosen because: 1) it was the primary outcome indicator in the ACRN's BAGS, BARGE, and SOCS studies and is the outcome on which the preliminary data are based; 2) it is recurrently measured by the subject and likely to reflect overall asthma control; 3) it is a test which most subjects can perform in a technically acceptable manner without continuous coaching; and 4) it would be a clinically significant outcome if there were a population-based change of the magnitude we expect.

The study has been powered to detect a minimum change in PEF of 25 liters/minute because this value: 1) is at the limit of what an individual subject can detect and 2) would clearly be a clinically significant outcome if there were a population-based change of this magnitude.

b) Other Physiological Outcomes:

The panel of physiological outcomes including PM PEF, peak flow variability, FEV_1, and methacholine responsiveness, represent standard measurements which we can use to compare our results to both our previous trials as well as to others' trials. Two additional physiological measurements have been added: the bronchodilator response to 4 puffs of inhaled albuterol and bronchodilator reversibility to 4 puffs of ipratropium. These outcome indicators have been added because the hypothesis to be tested relates to an adverse response to beta-agonist use, and each procedure measures distinct facets of the response to beta-agonists.

c) Symptom-Based Outcomes:

Symptom-based outcomes are standard in asthma treatment trials and are important from the perspective of patient adherence with treatment recommendations.

d) Inflammation Based Outcomes:

Because the regularly scheduled use of inhaled beta-agonists has been associated with airway inflammation (Wechsler, ME, presented at AAAAI, 2004), we have elected to

monitor "non-invasive" indices of airway inflammation. To do so, exhaled nitric oxide and exhaled breath condensates will be monitored according to established ACRN protocols for the performance of these tests. Our retrospective data in the SOCS trial suggested that increased airway inflammation, as detected by exhaled NO, occurred in B16-Arg/Arg subjects receiving salmeterol. These measurements would allow us to confirm this finding and characterize it further.

VI. SCREENING REQUIRED TO ACHIEVE DESIRED SAMPLE SIZE

We propose to compare individuals with the B16-Arg/Arg genotype (which occurs with a frequency of about 16%) to those with the B16-Gly/Gly genotype (which occurs with a frequency of 35%). Thus, to recruit 40 subjects in each of the genotypic categories, we will need to screen a minimum of 240 subjects. Allowing for exclusions, refusals to participate, matching by FEV_1 and racial category (Caucasian versus non-Caucasian), and withdrawals, we may need to screen 450 individuals.

Each center will screen subjects to achieve screening percentages of about 50% women and about 33% minority; screening will continue until the target population is achieved (approximately 12 randomized subjects/site). We recognize that, because of exclusion by genotype and genotypic variation among diverse populations (Martinez et al., 1997;Reihsaus et al., 1993;Weir et al., 1998), the enrolled cohort may not reflect the screened population. The enrollment period is projected to extend over 16 months.

VII. RECRUITMENT STRATEGY

Each clinical center involved in the ACRN was chosen based on documentation for patient availability, among other things. It is, however, worthy to note the specific plans of each center.

1. Harvard Clinical Center/Boston

The Boston Center has used a variety of recruitment methods to meet and exceed recruitment goals of previous ACRN studies.

Over the past five years, we have compiled an internal database of approximately 1500 individuals with asthma who are interested in participating in asthma studies. All of these individuals contacted us and expressed interest about asthma studies within the past year, and have been evaluated by our staff for participation in ongoing and future asthma clinical research studies.

The Boston site actively recruits subjects using a variety of external media. All methods are IRB-approved and include postcard mailings to area zip codes, newspaper advertisements, and broadcast e-mails and internet postings.

Brigham and Women's Hospital has introduced a new clinical research tool called the BWH Research Patient Database Registry (RPDR) that allows researchers with proper IRB approval to query the hospital's patient database for potential research subjects. We recently queried this system and identified approximately 30,000 patients with a

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diagnosis of asthma. With permission from their primary care physician, patients may be contacted about current asthma research. We are in the process of developing tools to reach these patients through their physicians. Access to the physician database will further expand our capability to recruit asthmatic patients of differing severities.

2. National Jewish Asthma Research Center, Denver, CO

There are over 400 asthma subjects (not followed in the National Jewish outpatient clinic) that have participated in research studies conducted at the Denver Center. Many of these subjects have been through various medication studies and bronchoscopies with lavage/biopsies. Their FEV_1s range from 30-110% of predicted.

1. Denver Health Medical Center – Dr. James Fisher, Head of Pulmonary Medicine, is supporting efforts of the Denver Center by helping to recruit from the asthmatic subject population at the Denver Health Medical Center. This is a large county hospital whose subject population comprises mainly Hispanic and African-American people.

2. Denver Veterans Administration Hospital – Dr. Carol Welsh, Pulmonary faculty member, will support this grant. The VA hospital has a large outpatient clinic of patients with asthma, but not chronic obstructive pulmonary disease.

3. Denver Kaiser Permanente HMO – Dr. Timothy Collins is the Director of Pulmonary Medicine and Dr. John Williams is the Director of Allergy at Kaiser. Drs. Collins and Williams have been actively involved in supporting research at National Jewish in the past by referring subjects. Their groups will continue to play an active role in clinical research support.

3. Washington University, St. Louis

The St. Louis site actively recruits subjects using a variety of external media. All methods are IRB-approved. They include newspaper advertisements in the local and minority newspapers, the University newspaper, posting fliers throughout the medical school campus, and the university website called "Volunteer for Heath." This is a service the University offers to match interested volunteers with current clinical trials at the medical school. This service has a website, and anyone can access this with the web address.

Over the past 10 years, Dr. Castro has compiled an internal database of more than 400 individuals with asthma who are interested in participating in asthma studies. All of these individuals have contacted us and have expressed an interest in participating in an asthma study. These individuals have been evaluated by our staff for participation in ongoing and future asthma clinical research studies.

4. University of California, San Diego

Recruitment activities at UCSD Clinical Trials Center is multi-faceted and includes a computerized database with current and previously enrolled subjects, direct advertising,

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and community outreach programs such as educational lectures on asthma and attendance at health fairs with staff conducting pulmonary screening tests. All activities, fliers and advertisements are approved by the UCSD Human Research Protection Program prior to initiation.

The UCSD Clinical Trials Center database has over 500 asthmatics who have been previously enrolled or expressed an interest in participating in a clinical trial. Interested subjects are entered into the database with fields for demographic, medical, medication, and pulmonary function tests. Quarterly newsletters and fliers are mailed to the subjects with specific information on trials and to maintain accurate contact information of the individuals.

In addition, this application is supported by the Naval Medical Center and Kaiser Permanente Healthcare whose directors (Drs. Warren Lockette and Michael Schatz) are faculty members at UCSD. The Clinical Investigation Department (CID), at Naval Medical Center, San Diego (NMCSD) is directed by Warren Lockette, M.D. and is dedicated to fostering training and research in both basic and patient-oriented research at the Naval Medical Center, San Diego. Dr. Lockette collaborates with the CTC recruitment program to recruit subjects from the active and retired navy community in San Diego for CTC studies. The NMCSD has 700,000 outpatient visits each year and serves as a provider of primary care to 260,000 patients living within an easy commute, i.e. a 40-mile radius of the hospital.

Kaiser Pemanente Healthcare: Dr. Schatz is the Director of the Allergy Division of the Kaiser Pemanente Healthcare of Southern California, Permanente Medical Group and a faculty member at UCSD. In San Diego alone, they serve over 600,000 members with over 11,000 identified asthmatic subjects. Kaiser-Permanente has a fully operational computerized pharmacy records system, which provides identification of patients using anti-asthma medications. This system will be used to access patients with asthma under the care of primary care physicians and nurses. In addition, because of freeway access to UCSD and traffic, the CTC has been successful in recruiting from southern Los Angeles, Orange and Riverside Counties. Kaiser members living in that region will also be recruited. Dr. Schatz has previously collaborated with Dr. Wasserman on NIH-sponsored research projects and will continue this active collaboration and contribute to the recruitment for the ACRN protocols.

5. <u>University of California, San Francisco</u>

Study population: The UCSF center's recruitment of asthmatic subjects relies on community advertising and on maintaining a database of subjects who have participated in previous studies, come for a "characterization" visit, or expressed interest in participating. They advertise in the San Francisco Chronicle, the Bay Guardian, and in neighborhood and college newspapers. They also advertise on "Craigslist," a Web-based bulletin board on local radio and television stations. They post fliers on neighborhood and campus bulletin boards, and present our studies to physician groups. Responses to these advertisements are made to a dedicated telephone number. A dedicated recruiter, Lila Glogowsky, responds to each inquiry to obtain basic information about demographics and about asthma severity, duration, and treatment. She

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schedules apparently qualified subjects for a "characterization visit" in which a coordinator obtains a detailed history and performs spirometry and skin testing.

Subject Characterization: The UCSF center's methods for characterizing subjects conform to national guidelines (e.g. spirometry), to widely accepted custom (e.g. methacholine challenge), or to its own standards as the center developing the method (eg., sputum induction and analysis). They have adopted standardized questionnaires for assessing asthma symptom severity, asthma control, and asthma-related quality of life. They have developed questionnaires on asthma history, patterns of health care utilization, and domestic exposure to allergens.

The recruitment/characterization program is supported by a database program ("File-Maker Pro") on a dedicated server. Phenotypic information is now stored on >5,000 potential subjects of a variety of ethnic backgrounds (64% Caucasian, 13% African American, 7% Hispanic, 10% Asian and 6% other).

Subjects at the University of California San Francisco: In addition to community advertising, subjects are recruited, especially those with severe asthma, from clinical programs overseen by UCSF faculty at Moffitt, S.F. Veteran's Administration, S.F. General, and Mt. Zion Hospitals. The faculty is responsive to approaches from colleagues conducting clinical trials and there has been collaboration with the Division of General Internal Medicine to recruit for specific protocols. This Division follows approximately 18,000 patients, of whom 8% (2,683) have a primary or secondary diagnosis of asthma (ICD-9 493.00, 493.01, 493.10, 493.11, 493.20, 493.21). Of these asthmatic patients, 48% are White, 20% Asian/Pacific Islander, 10% Latino, 16% African American, and 1% Native American. Sixty-four percent are female.

6.   University of Wisconsin/Madison

The Allergy Research Program of the University of Wisconsin maintains a file of potential subjects with mild to moderate asthma who are interested in future research participation. These individuals have been screened and/or participated in previous asthma studies. The following information is maintained: birth date, gender, ethnic background, age of asthma diagnosis, childbearing status, atopic status (including results of skin testing if performed previously), concurrent medical history, asthma and non-asthma medications. Approximately 85% of subjects in this database have "mild to moderate" asthma. This database of subjects will be used as the primary source of recruitment for this protocol. If additional subjects are needed, they will be recruited via U.W. Human Subjects committee-approved, newspaper advertising and from the U.W. Allergy Clinic subject population as well as the U.W. Sports Medicine Clinic, U.W. Student Health, V.A. Allergy Clinic, and the Northeast Family Practice Clinic.

7.   Wake Forest University Health Sciences Center, Winston-Salem, NC

The Cloverdale Clinical Research Center at Wake Forest University Health Sciences and the Center for Human Genomics maintains a screening database of approximately

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1075 subjects with asthma. These are subjects who have called our clinic expressing interest in participating in asthma research studies. Some have been screened for or have participated in past research studies at our site. The following information is maintained on these subjects as it is obtained: gender, age, ethnic background, medical history, asthma history, skin testing results, exhaled breath condensate results, exhaled NO results, methacholine challenge testing results, pulmonary function, sputum induction results, bronchoscopy results, chest x-ray results, and medication usage. Should additional subjects be needed beyond this database of potential subjects, we continuously advertise for potential subjects using television, radio, and newspaper and flier advertising (all advertising is IRB approved), as well as recruitment from the Wake Forest University Health Sciences Pulmonary and Allergy Clinics through our Primary and Sub-Investigators.

VIII. INCLUSION AND EXCLUSION CRITERIA

A. Screening Inclusion Criteria (for initial screen, Screen A, and for phlebotomy at Screen A)

1. Male and female subjects, ages 18 and older (no upper limit).

2. Clinical history consistent with asthma.

3. FEV_1:

   a) For subjects regularly using inhaled corticosteroids* an $FEV_1 \geq$ 50% of predicted.

   b) For subjects not regularly using inhaled corticosteroids, an $FEV_1 \geq$ 40% of predicted.

4. If on inhaled steroids, subjects must have been on a stable dose for at least 2 weeks.

5. Ability to provide informed consent, as evidenced by signing a copy of the consent form approved by the Committee on Human Research of the study institution.

6. Non-smoker (total lifetime smoking history < 10 pack-years; no more than five occasions of smoking any substance or using smokeless tobacco products in the past year).

7. No smoking or use of smokeless tobacco in the past 30 days.

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* Subjects will be considered to be regular users of inhaled corticosteroids if they report taking $\geq$ 50% of the prescribed doses over the 2 weeks preceding the screen visit.

B. Exclusion Criteria (for Screening and for entire study)

1. Use of greater than the equivalent of 1000 μg inhaled fluticasone daily (except as permitted in study; see ICS Equivalency reference card and study MOP).

2. Chronic use of any medication other than beta-agonists or inhaled corticosteroids, except:
   - oral contraceptives and other hormonal forms of contraceptives (i.e., DepoProvera-7, Norplant-7)
   - estrogen / progesterone replacement therapy for post-menopausal women
   - vitamins and calcium supplements
   - any nasal inhaled corticosteroid at a stable dose throughout the entire study (see study MOP)
   - acetaminophen
   - non-steroidal anti-inflammatory medications (e.g., aspirin, naproxen, ibuprofen, Cox_2 inhibitors)
   - thyroid replacement medications
   - lipid-lowering medication
   - stable dose medical therapy for well-controlled hypertension and well-controlled diabetes, except those meds specifically excluded in Table 1
   - medium and low potency topical cutaneous steroids
   - nasal saline spray
   - topical eye preparations for allergic eye symptoms (e.g. antihistamines, NSAIDs, or antiallergic compounds)
   - diuretics and specific antihypertensives (e.g. calcium channel blockers, clonidine, etc.)
   - acyclovir
   - antihistamines (48 hour washout prior to visits for fexofenadine, chlorpheniramine, desloratadine, loratadine and diphenhydramine; 72 hour washout for all others; see the Washout Periods for Allergy Skin Testing reference card for appropriate washouts prior to Visit 1 allergy skin test)
   - pseudoephedrine and oxymetazoline and other decongestants (48 hour washout prior to visits)
   - antibiotics for acne
   - stool softeners and bulk laxatives

- H_2 blockers and proton pump inhibitors for GERD
- Imitrex for migraines
- non-macrolide antibiotics
- Propecia (finasteride)
- SSRI class antidepressants
- non-SSRI antidepressants
- migraine analgesics (e.g., butalbital)
- antianxiety agents
- ACE inhibitors
- Librax
- CNS stimulants/appetite suppressants

3. Use of any drugs listed in Table 1 during the designated washout period prior to screening visits, Visit Pa, and/or Visit 1, or intention to take the drug during the study.

Table 1. Drugs to be withheld throughout the study	Washout prior to Screens, Pa and Visit 1
Leukotriene receptor antagonists	> 4 weeks
Inhaled steroids, except as provided in study	None
Intranasal steroids, except at stable dose throughout study	None
Oral steroids	> 6 weeks
Cromolyn/Nedocromil	> 2 weeks
Oral beta-adrenergic agonists	> 1 week
Monoamine oxidase inhibitors	> 4 weeks
Beta-adrenergic blockers	$\geq$ 2 weeks
Macrolide antibiotics	$\geq$ 6 weeks
Inhaled beta-adrenergic agonists (intermediate-acting, e.g., albuterol, terbutaline, metaproterenol, pirbuterol, bitolterol), except as provided in study	$\geq$ 6 hours
Salmeterol/formoterol, except as provided in study	$\geq$ 48 hours prior to Sc/Sd only; $\geq$ 24 hrs prior to all other visits
Anticholinergics, except as provided in study	$\geq$ 6 hours; $\geq$24 hours for methacholine challenge visits; $\geq$72 hours for tiotropium
Short-acting theophylline (e.g., Slophyllin tablets)	$\geq$ 12 hours
Long-acting theophylline (e.g., Theo-Dur, Slo-bid)	$\geq$ 24 hours
Ultra long-acting theophylline (e.g., Theo-24, Uniphyl)	$\geq$ 48 hours
Anti-IgE therapy (e.g., Xolair)	$\geq$ 6 months
Drugs withheld prior to pulmonary function and/or methacholine challenge, per MOP	**Specified time period**
Albuterol (study RESCUE or RESCUE 2 drug)	$\geq$ 6 hours
Anticholinergics (study RESCUE 1 drug)	$\geq$ 6 hours; $\geq$24 hours prior to visits including methacholine challenge
Salmeterol (blinded study drug)	$\geq$ 24 hours
Antihistamines	$\geq$48 hours in case of chlorpheniramine, diphenhydramine, fexofenadine, desloratadine and loratadine; $\geq$72 hours all others
Pseudoephedrine (Sudafed), Oxymetazoline (Afrin) and other decongestants	$\geq$ 48 hours
Methylxanthine-containing foods or beverages (e.g., coffee, tea) or medications	$\geq$ 6 hours
Alcohol-containing foods or beverages	$\geq$ 6 hours

4. Lung disease other than asthma.

5. Established or suspected diagnosis of vocal cord dysfunction.

6. Significant medical illness (other than asthma) that is not stable.

7. History of respiratory tract infection within the previous 6 weeks (only applies at screen visits Sa or Sc at which a methacholine challenge or albuterol reversal will be performed).

8. History of a significant exacerbation of asthma in the previous 6 weeks (see Section XIV for definition of asthma exacerbation and guidelines for treatment).

9. History of life-threatening asthma requiring treatment with intubation and mechanical ventilation within the past 10 years.

10. Hyposensitization therapy other than an established maintenance regimen.

11. Pregnancy or lactation. If potentially able to bear children, not using an acceptable form of birth control (see study MOP and Birth Control reference card).

12. Subjects who were randomized in the BAGS trial, BARGE trial, SOCS trial, or SLIC trial.

13. History of hypersensitivity to soya lecithin or related food products such as soybeans or peanuts.

C.   Inclusion Criteria for Screen B, C, D

1. Meeting general study entry inclusion and exclusion criteria (Section VIII A and VIII B)

2. Genotype eligible: B16-Arg/Arg or B16-Gly/Gly

3. At Visits Sc or Sd, as appropriate[*]: bronchial hyper-responsiveness or reversible airway obstruction as defined by:

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a.) For subjects regularly using inhaled corticosteroids:

   1.) If FEV_1 < 55% predicted, a $\geq$12% <u>and</u> 200 ml. improvement in FEV_1 after 2 puffs of inhaled albuterol.

   2.) If $FEV_1 \geq$ 55% predicted, a 20% reduction in FEV_1 in response to a concentration of inhaled methacholine $\leq$ 16 mg/ml (PC_{20} $FEV_1 \leq$ 16 mg/ml) <u>**OR**</u> a $\geq$12% <u>and</u> 200 ml. improvement in FEV_1 after 2 puffs of inhaled albuterol. [Methacholine challenge testing takes precedence over albuterol reversibility testing for those who qualify.]

b.) For subjects not regularly using inhaled corticosteroids:

   1.) If FEV_1 < 55% predicted, a $\geq$12% <u>and</u> 200 ml. improvement in FEV_1 after 2 puffs of inhaled albuterol.

   2.) If $FEV_1 \geq$ 55% predicted, a 20% reduction in FEV_1 in response to a concentration of inhaled methacholine $\leq$ 8 mg/ml (PC_{20} $FEV_1 \leq$ 8 mg/ml) <u>**OR**</u> a $\geq$12% <u>and</u> 200 ml. improvement in FEV_1 after 2 puffs of inhaled albuterol. [Methacholine challenge testing takes precedence over albuterol reversibility testing for those who qualify.]

* Centers may perform methacholine challenge testing or albuterol reversibility testing, as appropriate, at visit Sa at their discretion.

D.   Inclusion Criteria for Pre-Match

1. Successful completion of screen visits Sa-Sc/Sd

2. Genotype eligible (B16-Arg/Arg or B16-Gly/Gly)

E.   Inclusion Criteria for Visit 1

1. Meeting general study Inclusion/Exclusion criteria.

2. Genotype eligible (B16-Arg/Arg or B16-Gly/Gly) and matched by FEV_1 % predicted at Visit Pa within 10 percentage points of the predicted percentage points and on racial category (Caucasian versus non-Caucasian).

3. No history of respiratory tract infection within the previous 6 weeks.

4. No history of a significant exacerbation of asthma in the previous 6 weeks (see Section XIV for definition of asthma exacerbation and guidelines for treatment).

5. Ability of the subject to take his or her QVAR on schedule (according to self-report on pre-match diary cards) at least 80% of the time during the last two weeks of the pre-match phase prior to enrollment at Visit 1.

6. $FEV_1 \geq 50\%$ of predicted.

F.   Inclusion Criteria For Randomization (Visit 4)

1. Inclusion criteria for Visit 1.

2. $FEV_1 \geq 50\%$ of predicted.

G.   Exclusion Criteria For Randomization (Visit 4)

1. $FEV_1 < 50\%$ of predicted.

2. Significant exacerbation of asthma during the run-in period (see Section XIV for definition of exacerbation during run-in period).

3. Inability to comply with regular use of QVAR study medication (less than 80% compliance with overall dosing or less than 70% compliance with correct daily dosing in last two weeks of run-in (interval between visits 3 & 4) as reflected by Doser® device).

4. Inability to record peak flow measurements and symptoms in a symptom diary at least 75% of required times during the last two weeks of the run-in period (missed measurements more than 25% of the days between visits 3 & 4).

5. Presence at Visit 4 of any of the exclusion criteria stipulated for Visit 1 (see section VIII.B. above). Note: Respiratory tract infections that do not cause the subject to meet exacerbation criteria are <u>not</u> considered exclusionary.

6. Use of an average of ≥ 16 puffs of albuterol per 24 hours during last week of the run-in.

7. Inability to use an electronic peak flow monitoring (EPFM) device correctly for recording peak flow measurements.

8. Inability, in the opinion of the investigator or clinical coordinator, to coordinate use of the delivery devices (i.e., Diskus device or MDI) used in the study.

9. Participation of one of the subject's first-degree relatives in the post-randomization portion of the study.

IX. PROTOCOL DETAIL

A. Prescreening

Subjects will be interviewed prior to protocol entry (either by phone or in person) regarding their asthma and medical history. Specifically, status of asthma control, use of asthma and non-asthma medications, and health status in the previous six weeks will be determined. An overview of the goals of the study, the visit structure and procedures involved will be presented. If the subject appears to fulfill entry criteria and is interested in study participation, Visit Sa may be scheduled.

B. Screening

1. Visit Sa Screening
   a. Informed consent
   b. Brief medical history
   c. Spirometry
   d. Venipuncture for genotyping and measurement of IgE
   e. Bronchodilator reversibility assessment or methacholine challenge at each center's discretion

Subjects will visit their clinical center after having had verbal contact with one of the study investigators, or their representatives, concerning the general goal and outline of the trial. On this first visit, written informed consent will be obtained, using a document that has been approved by the ACRN as well as by the local IRB. (NB: Some centers may split the consent process into 2 distinct processes: one for genotyping, and one for the rest of the study. In those cases, only the genotyping consent process will be employed).

A medical history will be obtained and spirometry will be carried out according to protocols outlined in the ACRN MOP. All data will be recorded electronically and on forms supplied by the ACRN.

Depending on the subject, his/her medications, and each center's preference, qualification by bronchodilator reversibility or methacholine challenge may also be performed at this time.

If, based on the information gathered to this point, the subject meets the specific entry criteria (see Section VIII Inclusion and Exclusion Criteria), blood will be drawn for DNA extraction and genetic analysis, as well as for IgE measurement (to be analyzed only once subject is matched and enrolled in the main study).

2. Genotyping

    a. Genotyping Procedures for Blood Drawn at Visit Sa (Done at the Boston Center)

Details regarding genetic analysis blood draws and shipments are provided in the ACRN Genetic Analysis Manual. DNA will be extracted and stored in 500 µl aliquots at the Channing Laboratory (Boston, MA). Each subject will be genotyped on the basis of a 5 µg (100 µl at 50 ng/µl) aliquot of DNA transferred to Dr. Israel's lab (affiliated with the Boston Center) for single assay analysis. Samples collected in a given week will be processed and genotyped at the same time. Genotype results will be submitted to the DCC via a standard form for entry and verification. All DCC personnel, with the exception of two data entry clerks and the database programmer, will be blinded to the genotype results. Each subject's genotype-eligibility status will be reported to the clinical sites via the LARGE Subject Status Report, which will indicate only whether the subject is genotype-eligible or not (the specific genotype will not be supplied).

    b. Genotyping Methods

See section XIX, Genotyping Methods, for further details.

3. Visit Sb Screening

    a. Brief medical history and physical exam
    b. Spirometry
    c. Medication withhold if subject is taking long-acting beta-agonist regularly

Subjects who are genotype eligible (B16-Arg/Arg and B16-Gly/Gly subjects only) are contacted to return for another screening visit to determine whether or not they are eligible for participation in the trial. In those centers where a second consent form for study participation is employed, informed consent will be obtained for further study participation. Following a brief history and physical exam, spirometry is performed.

If the eligible subject is taking an asthma medication regularly that requires a specific washout prior to investigation (e.g. long-acting beta-agonist), the subject will be evaluated by a study investigator as to the appropriateness of drug withholding for the necessary washout interval prior to Visit Sc and dispensed an inhaled corticosteroid inhaler if he/she had been receiving combined ICS and long-acting beta-agonist.

If the subject does not require any drug withholding, then screening visit Sc may take place at that time.

4.   Visit Sc Screening
   a. Brief physical exam
   b. Spirometry
   c. Urine pregnancy test (for females)
   d. Methacholine challenge (or bronchodilator reversibility if FEV$_1$ <55% predicted)

Eligible subjects who meet criteria for methacholine challenge will undergo methacholine challenge according to ACRN MOP. Urine pregnancy test is performed on female subjects of childbearing potential. Other subjects may undergo bronchodilator reversibility testing with 2 puffs of albuterol according to the ACRN MOP.

5.   Visit Sd Screening
   a. Spirometry
   b. Bronchodilator reversibility if failed methacholine challenge at visit Sc

Subjects who fail to meet methacholine challenge criteria at visit Sc may return to the center for visit Sd and undergo bronchodilator reversibility testing with 2 puffs of albuterol according to the MOP.

Upon the successful completion of Visit Sc or Sd, individuals who have met all study entry criteria (see Section VIII) and who are either Arg/Arg or Gly/Gly will enter the pre-match phase of the trial.   Subjects will begin treatment with open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 80 mcg/puff, 3 puffs BID) and PRN albuterol (RESCUE) and will begin twice daily completion of a study diary card. Subjects will be instructed to return to the clinical center in 3 weeks.

C. Pre-Matching / Matching

1. Visit Pa
   a. Adherence review
   b. Diary review
   c. Spirometry

Subjects will return for Visit Pa after having been on a standardized dose of inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) for 3 weeks. At this visit diaries and adherence to medication dosing will be reviewed. Spirometry will be performed for purposes of identifying the subject's FEV$_1$ to be used for matching him/her to a subject bearing the opposite genotype. At the completion of Visit Pa, subjects enter the pool of match-eligible individuals and wait for a suitable match of the opposite genotype to be identified. Matched pairs must have Visit Pa FEV$_1$ values within 10% of predicted of each other and must match on racial status (Caucasian versus non-Caucasian).

Subjects who develop respiratory tract infections in the period leading up to Visit Pa must wait an additional 4 weeks after the infection has resolved before completing this

visit. These subjects should be given an additional supply of study inhaled corticosteroid (beclomethasone HFA (QVAR)) to accommodate the delay in Visit Pa.

Following completion of Visit Pa, subjects who were taking Advair prior to being screened for the LARGE study may resume taking this medication, in lieu of the study-supplied inhaled corticosteroid (beclomethasone HFA (QVAR)), if they desire. Subjects should wait 1-2 weeks following Visit Pa to resume taking Advair in order to allow adequate time to enter and process their study data and to determine if an immediate match will be identified for them. After this 1-2 week grace period, if no match has been identified, these subjects can begin using their own supply of Advair and discontinue taking the inhaled corticosteroid supplied by the study. No withhold period will be required for Advair prior to future pre-match visits (Pb …Px). If a match is identified for one of these subjects at a later date, he/she will need to discontinue Advair and begin taking study-supplied inhaled corticosteroid for a period of 4 weeks leading up to enrollment at Visit 1.

2. Visit Pb…Px
 a. Adherence review
 b. Diary review
 c. Spirometry

Subjects will return to the clinical center every 4 weeks for Visits Pb, Pc, …Pz for diary review, compliance review, spirometry and safety checks until a match is found.

Matched subjects (within 10 percentage points of percent predicted FEV_1 at Visit Pa and in the same racial category (Caucasian vs. non-Caucasian)) will be contacted to return for Visit 1 and will enter an 8-week run-in period during which they will continue to be treated with an open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and given inhaled albuterol (RESCUE) for use as a "rescue medication."

D. Run-in Period

1. Visit 1, week 0
 a. Asthma quality of life questionnaire
 b. Asthma Control Questionnaire (ACQ)/Asthma Symptom Utility Index (ASUI)
 c. Complete medical history
 d. Brief physical exam
 e. Exhaled nitric oxide collection
 f. Exhaled breath condensate collection
 g. Spirometry
 h. Allergy skin testing
 i. MDI technique assessment
 j. Dispensing of medications, diaries and PF device for study
 k. Safety review

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Matched subjects will return to the clinical center for the run-in period Visit 1. A brief physical exam will be performed and a complete medical history will be collected. Following completion of asthma quality of life, ASUI and asthma control questionnaires, exhaled nitric oxide will be collected according to standard techniques, exhaled breath condensates will be collected according to standard techniques and spirometry will be performed. Allergy skin testing will also be performed to characterize atopic status.

Subjects will be given an open-label inhaled corticosteroid inhaler (beclomethasone HFA (QVAR) 240 mcg BID) as well as an open-label albuterol (RESCUE) inhaler to be used for rescue treatment. MDI technique will be assessed and optimized if not performed correctly.

An electronic peak flow monitoring (EPFM) device and an asthma diary card will be distributed. Prior to distribution, the EPFM device readings will be checked using a Jones Flow-Volume calibrator. Only EPFM devices whose readings are within a specified range of the Jones will be distributed. Subjects will be taught how to use their EPFM devices. They will be instructed to measure peak flow and then use their corticosteroid inhaler immediately upon arising (between 0500 and 1000 hrs) and at bedtime (between 2000 and 0100 hrs.), and to wash their mouth out after each use. Subjects will be instructed to record and circle peak flow values obtained less than two hours after use of inhaled albuterol on their diary cards. The use of diary cards will be explained and subjects will be given an appropriate supply. After receiving further safety instructions with regard to asthma exacerbation criteria, when to use rescue medication, and reasons for physician and center notification, subjects will be instructed to return to the clinical center in 3 weeks.

2.   Visit 2, week 3

 a. Diary review
 b. Spirometry
 c. Ipratropium reversibility testing
 d. Dispensing of medication
 e. Asthma exacerbation criteria review and safety instructions

Subjects will return to the clinical center at the same time of day as on week 0 +/- 2 hours. If scheduling permits, all subsequent visits will occur within a +/- 2-hour window on the study day.

Diary cards will be reviewed for accuracy and compliance and new ones dispensed; EPFM device data will be uploaded. The subject's EPFM device will be tested against the Jones Flow-Volume calibrator and will be replaced if it does not meet defined quality control standards. Adverse events will be noted using the protocol outlined by the ACRN. A thorough assessment of exacerbation criteria will be carried out and identified exacerbations will be documented and treated according to protocol. (Subjects who experience an exacerbation at this point in the study will be terminated from further

study participation but will be allowed to re-enroll following treatment and appropriate washouts; subjects who exacerbate will be followed until their exacerbation conditions have resolved.). Clinical center personnel will assess adherence by reviewing the appropriateness of the timing of medication use, peak flow recording and symptom recording. Guidelines will be reviewed as needed. Spirometry will be performed. An assessment of bronchodilator reversibility to ipratropium bromide will be performed: subjects will be given 4 puffs of ipratropium and spirometry will be measured 30 minutes later. Open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) will be issued. Open-label albuterol (RESCUE) drug will be dispensed, if necessary. Asthma exacerbation criteria will be reviewed and safety instructions will be reviewed. Subjects will be instructed to return to the clinical center in 3 weeks.

3. Visit 3, week 6

a. Diary review

b. Nitric oxide collection

c. Spirometry

d. Bronchodilator response to 4 puffs albuterol

e. Dispensing of medication

Subjects will return to the clinical center at the same time of day as on week 0 +/- 2 hours. After diary review, nitric oxide collection, and spirometry, an assessment of bronchodilator reversibility to 4 puffs albuterol will be performed. Subjects will inhale 4 puffs of albuterol and have spirometry performed 15 minutes later. Open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) will be issued. Open-label albuterol (RESCUE) drug will be dispensed, if necessary. Subjects will be instructed to return to the clinical center in 2 weeks.

E. Randomization and First Active Treatment

1. Visit 4, week 8

a. Diary review
b. Asthma quality of life questionnaire
c. ACQ/ASUI
d. Nitric oxide collection
e. Exhaled breath condensate collection
f. Spirometry
g. Urine pregnancy test (for females)
h. Methacholine challenge
i. Asthma exacerbation criteria review and safety instructions
j. Diskus technique assessment
k. Dispensing of medication

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Subjects will return to the clinical center at the same time of day as on week 0 +/- 2 hours. After diary review, quality of life assessment, ASUI and ACQ, nitric oxide collection, and before spirometry, subjects will perform exhaled breath condensate collection according to the standard ACRN protocol. Eligible subjects who meet FEV_1 criteria (and with negative pregnancy test on that visit) will undergo methacholine challenge according to standardized ACRN protocol. Eligible subjects will then be randomized to treatment with either double-blind long-acting beta-agonist (salmeterol Diskus 50 mcg BID) or placebo in addition to open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID). Subjects will be trained on proper inhalation technique using a placebo Diskus labeled for this purpose. Subjects will receive study medications as well as RESCUE 1 (open-label ipratropium bromide) and RESCUE 2 (open-label albuterol) medications with instructions for their use. They will be instructed to return to clinic 2 weeks later for visit 5.

2. Visit 5, week 10

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Dispensing of medication

3. Visit 6, week 14

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Dispensing of medication

4. Visit 7, week 18

a. Diary review
b. Nitric oxide collection
c. Exhaled breath condensate collection
d. Spirometry
e. Ipratropium reversibility
f. Dispensing of medication

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5. Visit 8, week 22

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Bronchodilator response to 4 puffs albuterol
e. Dispensing of medication

6. Visit 9, week 26

a. Diary review
b. Asthma quality of life questionnaire
c. ACQ/ASUI
d. Nitric oxide collection
e. Exhaled breath condensate collection
f. Spirometry
g. Urine pregnancy test (for females)
h. Methacholine challenge

i. Asthma exacerbation criteria review and safety instructions
j. Dispensing of run-out medications (open-label beclomethasone HFA (QVAR) 240 mcg BID) and prn albuterol (RESCUE))

F. Run-out period, end of visit 9-beginning visit 12, weeks 27-34

Following 18 weeks of double-blind treatment with open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and either long-acting beta-agonist (salmeterol 50 mcg BID) or placebo, subjects will begin an 8 week run-out period which will also serve as the run-in phase of the second double-blind treatment phase. Like the initial run-in, subjects will receive open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID), as well as open-label rescue albuterol (RESCUE).

1. Visit 10, week 29

a. Diary review
b. Nitric oxide collection
c. Exhaled breath condensate collection
d. Spirometry
e. Ipratropium reversibility testing
f. Dispensing of run-out medication

2. Visit 11, week 32

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Bronchodilator response to 4 puffs albuterol
e. Dispensing of run-out medication

G. Crossover and Second Active Treatment Sequence (Visit 12-17, weeks 35-52)

    1.   Visit 12, week 34

    a. Diary review
    b. Asthma quality of life questionnaire
    c. ACQ/ASUI
    d. Nitric oxide collection
    e. Exhaled breath condensate collection
    f. Spirometry
    g. Urine pregnancy test (for females)
    h. Methacholine challenge
    i. Asthma exacerbation criteria review and safety instructions
    j. Dispensing of open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID), double-blind treatment medication (salmeterol 50 mcg BID or placebo) and open-label ipratropium rescue (RESCUE 1) with albuterol back-up (RESCUE 2)

Following completion of study procedures, subjects will be given the double–blind therapy with the alternative treatment regimen in addition to open-label RESCUE inhalers 1 and 2 and open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID).

    2.   Visit 13, week 36

    a. Diary review
    b. Nitric oxide collection
    c. Spirometry
    d. Dispensing of medication

    3.   Visit 14, week 40

    a. Diary review
    b. Nitric oxide collection
    c. Spirometry
    d. Dispensing of medication

    4.   Visit 15, week 44

    a. Diary review
    b. Nitric oxide collection
    c. Exhaled breath condensate collection
    d. Spirometry
    e. Ipratropium reversibility testing
    f. Dispensing of medication

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5.   Visit 16, week 48

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Bronchodilator response to 4 puffs albuterol
e. Dispensing of medication

6.   Visit 17, week 52

a. Diary review
b. Asthma quality of life questionnaire
c. ACQ /ASUI
d. Nitric oxide collection
e. Exhaled breath condensate collection
f. Spirometry
g. Pregnancy test (for females)
h. Methacholine challenge
i. Asthma exacerbation criteria review and safety instructions
j. Dispensing of run-out medications (open-label beclomethasone HFA (QVAR) 240 mcg BID) and prn albuterol (RESCUE))

H.   Run-out phase

Following the second treatment phase, subjects will undergo a 10-week run-out phase during which they will continue to receive open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 240 mcg BID) and albuterol (RESCUE). This will culminate in the end of study closeout visit at week 62

1.   Visit 18, week 55

a. Diary review
b. Nitric oxide collection
c. Exhaled breath condensate collection
d. Spirometry
e. Ipratropium reversibility testing

f. Asthma exacerbation criteria review and safety instructions
g. Dispensing of run-out medications (open-label beclomethasone HFA (QVAR) 240 mcg BID and prn albuterol (RESCUE))

2.   Visit 19, week 58

a. Diary review
b. Nitric oxide collection
c. Spirometry
d. Bronchodilator response to 4 puffs albuterol

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e. Dispensing of medication

3. Visit 20, week 60

a. Diary review
b. Asthma quality of life questionnaire
c. ACQ /ASUI
d. Nitric oxide collection
e. Exhaled breath condensate collection
f. Spirometry
g. Pregnancy test (for females)
h. Methacholine challenge
i. Dispensing of medication

4. Visit 21, week 62: End of study / closeout visit

Subjects will return to the center for their last visit and submit all medications, diary cards and any other study-related devices or materials.

a. Diary review
b. Brief physical exam
c. Asthma quality of life questionnaire
d. ACQ/ASUI
e. Nitric oxide collection
f. Spirometry
g. Collection of medications, equipment, and diary cards

X. PROTOCOL IN TABULAR FORM

Variable	Screen			Prematch			Run-in				Stage1 active treatment					Stage 1 run-out			Stage 2 active treatment					Stage 2 run-out			
Visit	Sa	Sb	Sc/Sd	Pa	Pb	Px	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Week	S	S	S	Sc/d +3	Pa+4	P(x-1)+4	0	3	6	8	10	14	18	22	26	29	32	34	36	40	44	48	52	55	58	60	62
Window (regular / extended)	-	-	-	-1d, +3d/ +7d	3d/ 5d	3d/ 5d	-	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d	3d/ 5d
Regular treatment dispensed	-	-	ICS	ICS	ICS	ICS	ICS	ICS	ICS	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS	ICS	ICS	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS/LAB vs. ICS/Plac	ICS	ICS	ICS	ICS	-
As needed treatment			alb	alb	alb	alb	alb	alb	alb	atrov	atrov	atrov	atrov	atrov	alb	alb	alb	atro v	atrov	atrov	atrov	atrov	alb	alb	alb	alb	-
Randomization										+																	
Informed consent	+																										
History	+	+					+																				
Physical		+	+				+																				+
Spirometry	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Venipuncture/ Genotyping	+																										
Allergy skin testing							+																				
Medication withhold		+																									
Urine pregnancy test			+							+							+			+			+			+	
Methacholine challenge			+							+							+			+			+			+	
Albuterol rev/BD4			+						+					+			+					+			+		

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Variable	Screen			Pre-match			Run-in				Stage1 active treatment					Stage 1 run-out			Stage 2 active treatment					Stage 2 run-out			
Visit	Sa	Sb	Sc/Sd	Pa	Pb	Px	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Week	S	S	S	Sc/d +3	Pa+4	P(x-1)+4	0	3	6	8	10	14	18	22	26	29	32	34	36	40	44	48	52	55	58	60	62
Window (regular / extended)	-	-	-	-1, +3/ +7d	3d/5d	3d/5d	-	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d	3d/5d
QOL/ASUI/ACQ questionnaires							+			+								+		+			+			+	+
Exhaled NO collection							+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MDI or Diskus (V4) technique assessment			+				+			+																	
Medication dispensing		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
EPFM dispensing							+																				
Diary card dispensing			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Diary review				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ipratropium reversibility								+					+			+					+			+			
Exhaled breath condensate collection							+			+			+		+	+		+			+		+	+		+	

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XI.   DRUG SUPPLIES

Drug supplies for this study will include an open-label inhaled corticosteroid (beclomethasone HFA (QVAR) 80 mcg 3 puffs BID), a long-acting beta-agonist (salmeterol 50 mcg 1 puff BID), an anticholinergic rescue (ipratropium) for use during the double-blind treatment phases, albuterol sulfate rescue for use during the pre-match, run-in and run-out phases, and placebo salmeterol. During the treatment periods, open-label inhaled corticosteroids will be administered in addition to double-blind long-acting beta-agonist or placebo. Ipratropium and albuterol inhalers will be administered as open-label rescue medications. During the pre-match, run-in and run-out phases subjects will be treated with open-label inhaled corticosteroids and prn albuterol. The ACRN will work with the pharmaceutical companies and contractors to coordinate with the DCC to ensure proper packaging, masking, and coding of drugs.

XII.   ADHERENCE AND MONITORING

Efforts will be made to determine subject adherence with medication dosing during the study. A Doser$^®$ device will be attached to each QVAR inhaler during the main study (Visits 1-21), and salmeterol Diskus inhalation counters will be examined. The Doser$^®$ device registers each actuation of the MDI and stores a daily history that will be reviewed at each clinic visit. The built-in counters on Diskus devices will be examined to determine the number of inhalations that were used. The Doser$^®$ and the Diskus counters allow for objective measurement of the number of puffs/doses used. A major limitation of these devices is their inability to discriminate between actual doses taken and "dose-dumping." They also do not yield much information regarding the timing of doses and the ability of subjects to comply with the dosing schedule required by the study.

As a secondary source of compliance information, subjects' diary cards will be examined for the number of puffs of each medication recorded for each day. This information will be compared to PEF measurements electronically recorded and date/time stamped from the EPFM device. Because subjects are instructed to perform their morning and evening peak flow maneuvers right before taking their study medications, timing of PEF monitoring can be used as a surrogate for timing of dosing with study medications. Limitations of this mechanism for monitoring adherence are accuracy of the subjects' recall and honesty, because the timing and confirmation of dosing cannot be verified directly.

XIII. RISKS/ BENEFITS

Overall Risks:

The LARGE study is a comparison of the effect of a long-acting beta-agonist compared to placebo in two cohorts of asthmatics receiving inhaled corticosteroids defined by their β_2-receptor genotypes. In both the BAGS and BARGE studies we found that in one subgroup defined by the B16-Arg/Arg genotype, the use of albuterol on a regularly scheduled basis resulted in a significant drop in AM PEF. Similarly, in the SOCS and SLIC studies, we found that the use of the long-acting beta-agonist salmeterol was associated with a detrimental effect on airway function in subjects with the B16-Arg/Arg genotype. Although these changes indicated a minor loss of asthma control, they were not associated with an increase in asthma exacerbations. Thus, there is a risk of an adverse outcome in this group, but it is minimal.

Overall Benefits:

There are no direct benefits to the individual subjects; however, there is a potential benefit to patients with asthma in general as the possibility of a more rational basis for therapy is devised.

Risks/Benefits Associated with Genotyping:

The purpose of LARGE is to assess the genetic basis for part of the observed variance in treatment responses in patients with asthma. At present, the clinical significance of the genetic component of variance in the treatment response is unknown. Thus, there are no clear recommendations for genetic counseling for patients who have been genotyped (Kimay-Asadi and Terry, 1997;Ober, 1998). Questions also exist about the potential harm to patients in the form of psychosocial consequences from classification with a genotype associated with adverse outcomes. Two areas warrant careful consideration: (a) the psychological stress on the patient and his or her family that comes from knowledge that one is a carrier of a genetic predisposition to an adverse outcome; and (b) the potential for genetic discrimination by employers and insurance companies who may cancel coverage by considering genetic test results proof of preexisting conditions (Kimay-Asadi and Terry, 1997).

The Americans with Disabilities Act of 1990 provides some protection for the "genetically disabled" from genetic discrimination. For example, the Equal Employment Opportunity Commission recently determined that the use of genetic testing results to deny employment is a violation of the Act (Kimay-Asadi and Terry, 1997).

To provide the best protection to research participants, respondents will be assured of complete confidentiality of the test results. As with all research data, information gathered by the study will be used only for aggregate analysis; it will not be released with any information that identifies research participants. Information about genotypes,

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in particular, will be coded and unlinked to individual respondent identifiers. The genotyping facility will not have access to patient-identifying information. The code to link respondents and their genotype will be securely stored and accessible only to the database programmer at the DCC. The DCC does not have access to the identities of patients. That information is retained at the centers.

Respondents will be informed that genotyping at loci related to asthma is valuable for research purposes, and in aggregate form only. Because of the lack of knowledge regarding the clinical significance of genotypes, subjects will not be supplied information about their genotype, and they will receive no genetic counseling (Welch and Burke, 1998).

Risks/Benefits Associated with Ipratropium Bromide as Rescue Medication:

Ipratropium bromide has been used in a number of asthma clinical trials, as the primary rescue therapy (GlaxoSmithKline, 2004;Israel et al., 2004;Polosa et al., 1991). In particular, it was used in the BARGE trial without any difficulties. While ipratropium bromide affords a comparable degree of bronchodilatation to that of albuterol, its onset of action is slower. The use of ipratropium as rescue medication is critical to studies where beta-agonist use is the primary exposure variable. While the Food and Drug Administration has not approved ipratropium bromide as primary rescue therapy, no adverse outcomes have been reported as a result of such use of this drug in any of multiple clinical trials. Despite the apparent safety of this drug as rescue therapy, we have designed the LARGE rescue algorithm (See Section XIV) to include albuterol as the superceding rescue medication.

XIV. ADVERSE EVENTS

A.   Definition

An adverse event shall be defined as any detrimental change in the subject's condition, whether it is related to an exacerbation of asthma or to another unrelated illness. Adverse events related to asthma exacerbation will be managed according to rescue algorithms outlined below.

B.   Adverse Events Unrelated to Asthma

Adverse events due to concurrent illnesses other than asthma may be grounds for termination from the trial if the illness is considered significant by the investigator or if the subject is no longer able to participate effectively in the study. Subjects experiencing minor intercurrent illnesses may continue in the study provided that the nature, severity, and duration of the illness are recorded and that any unscheduled medications required to treat the illness also are recorded. Examples of minor intercurrent illnesses include acute rhinitis, sinusitis, upper respiratory infections, urinary tract infections, and gastroenteritis. Medications are allowed for treatment of these conditions in accordance with the judgment of the investigator. However, because of the effect of inhaled corticosteroids on airway responsiveness, we will avoid escalating the dose of these drugs following enrollment into the trial.

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Documentation of an adverse event unrelated to asthma will be recorded on Clinical and Laboratory Adverse Event and Concomitant Medications for Asthma/Allergy and Adverse Events forms and will include the following information:

Description of the illness
Dates of illness
Treatment of illness (medications, doses, dates)
Whether hospitalization or emergency treatment was required
Treatment outcome

C. Adverse Events Related to Asthma: Asthma Exacerbation

During the course of the study, subjects may experience an increase in asthma symptoms. While an increase in asthma symptoms may be brief and self-limited, any increase in symptoms or changes in PEF should be carefully monitored by the subject, the clinic coordinator, and the physician. During the course of the study, symptoms may be of sufficient severity so as to warrant documentation as an asthma exacerbation.

1. Asthma Exacerbation: Definition

For this protocol, an asthma exacerbation is defined as the development of an increase in symptoms of cough, chest tightness, or wheezing in association with one or more of the following:

a.) Increased rescue use, i.e., an increase in "as needed" ipratropium and albuterol (combined, when applicable) use of ≥ 8 puffs per 24 hrs. over baseline use for a period of 48 hours or ≥ 16 total puffs per 24 hrs. for a period of 48 hrs. [During the first 3 weeks of the pre-match phase of the study, baseline is defined as the historical average daily albuterol use obtained at Visit Sc/d; during the remainder of the pre-match phase and for the first 3 weeks of the run-in (interval between Visits 1 &2), baseline is defined as the average daily albuterol use over weeks 1-3 of the pre-match phase. During run-in weeks 4-8, baseline is defined as the average daily albuterol use over weeks 1-3 of the run-in (interval between Visits 1 & 2). Baseline is defined as average daily albuterol use over weeks 7 & 8 (interval between Visits 3 & 4) for evaluation during the remainder of the study.]

b.) A PEF that does not increase to ≥65% of reference level after the first 60 minutes of rescue treatment (albuterol or ipratropium, as applicable during a given study phase). [During the first 3 weeks of the run-in (interval between Visits 1 & 2), reference level is the best PEF value obtained during three maneuvers following a successful peak flow performance check at Visit 1. During the last 5 weeks of the run-in, reference level is defined as the average AM PEF during weeks 1-3 established from diary cards returned at Visit 2. Following randomization, reference level is defined as the average AM PEF during weeks 7 & 8 of the run-in (interval between Visits 3 & 4)].

c.)   Symptoms that are not satisfactorily relieved after the first 60 minutes of rescue treatment (albuterol or ipratropium, as applicable during a given study phase).

d.)   Treatment with inhaled, oral, or parenteral corticosteroids as a result of rescue intervention or by the opinion of the treating physician.

Management of exacerbations

Asthma exacerbations that occur following randomization will be managed according to the rescue algorithms described below.   During medical management of the exacerbation, other trial medication will be continued, unless the treating physician considers it appropriate to suspend such therapy until the exacerbation resolves. Reinstitution of trial medications will occur when the exacerbation has resolved at the discretion of the investigator. A record of all medications, dosages, and frequency of occurrence will be kept during exacerbations.

Rescue Algorithms

Once an asthma exacerbation has occurred, the subject should contact the clinic coordinator and/or be evaluated at the study site or the nearest medical emergency facility as quickly as possible.

Because less significant changes in symptoms and/or PEF may precede more severe alterations in asthma stability, a series of rescue algorithms has been developed to address the various clinical presentations that may occur. Once any of these rescue interventions leads to the administration of corticosteroids in excess of the study dosage of QVAR, the subject also will be considered to have developed an asthma exacerbation. In addition, if in the opinion of the treating physician, corticosteroid therapy is warranted regardless of any antecedent measurements of pulmonary function (PEF, FEV_1, etc.), value for symptom score, or frequency of rescue beta-agonist use, the subject will be considered to have developed an asthma exacerbation.

The time at which an asthma exacerbation develops in relationship to the schedule of the LARGE protocol will affect the manner in which future clinic visits, medication adjustments, and diagnostic studies are scheduled or performed. The scheduling of these events is outlined below.

Subjects developing an asthma exacerbation during the pre-match phase of the study will be treated according to the rescue algorithm outlined below. They will continue to be followed for possible matching and enrollment in the main study. If a subject who has experienced an exacerbation becomes matched, he/she may enroll in the main study (Visit 1) after having washed out from the exacerbation and any rescue medications for at least 6 weeks' time.   The subject will continue to be seen for scheduled pre-match visits during the washout interval.

Subjects developing an asthma exacerbation during the initial run-in period will be terminated from study participation and may re-enroll after the exacerbation has fully resolved. Subjects developing an exacerbation during the second run-in period may

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require additional time in the run-in period to wash out from rescue medications in advance of entering the second double-blind treatment period. See Section XIV.E below for further details of visits related to this scenario.

Asthma exacerbations will be managed according to the following rescue algorithms. Subjects will be placed on open-label ICS at twice the dose used during the study (i.e., beclomethasone HFA (QVAR) 480 mcgs BID). During medical management of the exacerbation, other trial medication (i.e., double-blind salmeterol or placebo) will be continued, unless the treating physician considers it appropriate to suspend such therapy until the exacerbation resolves. Reinstitution of trial medications will occur when the exacerbation has resolved at the discretion of the investigator. A record of all medications, dosages, and frequency of occurrence will be kept during exacerbations. Additional visits and procedures will be scheduled as outlined in section XIV.E-F.

Rescue algorithms will be applied in cases where an exacerbation fails to resolve or PEF is not improved to $\geq$ 65% of reference level within 48 hours after increasing as-needed rescue use. Rescue algorithms are based on recommendations from the NAEP Guidelines for the Diagnosis and Management of Asthma. Ipratropium bromide, albuterol, inhaled steroids, and oral prednisone are the principal medications for rescue management. Subjects will be instructed in their use for home management, and supplies of ipratropium bromide, albuterol, and prednisone will be provided throughout the study. For severe acute episodes of asthma, treatment will be administered according to the best medical judgment of the treating physician.

a) Home Care

Asthma exacerbations will be recognized by an increase in symptoms and by a corresponding drop in PEF below reference level. Subjects will be educated to recognize exacerbations as early as possible to facilitate prompt treatment and to lessen morbidity.

- During the pre-match and run-in/run-out periods, subjects who recognize increased symptoms and/or a fall in PEF to < 65% of reference level will use albuterol by MDI, 2-4 puffs every 20 minutes up to 60 minutes if needed, and then every 4 hours, or less, if needed. Subjects will be instructed to use the as-needed RESCUE inhaler for treatment.

- During the double-blind treatment periods, subjects who recognize increased symptoms and/or a fall in PEF to <65% of reference level will use open-label ipratropium bromide MDI, 4 puffs initially, then 2 puffs every 20 minutes up to 60 minutes, if needed, and then every 4 hours, or less, if needed to reduce symptoms and normalize lung function. Subjects will be instructed to use their as-needed RESCUE 1 inhaler for this purpose. If the PEF does not increase to $\geq$65% of the reference level, or if symptoms are not improved after the first 60 minutes of ipratropium therapy, the subject will be instructed to use the same treatment scheme substituting open-label albuterol rescue (RESCUE 2) for the ipratropium bromide rescue.

- If the PEF does not increase to ≥65% reference level or if symptoms are not improved after the first 60 minutes of albuterol therapy, the subject should contact the investigator or their primary care physician or seek care in the emergency department.

- Failure of albuterol to control or maintain PEF ≥65% of reference level may necessitate the use of corticosteroids (see below).

b) Physician's Office or Emergency Room Treatment

- Subjects will be assessed by history, physical examination, and by physiological monitoring including spirometry or PEF. If the subject's PEF and/or FEV_1 is less than 25% predicted or if the subject shows evidence of altered mental status, cyanosis, labored breathing, or use of accessory muscles, sampling of arterial blood for respiratory gas analysis is indicated, with appropriate action taken depending on the results obtained.

- When treated in the physician's office or the hospital emergency room, subjects should initially be given albuterol by nebulization (0.5 cc of 0.5% solution) every 20 minutes over the first 60 minutes.

- If the PEF increases to ≥65% of reference level after the first 60 minutes, the subject can be discharged to continue treatment at home. Prednisone or open-label inhaled corticosteroids may be administered at the discretion of the physician to augment therapy.

- If symptoms persist and PEF remains <65% of reference level, nebulized albuterol should be continued as often as every hour and further treatment with oral or parenteral corticosteroids should be considered (prednisone, 60 mg orally; methylprednisolone, 60 mg iv bolus). Monitoring of PEF or spirometry should continue every hour. Within four hours of treatment, a decision should be made regarding subject disposition.

- If PEF increases to ≥65% reference level within four hours, the subject can be discharged to continue treatment at home. Home treatment should include an 8-day course of prednisone followed by open-label inhaled corticosteroid treatment (see below).

- If PEF remains >40% but <65% of reference level, an individualized decision should be made to hospitalize the subject for more aggressive therapy or to continue therapy at home with a course of prednisone followed by inhaled corticosteroids.

- If PEF is <40% of reference level after repeated albuterol treatments, the subject should be admitted to the hospital unless, in the physician's best judgment, alternative treatment could suffice.

c) Prednisone Treatment

In this protocol, prednisone will be used when, in the judgment of the investigator, acute exacerbations cannot be controlled by albuterol and inhaled corticosteroid therapy. Indications for prednisone therapy include the following:

- To achieve stable control of symptoms and optimize pulmonary function once asthma exacerbation status is achieved.

- For follow-up management after discharge from the physician's office, emergency room, or hospital for an acute exacerbation.

The dose of prednisone used during an acute exacerbation shall consist of 60 mg as a single dose every day for three days, followed by a 10 mg/day taper over the next five days. The decision to initiate or to continue a course of prednisone beyond eight days is left to the discretion of the physician.

d) Inhaled Corticosteroid Treatment

Inhaled corticosteroid dosing for worsened asthma symptoms during the LARGE trial will be the addition of open-label beclomethasone HFA 80 mcg/puff, 3 puff BID (or equivalent) to the treatment regimen for 2 weeks. This will represent a doubling of the study inhaled corticosteroid dose**.**

D. Adjustment of Trial Medications During Asthma Exacerbations

Subjects will be placed on open-label ICS at twice the dose used during the run-in and treatment periods (as indicated above). Trial drugs will be continued during exacerbations unless the treating physician considers it appropriate to suspend such therapy until the exacerbation resolves. Reinstitution of trial medications may occur when the exacerbation has resolved at the discretion of the investigator. A record of all medications, dosages, and frequency of occurrence will be kept during exacerbations.

E. Study Center Visits Following Exacerbations

If the subject receives open-label inhaled or systemic steroids for an exacerbation, regular follow-up evaluations will continue as outlined in the original protocol schema, Section IV (intent-to-treat). All medications used to treat exacerbations will be recorded and entered into the study database for the main study (Visit 1-21). For safety reasons,

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all subjects will be seen at the clinical center within one week (+3d) from the day they have been categorized as experiencing an asthma exacerbation. Following this "safety" visit, subsequent protocol visits will continue in accordance with the visit schedule established at Visit 4 (first treatment period) or Visit 12 (second treatment period).

However, subjects who experience an exacerbation in the weeks prior to Visit 12 (beginning of second double-blind treatment period) may need to delay entering the second treatment period until a sufficient period of time has passed to allow for washout of rescue medications. In particular, Visit 12 will be delayed until at least 8 weeks have passed since the documentation of exacerbation status and, if prescribed, until at least 6 weeks have passed since the last dose of oral prednisone and/or rescue inhaled corticosteroid (i.e., double the usual study dose of QVAR, per protocol). If a subject falls into the required washout period at the time that he or she was originally scheduled to complete Visit 12, then an additional safety visit will be performed prior to Visit 12. At that safety visit, spirometry will be performed, diary cards will be reviewed and dispensed, and the subject's asthma symptoms will be assessed. The subject will be given additional run-in medications to last for 2 weeks. If, after this safety visit, more than 2 additional weeks are required to meet the minimum washout period, then a second safety visit will be scheduled. If necessary, additional interim visits will be scheduled at 2-week intervals (+3 day window) during the washout period. Interim visits will be designated Visits 11A, 11B, 11C, etc., as needed. When less than 2 weeks are needed to meet all washout requirements, Visit 12 will be scheduled for 2 weeks following the current visit. All necessary washouts will be reviewed and confirmed prior to performing Visit 12 procedures.

F.   Criteria for Achieving Dropout Status

Patient becomes pregnant.
Patient withdraws consent.

G.   Criteria for Termination From Study Due to Asthma Exacerbations

For safety reasons, subjects will be terminated from the study if they have >2 exacerbations during either double-blind treatment period or during either post-randomization run-out period.

H.   Adverse Events as Outcome Variables

During exacerbations, the following variables will be recorded and used as outcome measures:

Hospitalization
Emergency room visits
Unscheduled physician/clinic visits
Number of subjects having an asthma exacerbation

XV. COST, LIABILITY AND PAYMENT

All tests will be performed without cost to the participating subjects. Since this is a trial using a well-established asthma treatment, liability for subject care costs incurred by subjects during the course of the trial will in most cases be borne by the subject or the insurer. Details of the National Institutes of Health policies concerning this issue can be found in NIH Documents # 5305 and 6352-2, *Research Patient Care Costs Supported by NIH Sponsored Agreements.*

Each subject will receive financial compensation within FDA guidelines for participation in an amount determined by the local center. For subjects who drop out, payments will be pro-rated for the length of time they stayed in the study, but payment will not be made until the study would have been completed had the subject not dropped out.

XVI. DATA RECORDING

Recording of all data including the informed consent, history, physical examination, results of allergy skin testing, vital signs, results of pregnancy tests, adverse events, confirmation of medication dispensation, methacholine challenge testing, and quality of life questionnaires will be recorded on forms prepared by the ACRN Data Coordinating Center (DCC). Initial data entry will be done at each Clinical Center and forms will be forwarded to the DCC for confirmatory entry. Results from pulmonary function tests will be transmitted electronically to Quantum Research, Inc. and then transferred (weekly) to the DCC for permanent storage. All data will be stored and analyzed at the DCC.

XVII. DISCUSSION OF ANTICIPATED RESULTS

Our anticipated results are influenced by our prior studies with short-acting beta-agonists and our retrospective analyses of genotype stratified outcomes with long-acting beta-agonists. To review, our retrospective analysis of the BAGS trial suggested that, in patients with mild asthma, those who are harboring the B16–Arg/Arg genotype experience detrimental effects with regular use of beta-agonists compared to as-needed therapy, or as compared to regular therapy, in patients bearing the B16-Gly/Gly genotype. Our prospective BARGE trial confirmed that a genotype-specific response to regular beta-agonists does occur. We found that the B16-Arg/Arg patients improved when they were withdrawn from beta-agonists and treated with ipratropium. In contrast, the B16-Gly/Gly patients experienced further improvement with regular beta-agonist use. These effects were not restricted to peak flow, but were also seen in FEV_1, symptoms, and supplemental reliever medication use. Our retrospective analysis of the SOCS and SLIC data extended our findings to long-acting beta-agonists. B16-Gly/Gly patients benefited from regular use of a long-acting beta-agonist while Arg/Arg patients did not. This difference was evident even in patients who were treated with inhaled corticosteroids, as they were in the SLIC study.

In this context we can consider the results that might be observed in our prospective comparison of long-acting beta-agonists in Arg/Arg vs. Gly/Gly patients who are treated with an inhaled corticosteroid.

First, our study may demonstrate that there is no significant difference in the improvement between the two treatment groups in response to treatment with a long-

acting beta-agonist. In this case, if both are improved and there is no difference between them, the null hypothesis will have been confirmed.

Secondly, we may find that both groups deteriorate with treatment with a long-acting beta-agonist. We believe this outcome to be unlikely in view of the available data in the literature suggesting that the population as a whole does improve with the addition of a long-acting beta-agonist (Pauwels et al., 1997). We therefore believe this outcome is unlikely and does not require further explication.

We believe the most likely outcome, based on the prior data with short-acting beta-agonists and our retrospective data with long-acting beta-agonists, is that we will observe differences in our outcome measures when we compare long-acting beta-agonist treatment to placebo, stratified by genotype. Our SLIC analysis suggests that we will most likely see no change, or an improvement, in the B16-Gly/Gly patients compared to placebo plus a PRN anticholinergic. The SLIC analysis, combined with the BARGE results, suggests that the B16-Arg/Arg patients will not have significant improvement, or will deteriorate, with long-acting beta-agonists. In fact, they may actually do better when treated with a placebo plus a PRN anticholinergic. These results would suggest that the B16-Arg/Arg patients would benefit from an alternate form of therapy other than long-acting beta-agonists.

If we do demonstrate such an effect, our exploration of markers of inflammation in the expired air and the exhaled breath condensate and the time course of changes in such markers may allow us to interpret the pathobiology of this effect.

Lastly, it is possible that the B16-Arg/Arg patients would do better on long-acting beta-agonists and B16-Gly/Gly patients would not. However, all our prior retrospective and prospective data make such a result unlikely.

It is important to note that, in order to minimize background beta-agonist use, patients in both arms of the study (as they did in the BARGE trial) will be using ipratropium for rescue therapy. From an ethical point of view, we must provide reliever therapy for these patients. Thus, it is theoretically possible that a portion of the difference we might observe might be related to a differential response of the two β-adrenergic receptor genotypes to anticholinergic therapy. While theoretically possible, we think this unlikely since in the BARGE trial the two genotypes also differed when one compared the different genotypes when they received regular albuterol. However, to explore this further, we are also examining whether there is a difference between the genotypes as it relates to the bronchodilator response to ipratropium. Additionally, we will once again perform a secondary analysis of the genotype-specific difference between the beta-agonist treatment arms.

In summary, considering the fact that combining long-acting beta-agonists with inhaled corticosteroids is becoming a rapidly expanding modality of therapy even for mild patients, our data will allow us to determine whether there is a genotype-attributable difference in response to the long-acting beta-agonist component of this therapy.

XVIII.   STATISTICAL DESIGN AND ANALYSIS

A. Data Collection and Data Management

Each clinical center will have a computer configuration that includes an Internet connection and a printer. This will give each center the capability of logging directly into the computing system at the Data Coordinating Center (DCC) over the Internet. Though this set-up is installed primarily to allow for distributed data entry into a centralized database on the ACRN project server at the DCC, menu options also include sending electronic mail, downloading study documents such as forms and reports, and viewing a calendar of ACRN events. A sophisticated security system limits access to qualified personnel and prevents corruption of the study database.

The DCC is responsible for generating the data collection forms based on input from the clinical centers. Once the data collection forms have been filled out and reviewed, the clinic coordinator will log into the DCC computer system and enter the data within 3 days of the subject's visit. The advantage of this distributed data entry system is that the clinic coordinators will review the data a second time as they are entering it, which serves as another level of quality control. The database management system will have range checks and validity checks programmed into it that execute upon entry. Clinic coordinators will be responsible for resolving any errors identified during the data entry process. Following error resolution, forms then will be forwarded to the DCC for second data entry (verification) and filing, which will be performed within 3 days of receipt. The DCC will be responsible for identifying problem data and resolving inconsistencies. At any time after entry, clinic coordinators will be able to view data entered in the database. If errors are noted and forms have already been sent to the DCC, data corrections must be submitted to the DCC through the database application.

Results from lung function tests will be sent to Quantum Research, Inc. for review/overreading by ACRN staff via modems in the computers attached to the spirometers. Data will be transferred from Quantum Research, Inc. to the DCC weekly for permanent storage and for use in tracking technician certification and overread grades.

B. Masking

Careful procedures are required in order to maintain triple masking of the study participants, clinical center personnel, and DCC personnel as to whether individual subjects are taking placebo or active long-acting beta-agonist. The DCC will work with the pharmaceutical companies to ensure that sufficient medication for a single subject is supplied for the duration of the study. The labels on the Diskuses will not indicate their contents (active salmeterol or placebo). Treatment medication for each subject will be packaged together in a kit that is labeled with a unique number. The contents of the kits will be known only to the project coordinator and database programmer at the DCC; all other DCC staff will remain blinded to each subject's treatment assignment. Drug kits and open-label beclomethasone HFA (QVAR) canisters for the pre-match, run-in and run-out periods will be delivered to the clinic coordinators. Triple-masking, i.e., masking

of the DCC personnel in addition to the study participants and clinical center personnel, will be employed so that the statistical analyses are not biased by preconceived notions. Until the time of manuscript preparation, DCC personnel will refer to the randomized groups in terms of codes and only the project coordinator and database programmer at the DCC will know the code identities.

In order to decrease the likelihood of incorrect drug distribution, each coded package designated for a study participant will have a sheet of removable labels attached to it. When the clinic coordinator retrieves a Diskus for the study participant, he/she will remove one of the labels and attach it to the medication distribution data collection form prior to mailing the form to the DCC. The clinic coordinator will initial across the label to indicate that he/she checked to make sure the appropriate Diskus was distributed to the participant.

C. Matching

Subjects will be screened for genotype at Visit Sa. The genotype information on all subjects will be maintained at the DCC so that clinical center personnel are masked to the genotype of each individual. When the DCC identifies a pair of B16-Arg/Arg and B16-Gly/Gly individuals who are "matched" and who meet study eligibility criteria, then the DCC will inform the clinical center that a pair of subjects is eligible for study entry. A B16-Arg/Arg individual and a B16-Gly/Gly individual are matched if (1) they both are Caucasian or non-Caucasian (by self–report and as determined by NIH race and ethnicity classification scheme) and (2) the difference in their percent predicted FEV_1 measurements at pre-match Visit Pa is less than 10%. To the extent possible, clinical personnel will be blinded with respect to information regarding which subjects are paired.

The purpose of the pair matching is to ensure that the two cohorts of genotypes are similar with respect to both an important prognostic variable (FEV_1) and ethnic/racial profile. The purpose of matching by race is that we wish determine whether there is a genotype-specific effect as opposed to a race-specific effect; we therefore want to assure that Caucasians are evenly distributed in both genotypic groups so that we can analyze whether an effect (if it occurs) occurs in a subgroup in which we would still have adequate statistical power to make such a determination.

When multiple matches are available for a given subject, within-center matches will be given priority. However, if a between-center match presents and is the only available match, the between-center match will be made immediately upon its identification. No waiting period for finding a within-center match will be enforced.

D. Randomization

The design of this trial is a 2×2 crossover within each genotype (B16-Arg/Arg and B16-Gly/Gly) with subjects randomized to the following sequences:

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(1)   8-week run-in period, 18-week treatment period with active inhaled corticosteroid (ICS) and active long-acting beta-agonist (LABA), 8-week run-out period, 18-week treatment period with active ICS and placebo LABA, 10-week run-out period;

(2)   8-week run-in period, 18-week treatment period with active ICS and placebo LABA, 8-week run-out period, 18-week treatment period with active ICS and active LABA, 10-week run-out period.

When a subject has completed the 8-week run-in period and is eligible for randomization, the clinic coordinator will log into the ACRN network server and indicate to the system that a subject requires randomization. If the subject's paired member already has been randomized to a sequence, then this subject will be assigned to the same sequence as its paired partner. Otherwise, this subject will be randomized to one of the two sequences. After entering the pertinent information with respect to eligibility criteria, the clinic coordinator will be asked to verify that all of the information has been reviewed carefully and the subject is eligible. If so, the clinic coordinator will be given a drug kit number, from which all medication for that patient will be dispensed. In order to maintain security of the randomization schedules, the data manager of the DCC will receive automatically a notice from the ACRN network server that the subject has been randomized. If no follow-up information is forthcoming on such a subject, the data manager will contact the clinic coordinators concerning the status of the subject.

As discussed in the section on sample size below, each clinical center will need to randomize approximately twelve subjects. Because this is a small number, it is not necessary to use permuted blocks in the randomization process.

If a member of a matched pair is deemed ineligible or withdraws during the run-in period, then the other member of the pair can continue in the study and be randomized. There will be an attempt to find another match for the remaining member of the pair, but this will not impede randomization if unsuccessful. If a member of a matched pair withdraws post-randomization, then the other member of the pair will continue in the study unimpeded.

E. Statistical Analysis

The primary question to be addressed by this study is whether the treatment regimens differ with respect to AM PEF at the end of the 18-week treatment periods. This primary question will be assessed separately within each genotype (B16-Arg/Arg, B16-Gly/Gly) and between genotypes. Secondary comparisons with respect to AM PEF (as well as with other outcomes) include the difference in this outcome variable between the end of 8 weeks of the Stage 2 run-out period vs. the end of 8 weeks of the Stage 1 run-out period. We will also assess whether such differences occur between a) the end of Stage 2 treatment period and the beginning of Stage 2 treatment period vs. the end of Stage 1 treatment period and the beginning of Stage 1 treatment period; and b) the end of 8 weeks of the run-out periods and the beginning of the respective treatment periods. Thus, change in AM PEF in both B16 genotype groups will be assessed at the beginning and at the end of each 18-week treatment period as well as after 8 weeks of run-out period.

Secondary response variables to be examined include other physiologic variables, asthma control and quality of life, and biomarkers of inflammation.

The other physiologic variables to be analyzed include PM PEF, peak flow variability ([PM PEF − AM PEF]/PM PEF), FEV_1, airway responsiveness (methacholine PC_{20}), ipratropium reversibility (AtRev), bronchodilator reversibility (BD4), and exhaled breath condensate (EBC). The asthma control variables to be analyzed include individual and total symptom scores, the number of occasions and actuations of rescue MDI, the number and occurrences of exacerbation, episodes of adverse asthma control, and asthma-related quality of life. The biomarkers of inflammation to be analyzed include exhaled nitric oxide (eNO) and exhaled breath condensate pH. Variables that are measured daily from the subject diary cards, e.g., PEF, PEF variability, symptoms, and rescue medication use, will be averaged between visits and weighted by the inverse of the squared standard error. The purpose of the weighting scheme for diary card data is to assign greater weight to means that are measured with low variability and less weight to means that are measured with high variability. A sensitivity analysis will be performed in which two other weighting schemes are invoked, namely, a weighting scheme that uses the number of observations and an unweighted scheme.

Because of the repeated measurements of the primary and secondary response variables over time, the most appropriate statistical analysis is longitudinal data analysis. In a longitudinal data analysis all of the data from the study participants are incorporated into the analysis. A variety of longitudinal data models have appeared in the statistical literature. We invoke the mixed-effects linear model (Laird et al., 1992;Vonesh EF, 1997). However, this is somewhat complicated due to the crossover nature of the trial. For this model, it is necessary to specify a function for describing the expectation of the chosen response variable over time. Given the design of this trial, an appropriate "within-patient" expectation function is

$E(Y_{ijkw}) = \beta_{ij0} + w\beta_{ij1}$ if $0 \le w \le 8$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + (w-8)\beta_{ij2}$ if $8 \le w \le 14$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + 6\beta_{ij2} + (w-14)\beta_{ij3}$ if $14 \le w \le 26$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + 6\beta_{ij2} + 12\beta_{ij3} + (w-26)\beta_{ij4}$ if $26 \le w \le 34$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + 6\beta_{ij2} + 12\beta_{ij3} + 8\beta_{ij4} + (w-34)\beta_{ij5}$ if $34 \le w \le 40$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + 6\beta_{ij2} + 12\beta_{ij3} + 8\beta_{ij4} + 6\beta_{ij5} + (w-40)\beta_{ij6}$ if $40 \le w \le 52$

$E(Y_{ijkw}) = \beta_{ij0} + 8\beta_{ij1} + 6\beta_{ij2} + 12\beta_{ij3} + 8\beta_{ij4} + 6\beta_{ij5} + 12\beta_{ij6} + (w-52)\beta_{ij7}$ if $52 \le w \le 62$

Where subscript i represents genotype (Arg/Arg or Gly/Gly), subscript j represents sequence assignment (ICS/active LAB $\Rightarrow$ ICS/placebo LAB or ICS/placebo LAB $\Rightarrow$ ICS/active LAB), subscript k represents patient, and subscript w represents week of the trial. Weeks 0-8 correspond to the 8 weeks of the run-in period, weeks 8-14 correspond to the first 6 weeks of the treatment period in the first stage, weeks 14-26 correspond to the last 12 weeks of the treatment period in the first stage, weeks 26-34 correspond to the run-out period in the first stage, weeks 34-40 correspond to the first 6 weeks of the treatment period in the second stage, weeks 40-52 correspond to the last 12 weeks of

the treatment period in the second stage, and weeks 52-62 correspond to the 10 weeks of the run-out period in the second stage. This particular expectation function is called a segmented or piecewise linear function because it consists of a set of connected lines.

Because of the one-to-one matching of B16-Arg/Arg and B16-Gly/Gly subjects prior to randomization, the two sets of repeated measurements for a matched pair (B16-Arg/Arg subject and B16-Gly/Gly subject) are assumed to be correlated. The correlation matrix is modeled as the Kronecker product of a 2×2 unstructured matrix for the matched subjects that allows for heterogeneous variances between Arg/Arg and Gly/Gly subjects, with either (1) an unstructured, (2) a compound symmetric, or (3) an autoregressive correlation matrix for repeated measurements over time. Although this is a complex correlation structure, it is necessary because a simpler model that assumes independence of all the observations from the matched pair is not valid. Akaike's information criterion will be calculated to assess which of the three correlation structures for the repeated measurements over time provides the best fit. Other baseline variables that are not involved in the matching criteria will be included as covariates in secondary data analyses, to adjust for their potential effects on the outcome variables.

PROC MIXED of SAS will be invoked to perform all of the longitudinal data analyses. The fit of the segmented linear model will be assessed via graphs of the fitted responses versus the observed responses. If the fitted model does not match the observed data very well, as indicated by the graphs and Akaike's information criterion, then the model will be modified to a "means" model rather than a segmented linear function.

The comparison of interest within each genotype is to determine whether the response variable for the ICS/active LAB with PRN anticholinergic differs from that of the ICS/placebo LAB with PRN anticholinergic at the end of the 18-week treatment regimen periods. This difference within genotype i corresponds to the contrast

$$0.5(8\beta_{i14} + 6\beta_{i15} + 12\beta_{i16}) - 0.5(8\beta_{i24} + 6\beta_{i25} + 12\beta_{i26})$$

which removes period and sequence effects. The contrast that compares treatment regimen differences between the two genotypes is

$$\{0.5(8\beta_{114} + 6\beta_{115} + 12\beta_{116}) - 0.5(8\beta_{124} + 6\beta_{125} + 12\beta_{126})\} -$$
$$\{0.5(8\beta_{214} + 6\beta_{215} + 12\beta_{216}) - 0.5(8\beta_{224} + 6\beta_{225} + 12\beta_{226})\}$$

The statistical approach is to estimate the β coefficients from the longitudinal data analysis and determine whether the estimated contrasts, relative to their standard errors, are significantly different from zero.

In the balanced and complete mixed-effects linear model, there are no missing data and subject visits are always exactly as scheduled. In reality, there are missed and/or mistimed subject visits and dropouts in nearly every clinical trial, yielding unbalanced and incomplete longitudinal data. Not only could valuable information be lost if the data from subjects who drop out or miss visits are not included in the analysis, but conclusions based on the statistical analysis could be biased. Therefore, we plan to

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reduce the bias as much as possible by incorporating all available data on randomized patients in the statistical analysis, regardless of whether a patient has missed some visits, dropped out, or been non-compliant. This is known as the "intent-to-treat" analysis. Subjects will continue to be followed throughout the trial as much as possible and their data will be included in the "intent-to-treat" analysis. A source of bias we will investigate is whether the occurrences of missing values is different for the sequences and/or genotypes.

The occurrence of exacerbation will be analyzed via McNemar's test because the crossover design yields paired binary data, i.e., presence/absence of exacerbation for active LAB and placebo LAB within each patient. A more sophisticated analysis for paired binary data that will be considered is generalized estimating equations (GEE), which can account for clinical center, other prognostic factors, and the pairing of patients from the two genotypes. If there is an adequate number of exacerbations (say, 10% of the sample), then time-to-occurrence will be analyzed via survival analysis methods. A special method is available to account for paired time-to-event outcomes (Huster et al., 1989).

The actual rates of exacerbation for the two treatment regimens is expected to be less than 10%, however, based on data from previous ACRN trials with ICS. For this reason, inclusion of post-exacerbation data will have a negligible impact on the intent-to-treat analysis of primary and secondary outcomes variables. Nevertheless, a sensitivity analysis that excludes post-exacerbation data will be performed.

F. Sample Size

80 randomized subjects (40 within each genotype) are required for this trial. Each of the seven clinical centers will randomize approximately 12 subjects to achieve this goal.

The justification for this sample size is as follows. The standard deviation of the estimated contrast of interest with respect to AM PEF from the BARGE trial is 24 L/min. To detect a difference of 25 L/min between Arg/Arg and Gly/Gly with a two-sided, 0.05 significance level test with 90% statistical power and accounting for a 15% drop-out rate, then 24 subjects per genotype are required. However, to attain 90% statistical power for the secondary outcome variable of spirometry FEV_1, a sample size of 40 subjects per genotype is required because the effect size is 0.15 L and the standard deviation is 0.19 L. As a result of the sample size of 40 subjects per genotype, the actual effect size for detecting between-genotype differences with respect to AM PEF is 15 L/min.

For a sample size of 40 subjects per genotype, the effect sizes for detecting treatment regimen differences within each genotype are 13.3 L/min for AM PEF and 0.13 L for spirometry FEV_1.

Therefore, the LARGE trial will have a target of 80 randomized subjects with the following treatment sequences*:*

Arg/Arg   Gly/Gly
A $\Rightarrow$ B   (n=20)   A $\Rightarrow$ B (n=20)
B $\Rightarrow$ A   (n=20)   B $\Rightarrow$ A (n=20)

G. Screening required to achieve sample size

We propose to compare individuals with the B16-Arg/Arg genotype (which occurs with a frequency of about 16%) to those with the B16-Gly/Gly genotype (which occurs with a frequency of 35%). Thus, to recruit 40 subjects in each of the genotypic categories, we will need to screen a minimum of 240 subjects. Allowing for exclusions, refusals to participate, stratification by FEV_1, and withdrawals, we may need to screen 450 individuals.

H. Haplotype analysis

In an exploratory manner, we plan to assess whether combinations of alleles aligned on the same chromosome (haplotypes) at different positions along the β_2-adrenergic receptor gene and its 5' leader and 3' terminal sequences will be associated with differential effects on asthma control, as defined by AM PEF and other outcomes, in the setting of regularly scheduled administration of an inhaled long-acting beta-agonist. Drysdale et al. (Drysdale et al., 2000) reported haplotype analysis of 13 SNPs in B2AR. Three common (>5%) haplotypes were identified in white subjects. An additional 12 SNP's have been detected and several are in the discovery phase. We plan to type for all available SNPs at the time we undertake the haplotyping. For example, working off the SNPs reported by Drysdale, although only 2 SNPs would be required to distinguish these common haplotypes from each other, we plan to analyze additional SNPs to distinguish the less common haplotypes from the 3 common haplotypes. To distinguish these 3 common haplotypes from the 9 other reported haplotypes, we will select 8 B2AR SNPs for genotyping: –709, –654, –47, +46, +79, +252, +491, and +523. This set of SNPs included 2 promoter SNPs (–709 and –654), a nonsynonymous SNP in the β upstream peptide (–47), 3 nonsynonymous SNPs within the B2AR coding region (+46 [Arg/Gly 16], +79 [Gln/Glu 27], and +491 [Thr/Ile 164]), and 2 synonymous SNPs within the B2AR coding region (+252 and +523).

With regard to the pharmacogenetic analyses related to these beta-adrenergic receptor haplotypes, we will use a program called PHASE (Stevens et al., 2001) to impute haplotypes. For statistical analysis of haplotypes, we will use CLUMP, which performs a permutation-based Chi square exact test and Monte-Carlo test for associations between disease and alleles at highly polymorphic loci (Sham and Curtis, 1995). This allows for the estimation of a global P value and the creation of contingency tables for estimation of haplotype specific odds ratios (OR). Finally, for covariate-adjusted analysis of continuous or categorical phenotypes with haplotype as the exposure we will use haplo.score (Schaid et al., 2002). Haplo.score output provides a scored estimation of both gene wide and haplotype specific effects. We have employed a modified version that allows for missing data by utilizing two approaches: a) incorporation of imputed frequencies using the haplo.score algorithm to allow for maximal likelihood estimation of an individual's haplotype; and b) a similar approach utilizing Phase outputs for the frequency estimation.

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XIX. GENOTYPING METHODS

For a given sample of DNA, the genotypes are assigned based on two methods: the Amplification Refractory Mutation System (ARMS) and Restriction Fragment Method (RFM). We will assign a genotype for each subject after both techniques are performed and there is concordance between the two methods. If we fail to get agreement (about 7% of allele calls), we will re-genotype the DNA using both techniques. If there was a technical or bookkeeping error, it will be corrected at this time. If the genotypes fail to agree on the second attempt, the genomic DNA will be cloned into a TA vector (Invitrogen) and 10 colonies will be directly sequenced using standard techniques to help determine the source of the error and the true genotype. If we are unable to assign the genotype at this time, we will extract new DNA from the subject's stored blood samples and repeat the above process. This procedure facilitates obtaining an accurate genotype assignment. Random sampling and re-genotyping of 10 subjects in the BARGE study showed 100% concordance with the original genotypes. The genotyping procedures are now described in detail.

(i) Genotyping by ARMS:

The sequence specific primers used to determine the A to G change at nucleotide 1633 (Genbank accession no. M15169) corresponding to the amino acid change at position 16, Arg to Gly (R to G) are:

Wild-type specific forward primer A1 (5 - GCCTTCTTGC TGGCACCCAA AA-3) corresponding to nucleotides 1612-1633, except that the penultimate base at the 3 end was changed from T to A.

Mutation specific forward primer A2 (5 - GCCTTCTTGC TGGCACCCAA AG-3), differs from the wild-type primer at the last nucleotide at the 3 end.

Common reverse primer Rev2 (5 - AGGATAACCT CATCCGTAAG G-3) corresponding to nucleotides 2483-2503 on the complementary strand.

Amplification by PCR of the genomic DNA of each sample includes two reactions for each allele assay separately: one for mutation detection at nucleotide 1633 with primers A1 and Rev2 for wild type, and the other with primers A2 and Rev2 for mutation type. Each PCR reaction contains: 5 μl template genomic DNA (250 ng), 2.5 μl PCR buffer (Boehringer Mannheim), 1.5 mM MgCl$_2$, 12.5 pmoles of forward and reverse primer, 20 mM dNTPs (Pharmacia), 1.5 units of Taq polymerase (Boehringer Mannheim), 0.05 units of Perfect Match, PCR enhancer (Stratagene) in a total volume of 25 μl. Conditions for PCR are: an initial hot start period of 5 min at 94°C; with temperature held at 80°C after the hot start, the Taq polymerase is added. This is followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C, with a final extension time of 5 min at 72°C. Thin-walled 96 micro-well plates (Costar) are used with mineral oil for amplification reactions in a PTC-100 thermal cycler (MJ Research). After

amplification, about 20 µl of reaction mixture are resolved by electrophoresis on a 2.0 % agarose gel and stained with ethidium bromide for analysis.

(ii) Genotyping by RFM:

Genotyping at BAR-16 will be carried out according to the method described by Martinez et al.[10] The BAR-16 containing region will be amplified using the primers: 5'-GCCTTCTTGCTGGCACCCCAT-3' and 5'-CAGACGCTCGAACTTGGCCATG-3'. The underlined bases were modified from the archival sequence to create a *Nco I* site. The reverse primer contains a *Nco I* site, and thus PCR products from both the Arg and Gly alleles are digested. Since the PCR product only from the Gly-16 allele contains the *Nco I* site in the forward primer, it will be cut twice by this enzyme. PCR reactions will contain 100 ng of DNA, 2.5 µl PCR buffer with 1.5 mM $MgCl_2$, 20 mM dNTPs and 10 pmoles of each primer in a total volume of 25 µl of reaction mix. Conditions for PCR will be: 94^oC for 6 minutes, followed by 35 cycles of 30 seconds at 94^oC, 30 seconds at 62^oC and 30 seconds at 72^oC with a final extension time of 5 minutes at 72^oC. For restriction enzyme digestion, 10 µl of the PCR product will be digested with 2 U of *Nco I* and Buffer 4 (New England Biolabs) for 2 hrs at 37^oC. The PCR products will be resolved by electrophoresis on a 4% NuSieve agarose gel and stained with ethidium bromide. The uncut PCR product is 167-bp in length. The enzyme digests the PCR product to produce a 145-bp fragment from the16-Arg allele and a 127-bp fragment from the 16-Gly allele; these differences allow allele assignments to be made.

(iii) Verification:

We have verified our methods by direct sequencing of TA clones derived from the PCR products. This was required because of unequal amplification of the mutant and wild type alleles. Moreover, to be sure of allele assignments during the trial, Dr. Eugene Bleecker will run about 10% of the samples to check our assignments as was done for the BARGE study. Samples sent to Dr. Bleecker for verification will be chosen, at random, from those that the ACRN identifies as B16-Arg/Arg or B16-Gly/Gly belonging to subjects who are matched and considered eligible to proceed with study enrollment at Visit 1.

Once an allele assignment is made, the files will be sent to the DCC which will then inform the clinical centers which of the subjects qualify for further screening. As stated in the protocol, to maintain the blind, the center is not informed of the specific genotype of the subject, only if the subject is genotype eligible or ineligible. Most of the staff at the DCC will remain blinded to genotype designation, as well. Only the database programmer and two data entry clerks who have no involvement in day-to-day activities of the LARGE trial will have access to the actual genotype results.

XX. EQUIPMENT

Manuals of OPeration (MOPs) have been developed and have been in use for performance of all ACRN procedures (spirometry, methacholine challenge, NO collection, etc), including ACRN equipment calibration.

Equipment to be used in the LARGE trial includes the following:
(1)   Skin Testing: the Multi-Test II provided by Lincoln Diagnostics, Inc. The Mulit-Test II device is a sterile, disposable, multiple test applicator used to administer skin-test substances. This device meets OSHA guidelines for technician protection, and it provides a lower coefficient of variation than similar devices and than a bifurcated small pox needle.
(2)   Exhaled breath condensate: equipment from Respiratory Research, Inc.
(3)   Exhaled nitric oxide: NIOX machine provided by Aerocrine, Inc.
(4)   Spirometry: equipment will be provided by QUANTUM Research, Inc. The spirometry equipment is customized for ACRN.
(5)   Peak-flow meter: AM1 device by Viasys. The AM1 device will provide daily measurements of peak flow and also provides compliance checks.

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