Allergen Inhalation Challenge, Refractoriness, and the Effects of Ibuprofen

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Department of Physiology and Pharmacology, University of Saskatchewan, Saskatoon

By

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ABSTRACT

Background: Bronchoprovocation challenges use direct and indirect acting stimuli to induce airflow obstruction. Indirect stimuli, either non-allergic/non-IgE mediated (e.g. exercise, mannitol) or allergic/IgE mediated (i.e. allergen inhalation model), trigger mast cells to release bronchoconstricting mediators (e.g. cysteinyl leukotrienes, histamine). Performing repeat challenges within a short timeframe (e.g. 3 hours) with non-allergic indirect stimuli results in a diminished refractory response to the second challenge. Cross refractoriness occurs between indirect stimuli. It follows that repeat bronchoprovocation with allergen might exhibit refractoriness that might be altered by ibuprofen. This study was designed to assess the response to a second allergen challenge performed 24 hours after an initial one to determine if the response is refractory. If refractoriness developed, the study aimed to determine whether a single dose of ibuprofen would alter the refractory response to the second allergen challenge. The study design also allowed for the assessment of the effect of ibuprofen on allergen challenge outcomes, including indices of airway inflammation.

Methods: Thirteen mild atopic asthmatics were enrolled in a randomized, double-blind, placebo controlled, cross-over study. Ibuprofen (400mg) or placebo was administered one hour prior to the first of two allergen challenges performed 24 hours apart. Blood and sputum eosinophils, airway responsiveness to methacholine, and levels of fractional exhaled nitric oxide were assessed before and seven hours after each allergen challenge. All data were log transformed, and differences in geometric means were analyzed by paired t-tests.

Results: After placebo, early asthmatic responses for the two challenges were not significantly different ($p = 0.82$). A single 400 mg dose of ibuprofen decreased both the early ($p = 0.03$; $n = 12$) and late asthmatic responses ($p = 0.03$; $n = 3$)

Conclusion: Allergen challenges conducted 24 hours apart do not exhibit refractoriness. A single dose of ibuprofen inhibits early and late asthmatic responses to allergen bronchoprovocation. Ibuprofen should be withheld for at least 24 hours prior to investigations utilizing allergen bronchoprovocation.

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List of Abbreviations

AC – allergen challenge

ACh - acetylcholine

AHR – airway hyper-responsiveness

AMP - adenosine 5'-monophosphate

APC – antigen presenting cells

AUC – area under the curve

BPT(s) – bronchoprovocation test(s)

cAMP – cyclic adenosine monophosphate

CBC – complete blood count

COPD – chronic obstructive pulmonary disease

COX - cyclooxygenase

EAR – early asthmatic response

EIB – exercise induced bronchoconstriction

FcεRI – high affinity IgE receptor

FDA – Food and Drug Administration

FeNO – fractional exhaled nitric oxide

FEV1 – forced expiratory volume in 1 second

FVC – forced vital capacity

G protein – guanine nucleotide binding protein

HDM – house dust mite

IgE – immunoglobulin isotype E

IL - interleukin

LAR – late asthmatic response

LT – leukotriene

M – muscarinic receptor

MCh - methacholine

MHC - major histocompatibility complex

OVA – ovalbumin

PC7 – provocative concentration causing a decrease in FEV1 of 7%

PC20 – provocative concentration causing a decrease in FEV1 of 20%

PD20 – provocative dose causing a decrease in FEV1 of 20%

PFT – pulmonary function test

PG - prostaglandin

ROS – reactive oxygen species

TH1 – T helper cell subtype 1 (involved in the innate immunological response)

Th2 – T helper cell subtype 2 (involved in the adaptive immunological response)

Tmax - the time after administration of a drug when the maximum plasma concentration is reached

TNF – tumor necrosis factor

TSLP – thymic stromal lymphopoietin

TX - thromboxane

CHAPTER 1 – GENERAL INTRODUCTION

1.1) Brief Overview of Asthma

Asthma is an obstructive lung disease which is characterized by its reversibility. Symptoms include shortness of breath, cough, wheezing, and chest tightness. According to the World Health Organization it is the most common chronic disease in children and affects approximately 235 million individuals worldwide. There is a higher prevalence in less developed countries, as well as a greater death rate from acute exacerbations (1). In 2010 it was estimated that 8.5% of individuals aged 12 and older in Canada were diagnosed with asthma by a medical professional, and approximately 250 people succumb to this condition every year (2, 3).

Classically asthma has been stratified into two groups; non-allergic/intrinsic and allergic/extrinsic/atopic asthma. Although both types share similar pathophysiologic characteristics, they have different triggers and responses to medications. Intrinsic asthma is rare, more treatment refractive, and does not have known environmental inducers unlike its counterpart. Common environmental triggers associated with atopic asthma include animals, cold, seasonal allergens (dust, pollen), and molds (4). It is important to note that the classification of different subtypes of asthma is evolving, especially as researchers are learning more about the underlying mechanisms of the disease.

1.2) Pathophysiology

Bronchial narrowing predominantly occurs by 3 different mechanisms; smooth muscle bronchoconstriction, inflammation, and mucus hyper-secretion. A pictorial depiction of a normal and a constricted airway is shown in Figure 1.1.

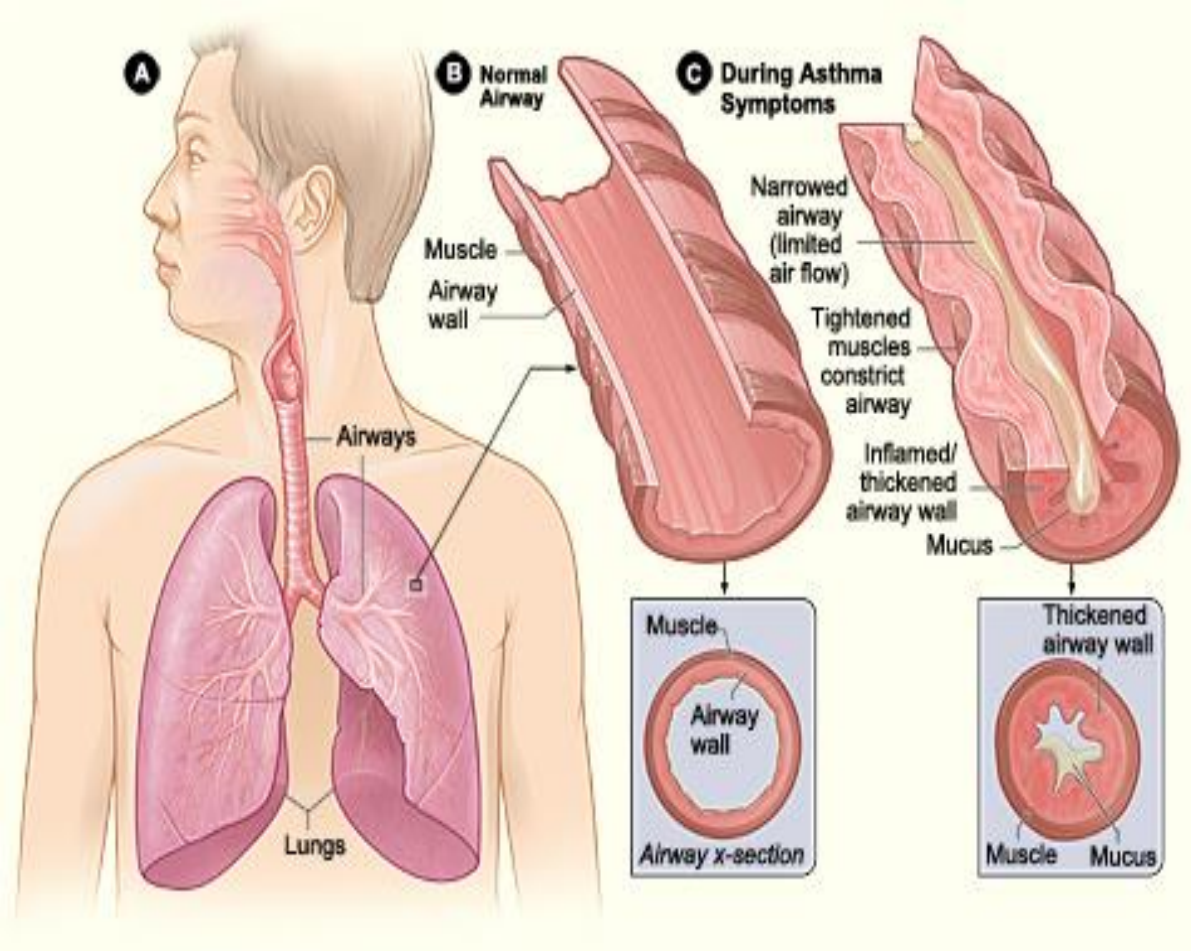

Figure 1.1: Diagram of a healthy and asthmatic bronchiole. (Permission for this figure was not needed as it came from an open source. Retrieved from http://www.nhlbi.nih.gov/health/health-topics/topics/asthma).

1.2.1) Bronchoconstriction

Contraction of human bronchial smooth muscle predominantly occurs through parasympathetic innervation of the muscarinic (subtypes M2 and M3) receptors which are bound by acetylcholine (ACh). Although M2 receptors outnumber M3, it is thought that the latter plays a greater role in constriction, while the M2 primarily helps inhibit smooth muscle relaxation. Once ACh binds to the M3 receptors they in turn couple with Gq protein and activate phospholipase C, resulting in an increase in intracellular calcium, thus leading to a tightening of the airway smooth muscles. The function of the M2 receptor, which binds to Gi, is thought to counteract the effects of adrenergic beta-receptor mediated relaxation by inhibiting the generation of cyclic adenosine monophosphate (cAMP) (5, 6). The result is an increase in parasympathetic tone.

Individuals who suffer from asthma generally have changes in their neuronal control which leads to increased constriction in response to vagal stimulation (7). Evidence of this increased sensitivity and the importance of these muscarinic receptors arises from gene knock-out mice studies, where dual elimination of M2 and M3 led to an almost complete loss of contractility (8). In addition, T helper subtype 2 cells (Th2) can lead to vagal stimulation resulting in increased ACh release and binding on muscarinic receptors on smooth muscle cells resulting in lumen narrowing (9). Finally, the fact that the asthmatic lung is more sensitive to ACh analogs such as methacholine is further evidence of the importance of muscarinic receptors located on the airway lumen causing bronchoconstriction.

1.2.2) Airway Inflammation

The inflammatory process is quite complex and involves the interaction between numerous cell lineages and mediators. Current asthma therapeutic research has focused on finding targets in the inflammatory pathway to curb airway hyper-responsiveness due to inflammation. Progress in molecular and gene expression techniques have allowed investigators to uncover numerous "players" in this process.

Although the precise role of the various cellular players is still uncertain (due to the multitude and complexity of their interactions with others), investigators have been able to determine which ones are predominant, and have been able to gain an appreciation of their functions. Mast cells are both directly and indirectly responsible for a great deal of the pathology involved with asthma. These cells reside at the external interface between the bronchiole lining, environment near blood vessels, and nerve endings. Mast cells contain preformed mediators including histamine, proteases, numerous interleukins including IL-4, 5, 13, tumor necrosis factor (TNF)-alpha, and lipid derived mediators including prostanoids and leukotrienes. Activation of mast cells predominantly occurs by the binding of allergen to IgE which results in the crosslinking of these antibodies. IgE is attached to the FcεRI on the mast cell surface. The result is direct bronchiole smooth muscle contraction (via degranulation and mediator production), increased vascular permeability, and recruitment of other inflammatory mediators. In addition, proteases released from mast cells are involved in lung remodeling associated with chronic disease (10).

Eosinophils, basophils, and neutrophils are also contributors to airway inflammation. All of these cell types are produced in the bone marrow, contain granulocytes, are recruited to lung tissue, and are involved in the inflammatory pathway. Eosinophils further contribute to airway inflammation by releasing major basic protein, reactive oxygen species (ROS), cytokines, enzymes, and lipid mediators (11). The role of basophils is somewhat unclear in asthma, however, it is thought that allergen also crosslinks IgE bound to FcεRI receptors on the basophil surface causing degranulation of mediators including histamine, proteases, and liposomal enzymes. This cell line comprises less than 1% of peripheral blood and is greatly outnumbered by eosinophils (12). Although not predominant in many individuals who suffer from asthma, neutrophils can also play a role in the inflammatory response by mediator release (13)

Dendritic cells are a specialized type of macrophage which act as an antigen presenting cell (APC). They line the epithelium of the respiratory tract. Dendritic cells take up antigens and process them into peptides, and then present them to naïve T-cells, resulting in these T-cells differentiating and maturing. These APCs are essential in both the priming and development of the allergic response, as they are responsible for introduction of the irritant (14).

T and B lymphocytes also play important roles in the inflammatory response. Naïve T-cells get programmed to predominantly follow a Th2 response, although there is some evidence to show that the Th1 cellular immunity arm may also play a minor role (15). B-lymphocytes are important for leading to the production of IgE antibodies (11).

Important interleukins (IL's) involved in airway inflammation include 4, 5, 13, and thymic stromal lymphopoietin (TSLP). IL-4 is a significant pro-inflammatory mediator in asthmatics

which has been heavily examined in the literature. Its functions include; immunoglobulin isotype switching leading to the production of IgE, maturation of naïve helper T-cells (Th0 to Th2 lymphocytes), mucus secretion, eosinophil transmigration, expression of vascular cell adhesion molecule 1, and activation of cells to up-regulate the release of other cytokines, including itself. Animal studies have shown that when IL-4 is neutralized in mice, either pharmacologically or in gene knock out models, there is a decrease in airway eosinophilia and bronchial reactivity. In addition, humans who suffer from this disease had higher concentrations of this cytokine in both their peripheral blood and bronchoalveolar lavages compared to controls (16).

The sole function of IL-5 is for growth, maturation, activation, and release of eosinophils, and to a lesser degree basophils, from myeloid precursors in the bone marrow. These two granulocytes are the only cells in humans which contain receptors for IL-5 (17). IL-13 is produced by helper CD4+, CD8+ cells, natural killer cells, and to a lesser degree by mast cells, basophils, and eosinophils. IL-13 has very similar effects as IL-4 as it can lead to the production and release of hematopoietic stem cells, as well as numerous other mediators (18). This cytokine has also been related to an increase in airway hyper-responsiveness (AHR), susceptibility to infection, glucocorticoid resistance, goblet cell hyperplasia, and mucus hyper-secretion (19).

TSLP is a cytokine released from respiratory epithelial and stromal cells in response to allergen exposure. This cytokine functions to cause dendritic cells to upregulate major histocompatibility complex (MHC) II molecules, as well as release chemo-attractants to recruit other inflammatory cells, including eosinophils, neutrophils, and Th2 cells. Dendritic cells which

are activated by TSLP drive Th2 differentiation by expressing large amounts of OX40L, which interacts with OX40 on T cells (20).

Atopic asthma resulting in a Th2 response is the most common mechanism leading to airway inflammation. A general overview of the process is shown in Figure 1.2.

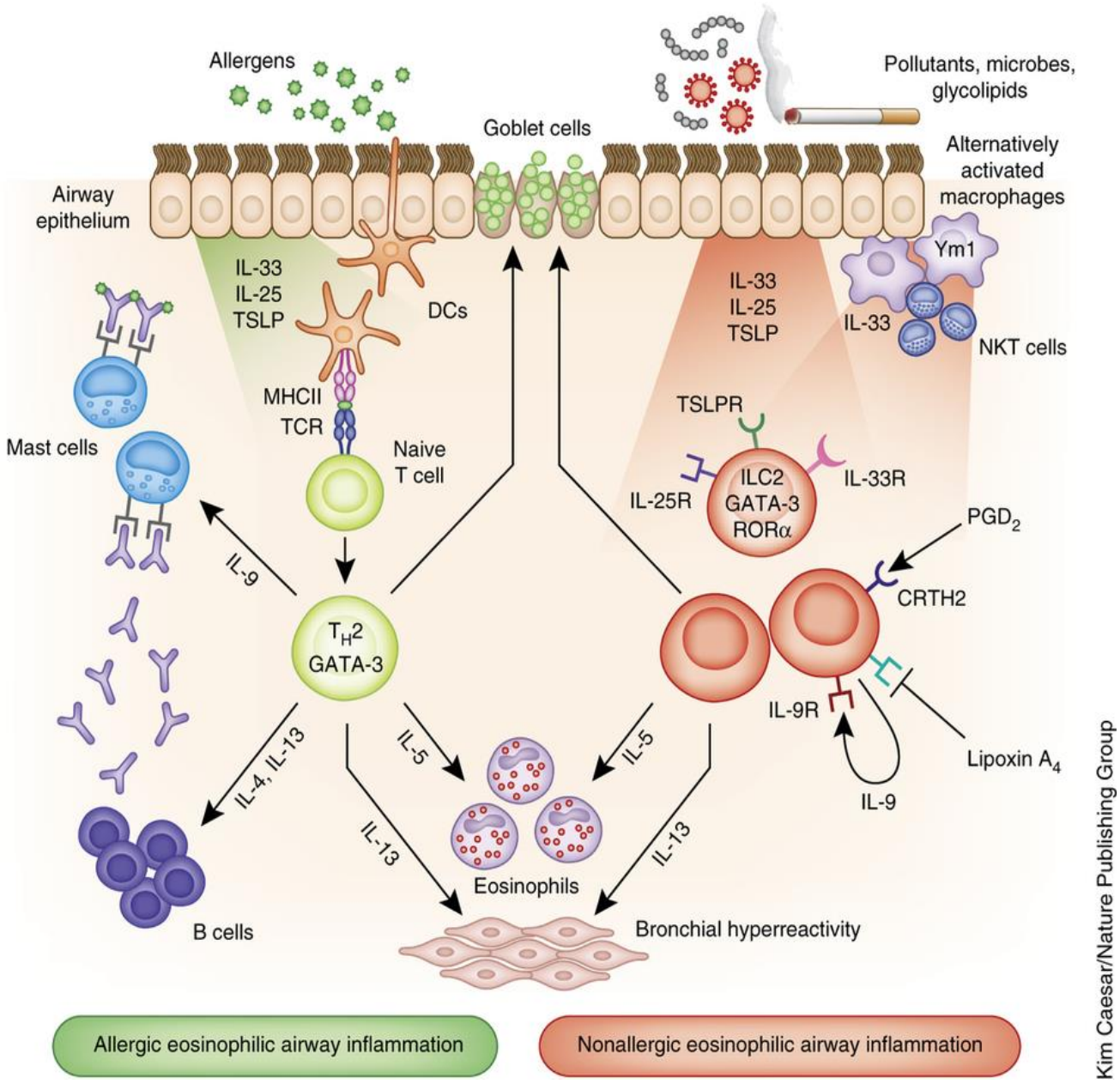

Figure 1.2: Airway Inflammation. Depiction of the Th2 reaction which causes airway inflammation. Reproduced with permission from "Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol. 2015 Jan; 16(1):45-56".

After an initial sensitization to a foreign compound, repeat exposure to this agent results in it being taken up by dendritic cells which process it into peptides that bind to naïve T-cells. The naïve T cell then differentiates to mature Th2 cells. In addition to this mechanism, allergen exposure to epithelial cells results in the release of TSLP which activate dendritic cells to produce chemoattractants for mature Th2 cells. With the aid of interleukins Th2 cells differentiate plasma cells which release IgE, cause mast and eosinophil cell production, recruitment from the bone marrow to the lungs, and degranulation, which results in bronchiole constriction (15, 21, 22, 23).

1.2.3) Mucus Hypersecretion

Often overlooked for its contribution to bronchoconstriction, mucus hypersecretion plays a significant role in the pathology of airway narrowing. In the lungs mucus is produced and released from goblet cells which are interspersed throughout the airway epithelium, and act to protect the lungs from foreign substances. Asthmatic patients tend to have goblet cell hyperplasia which results in increased mucus production (Figure 1.3), as well as increased mucin protein constituents which cause it to be more viscous (24). Mucus clearance also tends to be slower in certain asthmatics who have a predisposition for increased airway mucus production leading researchers to advocate that it should constitute a specific phenotype of this disease (25).

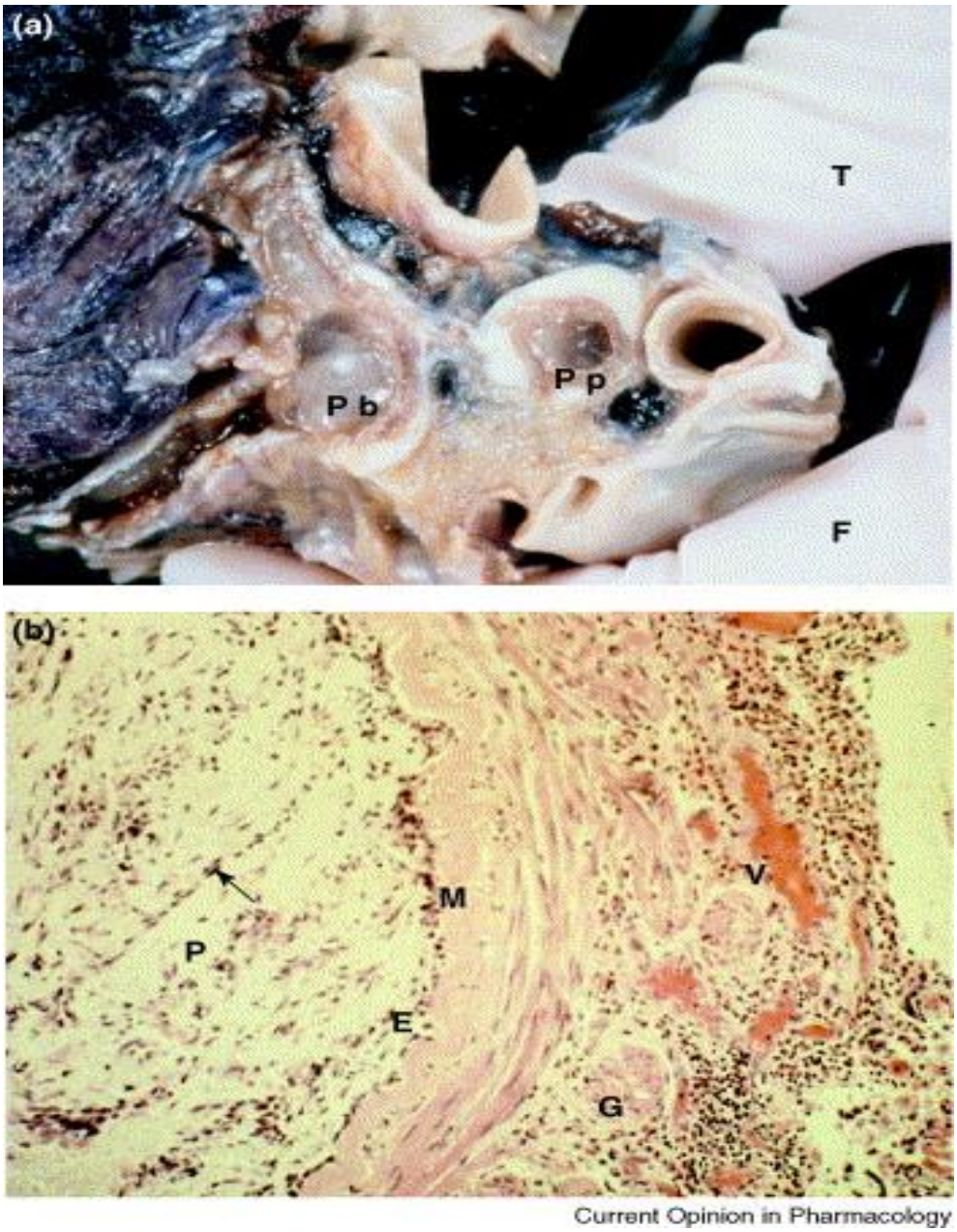

Figure 1.3: Mucus Hypersecretion. The top figure shows a gross bronchiole with a mucus plug, while the bottom shows a histological representation of mucus filling. a) Gross pathology; T – thumb, F – finger, Pb – gelatinous plug, Pp – large airway. b) Histology; P – mucus plug with infiltrated inflammatory cells, E – damaged epithelium, G – submucosal gland, M – thickened reticular basement membrane, V- blood vessel.

Reproduced with permission from “Rogers DF. Airway mucus hypersecretion in asthma: an undervalued pathology? Curr Opin Pharmacol. 2004 Jun; 4(3):241-50”.

1.3) Early and Late Asthmatic Response (EAR and LAR)

The asthmatic response varies between individuals, but three general types have been seen among those who suffer from this condition. The first is the early asthmatic response, where a maximal response occurs 15 -30 minutes after exposure, and tends to completely resolve after 2-3 hours. Experimentally it is characterized by a drop in forced expiratory volume (FEV1) of 20% which resolves within these time points. The early asthmatic response is considered to be a type I hypersensitivity reaction and occurs as a result of allergen binding IgE on mast cells, thereby triggering mediator release (26, 27).

In addition, there are also the dual responders who have both an early and late asthmatic response (Figure 1.4). The late asthmatic response is characterized by bronchoconstriction 3 - 8 hours after exposure to the allergen (and is also IgE dependent). In those who are dual responders there is a plateau period of normal (or close too) FEV1 between the EAR and LAR. The mechanism underlying the late asthmatic response is still not clearly understood, however, it is thought that Th2 immune cells and mediator driven. Early and dual responders make up the majority of allergic asthmatics, although, isolated late responders have been documented (27, 28, 29).

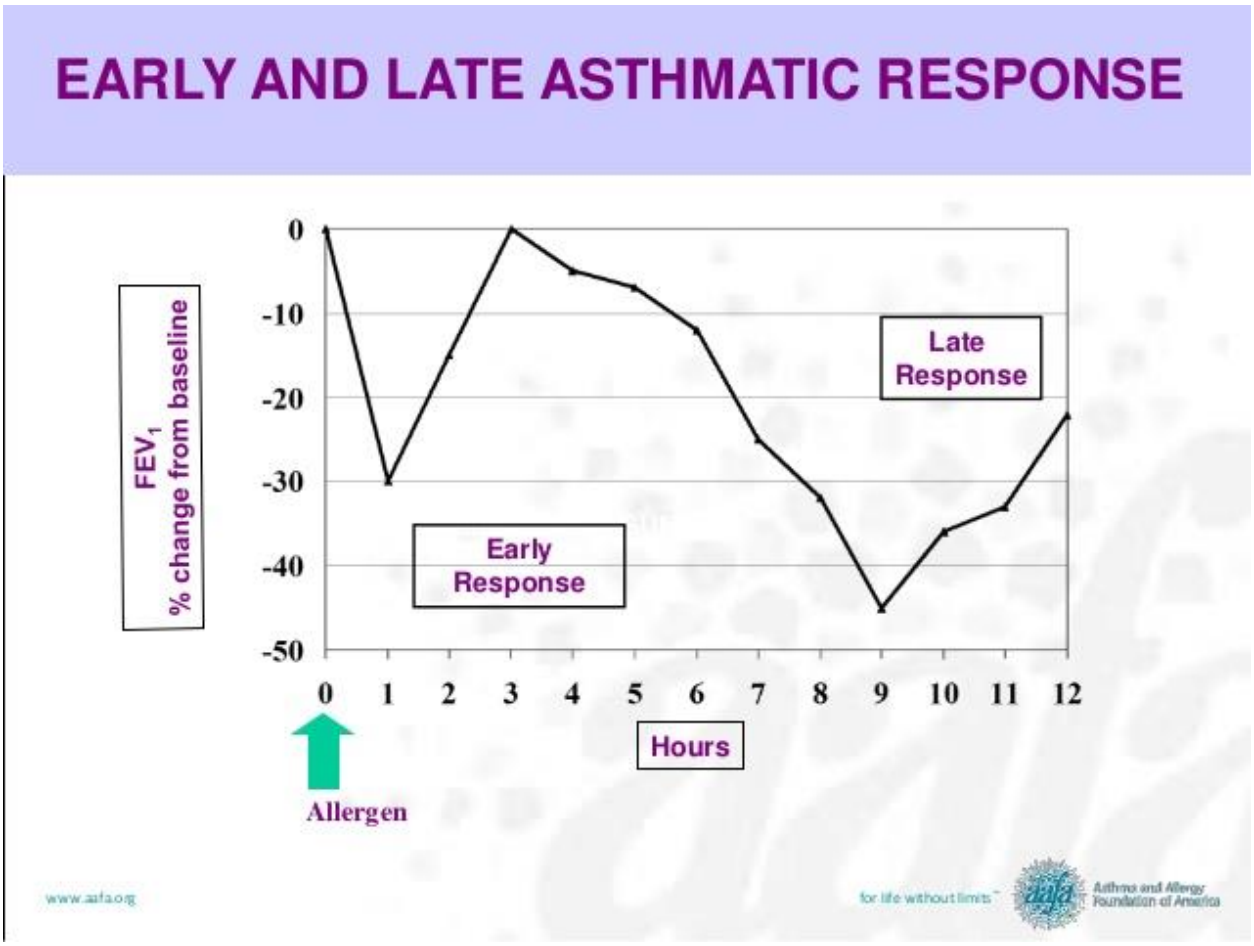

Figure 1.4 – Time course of early and late asthmatic response. Diagram showing FEV1 vs time graph which depicts the early and late response. (Permission for this figure was not needed as it came from an open source. Retrieved from http://www.aafa.org).

1.4) Bronchoprovocation Tests (BPTs)

Direct and indirect challenges are mostly used for research purposes, however, they also have clinical value as they can help a physician diagnosis asthma. The two direct challenges use the pharmacological agent's methacholine (MCh) and histamine. These compounds are labeled as such because they cause airway narrowing by 'directly' binding to receptors on airway smooth muscle, and causing bronchoconstriction. Currently MCh is used most often (30).

The MCh challenge is considered positive when there is a 20% reduction in FEV1 after inhaling 16 mg/ml or less of methacholine. It is referred to as the provocative concentration causing a fall in FEV1 of 20% (PC20), and is calculated by the following equation:

PC20 = antilog [logC1 + (log C2 – logC1) x (20 – R1)] / (R2-R1)];

where C1 refers to the 2nd to last concentration given, C2 is last concentration given, R1 equals percent fall in FEV1 after C1 and R2 is % fall in FEV1 after C2. This equation is used because rarely will an individual fall exactly 20% after inhaling a concentration (31).

However, if after inhaling one single concentration the FEV1 is close to or greater than 20% than it can be calculated by a single point formula, which is as follows:

(20 / % fall in FEV1) x [MCh concentration] (32).

During testing an individual inhales vaporized MCh at doubling concentrations (usually starting at a low dose of about 0.25 or 0.5 mg/ml if prior responsiveness is unknown) for two minutes, then the individual blows into the spirometry machine at 30 and 90 seconds. The drop in FEV1 is calculated by the difference in the lower of these two recorded exhalations and the lower post diluent FEV1 divided by the lower post diluent FEV1. This challenge is continued until FEV_1

declines by ≥ 20% or until a concentration of 16 mg/ml is inhaled. If a fall of 20% is not seen after this concentration then the test is deemed negative. It should be noted that there are other protocols for how to administer this test, which is usually country dependent, but this is the most common way in North America. A histamine challenge would be performed in a similar fashion (33).

The MCh challenge has a high sensitivity and thus high negative predicative value, so a negative challenge is useful in ruling out asthma. Other advantages of MCh challenge testing is that it has been widely approved for use in humans and proven to be quite safe. Drawbacks include; it fails to identify those who have exercise induced bronchoconstriction (EIB), baseline FEV1 must be close to normal to have an accurate test, and it has a low specificity (not a good rule in test). In general, the MCh challenge has been found to be quite useful, and provides valuable information not only in the research setting but also as a tool to help a clinician make the proper diagnosis (32 - 34).

Indirect challenges include physical and chemical stimuli which induce airflow obstruction by causing the release of a variety of mediators from inflammatory cells (e.g. mast cells). There are numerous different indirect bronchoprovocation tests, including exercise, adenosine monophosphate (AMP), dry air, hypertonic saline, and mannitol. They can act by increasing the osmolarity of the airway surface liquid which results in the release of mediators from inflammatory cells as well as neuropeptides from sensory nerves. Similar to direct challenges, increasing concentrations of indirect stimuli are administered and responses are measured by spirometry changes (35, 36).

The main advantage of indirect challenges is that they have a higher specificity and positive predictive value than direct challenges, and are therefore better at "ruling in" asthma. Other benefits include; less equipment is required to identify EIB, the response correlates with eosinophilia (i.e. correlates with airway inflammation), and hypertonic saline as an indirect stimuli can also be used to induce sputum for analysis of airway inflammation. Currently they are not routinely used as diagnostic tests in North America, although they are gaining traction in other areas of the world, such as Europe. The main drawbacks are cost, low sensitivity, can be uncomfortable at high doses, and lack of approval by the Food and Drug Association (FDA) for use in North America (34, 35, 36).

The allergen challenge model is considered to have originated in the early 20th century, and is a commonly used tool to assess the pathophysiology of asthma in the laboratory setting, as well as test novel therapeutics. By definition, allergen acts as an indirect stimuli, however, the response to allergen has properties which are unique to itself, such as being an IgE mediated driven process. This test is considered to be extremely efficient at assessing the LAR, which is important for pharmaceutical trials.

The allergen challenge is conducted similarly to the methacholine challenge described above (i.e. an individual inhales doubling concentrations for two minutes each until there is a ≥20% fall in FEV1). Then the test subject is generally followed for 7-8 hours over the course of the day to assess their lung function as well as any other parameters one may wish to investigate. Advantages of this method are that it mimics what happens in nature, it can be reproduced within the confines of the laboratory setting, it has been shown to be safe, and the methods have been standardized (37, 38).

1.5) Refractoriness and Increased Airway Hyper-Responsiveness (AHR) with Indirect Challenges

An interesting phenomenon seen when indirect challenges have been performed within a relatively short time frame (1-3 hours) is refractoriness. Refractoriness refers to a decreased airway response when exposed to a second challenge. This has been shown for EIB, AMP, sodium metabisulfite, and mannitol among others (39- 42). In addition, experiments have shown cross-refractoriness when indirect challenges are performed consecutively with two different indirect acting stimuli (43). A study conducted in our laboratory showed that 24 hours after an allergen challenge there was a diminished response to mannitol (44). The cross-refractory/refractory phenomenon has led researchers to not only hypothesize that there is a shared mechanism between the challenges, but also that the body is producing something to protect itself from the subsequent insult. One of the proposed theories is that protective (anti-inflammatory) prostaglandins are synthesized, released, and protect the airways (45).

Experiments have been conducted where repeated allergen challenges have been done on individuals both receiving low and high doses of allergen. The low dose study was done to mimic what an individual would be exposed too in the natural environment, and investigators discovered this to be safe, and cited that lung functions were equal through-out (46). Grainge and Howarth found that when repeated high dose challenge was administered 48 hours apart there was no significant change in pulmonary lung function (47). Another high dose 24 hour study revealed diminished FEV1's and worse symptomology with subsequent allergen challenges. However, this study may have had confounded results as it was assessing the use of an autologous *E. coli* auto-vaccine for treatment of HDM(48). As is evident by these experiments there is still a gap in knowledge about the effects of performing repeated allergen

challenges within a short time frame and whether allergen, as an indirect acting stimuli, exhibits a refractory state.

Increased AHR has been shown in numerous studies when performing a MCh challenge after an indirect or allergen challenge test. This is exemplified by having an increased response to the same amount of MCh or by having a ≥20% reduction in FEV1 at a lower dose of MCh (49, 50). It is thought that the airways get primed during the initial exposure which results in an increased sensitivity to the effect of the direct acting stimuli, such as MCh. This finding has mechanistic significance which could relate to refractoriness, as it represents the other end of the spectrum.

1.6) Hypotheses and Rationale

There were three hypotheses which we set out to test in this experiment, which included:

1) That a refractory period will occur when performing allergen challenges 24 hours apart. Indirect challenges have shown a decreased response when done within a short time frame, and we wanted to see if this would occur with allergen challenges. The few studies which have been published have shown performing allergen challenges within 24 hours or more is safe, so we were confident it would not be harmful to individuals. The importance of assessing whether refractoriness is present is because it could provide insight into the underlying pathophysiology of airway inflammation. In addition, the allergen challenge model is commonly used in studying novel therapeutics, so by proving it can be safely performed within close time points could alter the way these trials are designed. This will be tested by conducting allergen challenges 24 hours apart.

2) Ibuprofen will inhibit the refractory response (if one does exist) to allergen challenges performed 24 hours apart. One hypothesis holds that indirect challenges have a refractory period because the body is producing a greater amount of protective mediators, such as the prostaglandins E2 and I2. Evolutionarily this makes sense as our bodies would try to adapt in order to curb the deleterious effects of environmental agents. Hypothetically, ibuprofen should non-selectively reduce the amount of all circulating prostaglandins (PG's). If the protective PG's are overexpressed and ibuprofen inhibits their production, as well as the production of pro-inflammatory PG's, it is conceivable that we could pharmacologically control the refractory period. This inhibition will be tested by comparing the placebo and treatment arms of this study.

3) Ingestion of 400 mg of ibuprofen taken 1 hour prior to allergen inhalation challenge will alter the airway response (either better or worsen) to allergen inhalation. NSAIDs, like ibuprofen, are commonly used medications, however, the effect of ibuprofen on airway responses to allergen in individuals with atopic asthma has not yet been fully documented. It is assumed to be safe as there are no contra-indications for taking this drug in those who suffer from reactive airway disease. Our primary interest was to test how this drug would affect lung function, as well as inflammatory cells and mediators. This will be tested in a randomized double blind placebo controlled cross-over fashion, where prior to one allergen challenge participants will swallow 400 mg of ibuprofen.

CHAPTER 2 – MATERIALS AND METHODS

2.1) Ethics Approval to Conduct Research in Human Participants

A Research Ethics Board Application (Appendix A) was submitted to the University of Saskatchewan Biomedical Research Ethics Board for review and approval. The Consent Form (Appendix B) and the REB Certificate of Approval (Appendix C) to conduct the study are also appended.

2.2) Participants

Individuals of either gender were eligible to participate in the study. Participants needed to have mild atopic asthma as defined by not requiring medication on a daily basis or corticosteroids, as well as not having a significant airway response or infection in past 4 weeks. The severity of a participant's condition was assessed during initial screening by obtaining baseline lung functions by spirometry, airway responsiveness to MCh, and a skin prick test. In those who participated in previous studies we confirmed the status of their airway hyper-responsiveness by repeating their baseline lung function and MCh challenge testing.

Additional inclusion criteria was as follows:

i) Being between the ages of 18 – 70 years old

ii) Be diagnosed with asthma by a medical professional

iii) Have a MCh PC20 <16mg/mL

iv) Have baseline FEV1 >70% on initial screening

v) Not require daily controller medications (e.g. inhaled or systemic corticosteroid or other anti-inflammatory treatment)

vi) Have a clinically relevant positive allergy skin test (i.e. formation of a wheal 2mm or greater to at least one allergen administered during skin prick test).

Individuals were not eligible to participate in the study if any of the following exclusion criteria were applicable:

i) Respiratory infection within 4 weeks of starting the study

ii) Hypersensitivity to ibuprofen

iii) Immune system disease (excluding allergies)

iv) History of cardiovascular disease or neurological disease

v) Respiratory disease other than allergic asthma

vi) Bleeding diathesis

vii) Recent urinary tract infection or urogenital problems

viii) Recent surgery (thoracic, abdominal, eye, etc.)

ix) History of anaphylaxis to allergens being tested

x) Severe asthma or an exacerbation within the past 6 months

xi) Severe skin reaction to allergens being tested

xii) Allergen exposure within 4 weeks of starting the study

xiii) Pregnant or lactating females

xiv) Vaccination with live attenuated virus within 6 weeks of starting the study

xv) Currently undergoing allergy-specific immunotherapy.

Study participants could be withdrawn from the study at any time if:

a) the individual voluntarily withdrew consent. If for any reason a participant did not wish to continue in the study they were made aware that they could stop testing at any time without any consequences or the need to justify their decision. No individuals withdrew from this study.

b) the principal investigator or other research personnel conducting the study could cease testing on an individual if they believed that it would be in the best interest of the participant, or if something happens so that the test subject now meets one of the exclusion criteria. Two individuals were removed due to concerns about their worsening symptomology. Both participants agreed to discontinue testing.

Participants were allowed the use of a rescue inhaler (e.g. salbutamol) as needed before and during the trial, but preferably not within 8 hours of a scheduled visit. No re-scheduling was required due to salbutamol use. Asthma therapy restrictions were as follows:

i) 8 hours for short-acting beta2 agonists (e.g. salbutamol, terbutaline)

ii) 4 weeks for inhaled corticosteroids (e.g. fluticasone propionate, budesonide)

iii) 4 weeks for combination inhaled corticosteroids plus long acting beta agonists (e.g. fluticasone/salmeterol and budesonide/formoterol)

iv) 12 hours for ipratropium bromide (an anti-muscarinic)

v) 24 hours for long acting beta agonists and theophylline

vi) 72 hours for tiotropium bromide and antihistamines

vii) 4 days for leukotriene receptor antagonists (e.g. montelukast sodium)

The following lifestyle considerations were also asked of each subject to ensure optimal testing:

i) Refrain from participating in any vigorous exercise 24 hours prior to testing.

ii) Refrain from eating breakfast prior to allergen challenge days when ingestion of a pill was required (after the first hour of testing there was an opportunity to eat). The rationale was to try to control for any effect food may have had on the pharmacokinetics of the medication.

iii) Not consume any tea or coffee within 4 hours of visits 2 and 5 (including any caffeine containing products such as chocolate and cola drinks)

iv) Be a lifetime non-smoker or greater than one year ex-smoker with less than a 10 pack year history

Follow-up emails, phone calls, and text messages were conducted with each participant to ensure there were no long term adverse effects. Subjects were contacted 2 – 4 weeks after the study was completed. No issues were noted by any of the participants.

2.3) Methods

This experiment was a double blind randomized cross over placebo controlled study. Upon initial screening the testing parameters and consent were reviewed, a baseline MCh was performed, and a SPT was conducted to assess atopy. Testing days involved 2 sets of 3 days with a minimum 13 day wash-out period in-between. On day one of each triad we assessed the individual in the afternoon and performed a MCh challenge, obtained sputum, blood, and urine, as well measured FeNO. These visits lasted approximately one hour.

Day 2 began in the morning (between 7 and 9 am) where baseline FEV1 was assessed, followed by performing the allergen inhalation challenge. One hour prior to testing we required the test subjects to take 2 pills, which were double blinded to both the participants and investigators, and were either ibuprofen or a sugar pill. We then measured lung function over the next 8 hours, assessed FeNO, took blood, urine, and sputum samples, and performed a MCh challenge. Test day 3 mirrored day 2, except no pill was required to be taken prior to testing, and a FeNO level was taken initially in the morning prior to beginning allergen inhalation. Days 2/3 and 5/6 took approximately 9 hours. Each set of 3 days were arranged so that the time when each individual was being tested was similar.

Prior to arriving in the lab the participants ingested either ibuprofen or placebo pills (blinded to both the individual and investigator) during one set of testing, and then the other during the next triad. Each subject was required to return their empty pill bottles as a means of monitoring ingestion. By taking both the placebo and ibuprofen pills each individual acted as a

control for themselves, as well as allowed us to determine the effects of ibuprofen on allergen challenges while limiting bias. A study schematic is presented in Figure 2.1.

Visit 1		Triad 1			Washout	Triad 2		
Screening n= 16	Randomization n = 13	Visit 2	Visit 3	Visit 4		Visit 5	Visit 6	Visit 7
		Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
		Pre AC	AC1	AC2		Pre AC	AC1	AC2
Consent MCh Skin Prick Test	n = 5 to placebo first n = 8 to ibuprofen first	Blood FeNO MCh SI	Dose AC Blood FeNO MCh SI	FeNO AC Blood FeNO MCh SI	Minimum 13 days	Blood FeNO MCh SI	Dose AC Blood FeNO MCh SI	FeNO AC Blood FeNO MCh SI
MCh = methacholine challenge; FeNO = fractional exhaled nitric oxide; SI = sputum induction; AC = allergen challenge; Dose = placebo or 400mg ibuprofen 1 hour pre AC1								

Figure 2.1 – Schematic presentation of the study

Tests performed on participants in this study included:

i) Pregnancy testing

Sexually active women in child bearing years (a sexually mature woman who has not undergone a hysterectomy or who has not been post-menopausal for 24 consecutive months), who was not using a medically approved effective method of birth control, or who has had sexual intercourse that could result in pregnancy, were required to complete a urine pregnancy test prior to study entry. Acceptable methods of birth control included the following, and should have been started at least one month prior to beginning testing:

-birth control pills, patch, or ring

-condoms plus spermicidal cream or jelly or foam

-diaphragm plus spermicidal cream or jelly

-cervical cap plus spermicidal cream or jelly

-vasectomy with an appropriate follow up at 3 months to confirm azoospermia

-tubal ligation

-IUD (a.k.a. intrauterine device)

-Depo-Provera

ii) Skin Prick Test and Skin Test Endpoint

Each individual was skin tested to determine their sensitivity to common aero-allergens. Those who had previously undergone skin testing (participated in past studies) were exempt if we had

their results. Drops of various liquid allergens, including foods, animals, pests, and fungi, were placed on the volar side of an individual's arm, inoculated using a small metal lancet, and then was observed for 15 minutes to see if a wheal developed. Wheal sizes were evaluated and recorded.

An appropriate allergen was chosen based on the response to the skin prick test and clinical history. Doubling dilutions (1:8 to 1:1024 or lower if necessary) of the chosen allergen was prepared and used to perform the skin test endpoint (the skin test endpoint was performed in all subjects regardless if they participated in past investigations). The skin test endpoint is defined as the dilution of allergen that produces a wheal of 2mm in diameter. This is used in conjunction with the results of the methacholine challenge to determine the predicted allergen PC20 for the allergen challenge. The following formula was used to extrapolate at what concentration an aero-allergen inhalation would cause a 20% decrease in FEV1:

Predicted allergen PC20 = anti-log [0.68 x log (methacholine PC20 x skin test endpoint)] (51, 52)

iii) Methacholine Challenge Test

The procedure for a methacholine challenge test is outlined in the American Thoracic Society Guidelines (1999), and involves:

1. Methacholine prepared at the following concentrations: 0.03, 0.06, 0.125, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00, and 16.00 (mg/ml).

2. Baseline spirometry performed.

3. By means of a Bennett-Twin nebulizer (calibrated to an output of 0.13 mL/min) and with the use of nose clips the first concentration (diluent) was administered for a period of 2 minutes. Patients were asked to breathe normally. After 2 minutes the nebulizer was removed.

4. Target fall in FEV1 was calculated at 80% of the patients' baseline FEV1 (i.e. a 20% drop).

5. FEV1 was measured by spirometry at both 30 and 90 seconds after the nebulization ended.

6. The FEV1 values were recorded and the lowest FEV1 post methacholine inhalation value was compared to the lowest FEV1 post diluent inhalation in order to calculate the fall in FEV1.

7. Steps 4 through 6 were repeated for each of the doses listed above until the target FEV1 was achieved, or a dose of 16 mg/mL was reached (31).

In addition to the screening methacholine challenge, six more MCh tests were conducted. One occurred in the afternoon on the day prior to allergen challenge, and then approximately 7 hours after each allergen provocation testing. The purpose of these MCh challenges was to assess allergen induced airway hyper-responsiveness to direct stimuli.

iv) Allergen Challenge

The allergen challenge was performed as follows:

1. Baseline spirometry was conducted, and the highest FEV1 was used for comparison with the post allergen inhalation FEV1 (this is in contrast to the MCh where the lowest value was used).

2. Target FEV1 was calculated at 80% of the patients' baseline (highest) FEV1 (i.e. a 20% drop).

3. By means of a Wright nebulizer (calibrated to an output of 0.13mL/min) and with the use of a nose clip the first concentration was administered for a period of 2 minutes exactly. A Wright nebulizer was used because it can be fitted with a two-way Hans-Rudolph valve and filters can be attached to the expiratory port which prevents nebulized allergen from contaminating the lab environment. The first dose was 3 - 4 concentrations below the predicted allergen PC20. Participants were asked to breathe normally via a mouthpiece during aero-allergen inhalation.

4. After nebulization had ended we measured FEV1 at 10 minutes and once again 1 minute later for each dilution. If the target fall in FEV1 had not been reached, the next concentration of allergen was administered.

After the FEV1 had fallen 20% or more, no more allergen was provided and the FEV1 was measured at 20, 30, 45, 60, 90 and 120, 180, 240, 300, 360 and 420 minutes to assess if an individual developed a late asthmatic response (53).

v) Samples Collected

During this study we collected sputum, blood, and urine for the purpose of assessing mediators/products of inflammation. In asthma research commonly measured cells include; eosinophils, basophils, tryptase, interleukins 4, 5, 13, 9-alpha, 11-beta prostaglandin F-alpha (a by-product of PGD2), prostaglandin E2, among others (54). The samples were frozen at minus 80 degrees Celsius, stored in the asthma lab, and will be used for future mechanistic studies in allergic asthma. It should be noted that participants were made aware of this in the consent form.

vi) Exhaled Nitric Oxide Measurements

FeNO was measured using a chemiluminescence gas analyzer (Niox, Aerocrine Inc., New York, NY). Subjects performed inhalation to total lung capacity followed by an exhalation with a constant flow rate of 50 mL/sec via a filter/mouthpiece. The procedure was performed in duplicates unless 2 measurements were not reproducible within 10%, in which case an additional reading was taken and the three reproducible values were averaged. FeNO was collected prior to MCh challenges and after allergen challenges (AC), except on the third day of each triad where a measurement was also taken first thing in the morning prior to the AC.

2.4) Statistical analysis

Data were analysed using Statistix 10.0 software (Tallahassee, Florida). Early asthmatic responses (EAR) PC20, late asthmatic responses (LAR) PC15, methacholine PC20, blood eosinophils, sputum eosinophils and FeNO data were log transformed prior to paired t-test comparison. Data are reported as geometric mean with 95% confidence intervals.

CHAPTER 3 – RESULTS

3.1) Participants

Sixteen individuals were screened for participation in the study. Of the 16 people screened, 13 were recruited for the investigation. Eleven individuals completed the entirety of testing, while 2 test subjects had to be prematurely withdrawn. Testing on one subject (#11) was stopped on their last day because their baseline lung function was below 70%, which was a minimum requirement for being exposed to allergen inhalation. In addition, although this subject was willing to conclude testing we decided not to conduct the last allergen challenge as they were experiencing increased chest tightness and fatigue. Data collected from the previous visits was included in the analysis for this individual.

The other individual (#13) suffered from 2 respiratory tract infections between triads, and developed an intractable cough. They did complete the first arm (i.e.) first triad, including two repeated allergen challenges. Prior to the undergoing further testing their baseline FEV1 was well under 70%. It was thought that this individual's asthma progressed to moderate severity, therefore requiring a more intensive treatment regimen. Proper medical follow-up with a physician was arranged for this individual. There was no concern that the worsening of their symptomology was due to participating in the study. Data previously collected on this individual was not included in the analysis as they were randomised to receive the ibuprofen treatment first.

A demographic representation of the study participants is shown in Table 3.1:

Table 3.1: Participant demographics.

Participant	Age (years)	Gender	Height (cm)	FEV_1 (L)	FEV_1 (% predicted)	Baseline MCh PC_{20} (mg/mL)	Allergen inhaled	EAR or DAR
001	45	F	163	2.53	87	6.0	Timothy Grass	EAR
002	27	F	159	2.78	90	3.2	HDM-DP	EAR
003	49	F	178	2.54	74	5.9	Cat	DAR
004	30	M	196	5.39	100	0.97	Cat	EAR
005	38	M	178	3.56	83	0.35	Cat	EAR
006	22	F	168	3.15	90	14.2	Cat	EAR
007	29	F	163	2.86	89	1.4	Cat	EAR
008	22	M	185	4.60	92	7.3	Cat	EAR
009	20	F	170	3.17	88	2.8	Cat	DAR
010	21	M	183	4.30	87	8.5	HDM-DP	EAR
011	24	M	180	3.93	83	0.48	Cat	DAR
012	68	M	168	2.17	77	0.53	Timothy Grass	EAR
013	31	F	163	2.25	71	0.13	Cat	EAR

Standardized Timothy Grass 100,000 BAU/ml; Standardized HDM- DP 30,000 AU/mL; Standardized Cat Pelt 10,000 BAU/ml; EAR – isolated early asthmatic response; DAR – dual asthmatic response (with decrease in FEV1 of 15% during the late response).

3.2) Development of Refractoriness

We discovered that a refractory period did not develop. After placebo, the EAR PC20 of the second allergen challenge was not significantly different from the first allergen challenge [250 units/ml (79-790) versus 225 units/ml (82-617); p = 0.82]. This p value indicates there was no significant difference between the amount of allergen required to cause a reduction of FEV1 by 20% between the first allergen challenge and the second allergen challenge conducted 24 hours later.

3.3) Effect of Ibuprofen on Refractoriness

Our second hypothesis was to assess the effects of ibuprofen on the refractory response induced by repeating a second AC within 24 hours of the first AC. Since there was no refractoriness seen, assessing the effects of ibuprofen was moot, and therefore not done.

3.4) Effect of Ibuprofen on the Early Asthmatic Response

The final hypothesis we wanted to evaluate was the effect of ibuprofen on the allergen challenge. We found that the concentration of allergen required to cause a fall of 20% in FEV1 was significantly greater for the ibuprofen group compared to the placebo. The PC20's were 356 units/ml (125-1017 units/ml) compared to 225 units/ml (82-617 units/ml) with a p of 0.027. The EAR PC20 of the second post ibuprofen allergen challenge was 213 units/ml (73-619 units/ml) and not significantly different from the first or second allergen challenge EAR PC20 after placebo. This suggests the inhibitory effect of ibuprofen was gone at 24 hours.

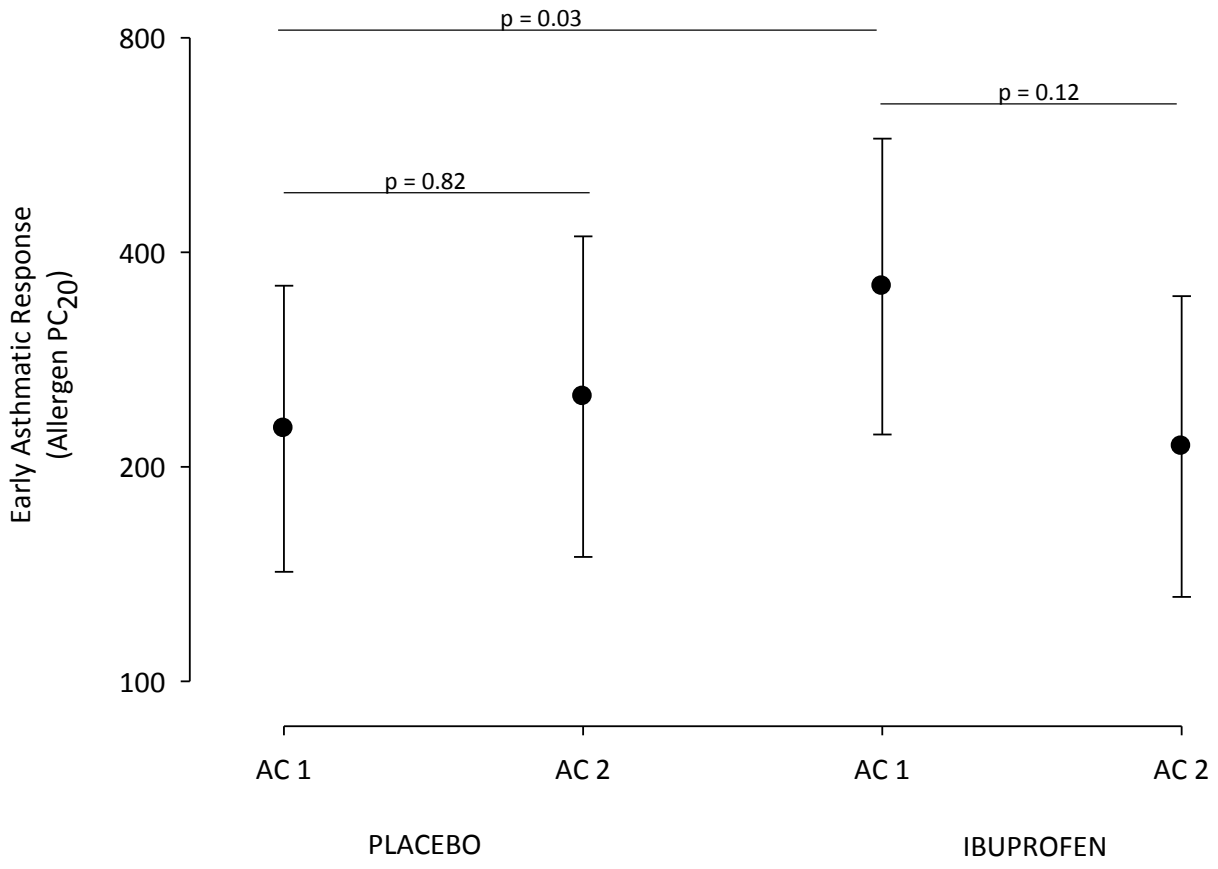

Figure 3.1 Early asthmatic responses (EAR PC20's) for allergen challenges after placebo (left) and ibuprofen (right) treatments. AC1 and AC2 after placebo are similar indicating there is no refractory period when allergen challenges are done 24 hours a part. This graph also shows that an increased amount of allergen was required to cause a drop of in FEV1 of 20% during AC1 in the ibuprofen arm.

3.5) Effect of Ibuprofen on the Late Asthmatic Response

An incidental discovery we discerned was the effect of ibuprofen use on dual responders. As mentioned previously, dual responders have an EAR which is maximal in the first 15 – 30 minutes which resolves back to baseline and plateaus for the next few hours, and then another drop in FEV1 is experienced 3 – 8 hours later. The late response is generally defined as a fall in FEV1 of 15% or more after an initial drop and resolution to baseline, usually greater than 3 hours after the initial exposure.

We had 3 subjects who fell into this category, and found that ibuprofen attenuated the late asthmatic response. Using the equation for PC15 to represent the late response, we found that after ibuprofen treatment, the LAR PC15 was 208 units/ml (5.2-8318 units/ml), whereas after placebo treatment the LAR PC15 was 57 units/ml (1.3-2630 units/ml). The protection from pre-treatment with ibuprofen was significant with a p value of 0.03.

However, if we include the two participants who had decreases in FEV1 of greater than 7.5% over the 3-7 hours post allergen inhalation, our LAR sample size would increase from 3 individuals to 5 individuals. The inclusion of a 7.5% or greater response is reasonable as isolated early responders tend not to show any worsening of their lung function following recovery from the EAR. The inhibitory effect on the LAR was even more pronounced with this cohort as the ibuprofen treatment had a PC15 of 599 (57-6283 units/ml), while the placebo had one of 211 units/ml (12-3724 units/ml), with a p value of 0.01.

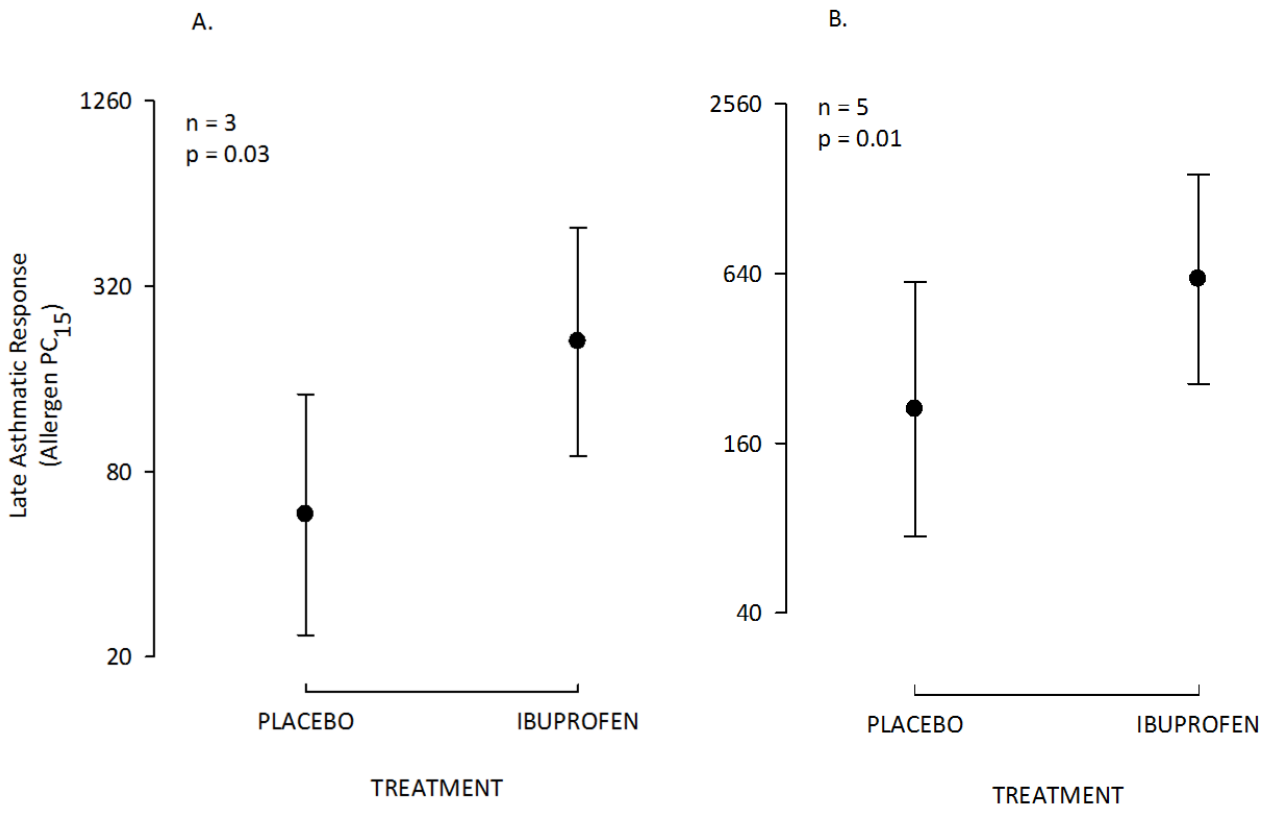

Figure 3.2 Late asthmatic responses (LAR PC15) in the two different sample sizes (A, left, n=3; B, right, n=5).

3.6) Peripheral eosinophil counts and markers of airway inflammation

Peripheral blood eosinophils numerically increased after both allergen challenges following placebo treatment. The increase was significant compared to baseline levels following the second allergen challenge only (p=0.001). Peripheral blood eosinophils also numerically increased after both allergen challenges following ibuprofen treatment. The increases were significant versus baseline as well as between the first and second allergen challenges (p = 0.003, p = 0.0005 and p = 0.005 respectively).

Following placebo treatment, sputum eosinophils increased after the first allergen challenge and further increased after the second allergen challenge but neither increase reached statistical significance versus baseline. After ibuprofen treatment, sputum eosinophils had a similar pattern of increases following the allergen challenges which reached statistical significance versus baseline and between the first and second allergen challenges (p = 0.05, p = 0.007 and p = 0.02 respectively).

Levels of FeNO were significantly increased versus pre allergen challenge values for both the placebo (p = 0.005) and the ibuprofen (p = 0.005) treatment arms at 7 hours after the second allergen challenges. Allergen exposure did not alter airway responsiveness to methacholine following placebo treatment. After ibuprofen, however, airway responsiveness to methacholine only significantly increased after the first allergen challenge. The pre challenge methacholine PC20 was 2.4 mg/ml versus the methacholine PC20 of 1.6mg/ml at 7 hours after the first allergen challenge (p=0.04).

Table 3.2: Changes in indices of airway inflammation

Treatment	Time point	Blood EOS ($x10^9$/L)	Sputum EOS (%)	FeNO (ppb)	MCh PC_{20} (mg/mL)
Placebo	pre AC (baseline)	0.17 (0.11-0.26)	5.1 (2.7-9.6)	29.5 (19.7-44.2)	1.9 (0.95-3.8)
	7h post AC1	0.20 (0.14-0.28)	8.2 (3.1-21.9)	33.2 (22.3-49.4)	1.9 (0.95-4.1)
	7h post AC2	0.23 (0.16-0.34)	12.1 (4.2-35.0)	39.0 (25.4-59.9)	1.7 (0.96-3.1)
Ibuprofen	pre AC (baseline)	0.14 (0.08-0.25)	4.0 (2.1-7.8)	29.7 (19.6-44.8)	2.4 (0.99-5.8)
	7h post AC1	0.21 (0.14-0.31)	8.6 (3.4-21.8)	30.5 (17.9-52.1)	1.6 (0.70-3.5)
	7h post AC2	0.26 (0.18-0.39)	14.1 (7.0-28.5)	40.8 (24.4-68.3)	1.5 (0.73-3.2)

Data are presented as the geometric mean (95% confidence intervals). AC = allergen challenge; EOS = eosinophils; FeNO = fractional exhaled nitric oxide and MCh PC_{20} = concentration of methacholine causing a 20% fall in FEV_1.

Urine and plasma was collected in order to quantify markers of inflammation at different stages of the investigation. We were especially interested in assessing both inflammatory and anti-inflammatory prostaglandins. Unfortunately we were unable to evaluate them for the purposes of this experiment due to cost and technical issues, however, samples were frozen, labelled, and stored in the asthma lab, and can be used for future studies.

CHAPTER 4 – GENERAL DISCUSSION

4.1) Analysis of Findings

The primary objective of our study was to determine whether airway responses to a second allergen challenge performed 24 hours after the first exhibit a refractory state. We observed that the early asthmatic response to a second allergen challenge was unchanged compared to that of the first, indicating a refractory state does not develop when allergen challenges are performed 24 hours apart. Refractoriness has been seen with various non-allergic indirect stimuli, such as exercise (39), adenosine monophosphate, (40) and mannitol (44). Cross-refractoriness has been shown between exercise and metabisulphate (41), exercise and AMP, (55) and allergen and mannitol (50, 44). Both allergic and non-allergic indirect acting stimuli trigger the release of bronchoconstricting mediators from mast cells. Considering their similar mechanisms of action, as well as the presence of cross refractoriness, it is conceivable that allergic stimuli might also exhibit a refractory period with repeat challenges.

Although both stimuli trigger mast cell degranulation, the IgE mediated allergic pathway (a type I hypersensitivity reaction) is much more complex than that of non-allergic stimuli. IgE mediated mast cell degranulation also has a lot of downstream effects on other inflammatory mediators. This mechanistic difference may explain the lack of refractoriness with repeat allergen challenge.

Another possible explanation is that the time frame between challenges (i.e. 24 hours) needs to be shorter such that the second challenge is performed soon after recovery. The importance of duration between challenges may relate to depletion of mast cell mediators as a potential

mechanism leading to refractoriness. A longer duration between challenges would favor the absence of a refractory state, although recent data from Larsson et al suggests mast cell mediator depletion is not a causal factor in the development of refractoriness (42). Additionally, cross refractoriness with mannitol has been shown to be present at both 3 hours and 24 hours after an initial allergen challenge (44, 50). Future studies that could provide additional insight include an investigation of mannitol challenges performed 24 hours apart, an investigation of airway responses to allergen challenge after recovery from mannitol challenge, and an investigation where repeat allergen challenges are performed within a shorter timeframe.

Given that we did not observe a refractory response with repeat allergen challenge, the effect of ibuprofen on this phenomenon is moot. However, in addition to the role of protective prostaglandins in the mechanism of refractoriness (56), it is possible inhibition of protective prostaglandins with an NSAID may lead to the development of late asthmatic responses in isolated early responders. It may be of interest to note, as an ad hoc assessment, that late responses did not occur following ibuprofen treatment in those with isolated early responses in our experiment.

Our study design allowed for the assessment of ibuprofen on airway responses to allergen challenge. We discovered significant inhibition of the EAR after a single 400 mg dose of ibuprofen administered one hour prior to allergen exposure. The protection was gone at 24 hours, consistent with the pharmacokinetics of ibuprofen.

We reviewed five investigations in which the effect of NSAID's on allergen challenge had been reported (57-61). With the exception of the Joubert et al (58) study, which treated participants with 100 mg/day of indomethacin for three days, treatment with NSAID's were ineffective in decreasing the early asthmatic response. Conversely, with respect to the LAR, four of the five studies we reviewed documented significant inhibition of the LAR (57, 60, 61), which is consistent with our findings. We showed significant inhibition of the LAR in our three *bona fide* late responders (i.e. decrease in FEV1 ≥ 15% in the 3-7 hours post allergen challenge). By including participants that had a fall in FEV1 of greater than 7.5% in the 3-7 hours after allergen challenge, our LAR sample size increased to 5, and the inhibition of the LAR showed an even greater statistical significance ($p=0.01$ versus $p = 0.03$). The LAR is commonly reported as the maximal fall in FEV1 or area under the curve (AUC). Meaningful interpretation of the LAR using these endpoints requires that the same dose of allergen be administered across all allergen challenges.

Due to safety considerations, we were unable to administer the same dose of allergen during all four allergen challenges in 58% of our participants (i.e. FEV1 fell ≥ 20% at a weaker concentration of allergen than that given during the first allergen challenge). We controlled for differences in the dose of allergen administered by assessing and reporting the airway responses to allergen as the EAR PC20 and LAR PC15. This methodological difference may explain the discrepancy in the effect of NSAID's on the EAR between our current data and that previously reported.

Certain studies that employ repeat allergen exposure (e.g. low dose allergen challenge methodology) have shown worsened asthma outcomes, including increases in symptoms,

rescue therapy, inflammatory markers, and worsened airway responses [i.e. FEV1, EAR, LAR] (47, 48, 62). The similar EAR PC20 data following the placebo treatment in our current study do not suggest a priming effect of the first allergen challenge on airflow responses to a second allergen challenge. The study populations in which worsened responses have occurred following repeat allergen challenges focus on late responders. Our mixed study population of both early and late responders, predominantly early, may explain the absence of a priming effect.

Well documented consequences of allergen exposure in dual responders include increased airway responsiveness to methacholine, levels of fractional exhaled nitric oxide, peripheral blood and sputum eosinophils. The presence of a late asthmatic response was not an entrance criteria in the current study, as previously mentioned, and dual responders accounted for only a small portion of our subject demographic. Nonetheless, our study population as a whole, following both placebo and ibuprofen treatment, had greater levels of sputum and peripheral blood eosinophils, as well as FeNO increased after the first allergen challenge. These were further increased after the second allergen challenge. The magnitude of the elevations tended toward statistical significance following ibuprofen.

In addition, airway responsiveness to methacholine did not increase significantly after placebo, but did increase significantly after ibuprofen. The increase in methacholine responsiveness following ibuprofen was less than one concentration, which is probably not clinically relevant as the MCh challenge has a reliability/reproducibility of plus/minus one concentration when repeated on an individual. The sequelae data must be interpreted with caution for two reasons. First, the amount of allergen administered across all allergen challenges was not

consistent. It is worth noting, however, that the amount of allergen delivered was always less and this would not intuitively translate to the observed increases in airway responses. Second, our study population includes individuals with either isolated early or dual responses, and much of what we appreciate about airway inflammation following allergen challenge is based on findings in dual responders.

Ibuprofen and other NSAID's non-selectively inhibit cyclooxygenase enzyme activity and decrease the production of prostaglandins and thromboxanes. These eicosanoids, along with other arachidonic acid metabolites generated by lipoxygenase enzymes have a wide range of physiological effects, many of which are relevant to the airway responses induced by allergen exposure in atopic asthmatics. In the absence of mechanistic data it is difficult to postulate how a single dose of ibuprofen led to a decrease in early and late asthmatic responses. We have previously reported inhibition of early and late asthmatic responses following single dose montelukast, which targets the lipoxygenase pathway of eicosanoid production (63, 64). If we consider downstream effects of cyclooxygenase inhibition, we anticipate a decrease in the production of the different prostaglandin isoforms and a subsequent decrease in their related effects. For example, PGD2 is known to cause bronchoconstriction and blocking its production should therefore produce an inhibitory effect on the early asthmatic response. Conversely, PGE2 is bronchoprotective (65, 66), and decreasing levels of PGE2 might be expected to worsen airway responses to allergen challenge. Another possible outcome of cyclooxygenase inhibition is a shift in eicosanoid production away from prostaglandin synthesis toward lipoxygenase generated eicosanoid synthesis. This has been proposed as a mechanism by which worsened asthma responses are observed following the use of the COX-1 inhibitor aspirin (67). If

leukotriene production increases following ibuprofen treatment one may expect a greater or at least similar response to allergen challenge as has been observed in a study which discovered this when pre-treated with etoricoxib (68).

4.2) Critical Appraisal of the Study

In general this study was successful as we were able to recruit the appropriate number of participants, test all of our hypotheses, and gained insight into future investigations. However, there were a few aspects which may have been improved upon. First, many studies which use the allergen challenge model do a screening allergen challenge to determine the sensitivity of an individual and decipher the optimal concentration at which to begin the test. The importance of this initial assessment would be to confirm that their actual PC20 to inhaled allergen correlates with that predicted from their response to methacholine and skin test endpoint (recall equation page 35).

A screening allergen challenge would also be useful in determining whether a participant was an isolated early responder or a dual responder. This information could reduce the length of time required for each subsequent challenge, and correspondingly the length of each lab each visit, because if found to be an isolated early responder (especially those who have been in past studies) we could potentially stop following them an hour after they return to baseline (assuming there was not an artificial creation of a late response, which we did not witness). We chose not to perform a screening challenge because it would have added another full day of testing, and we thought this may dissuade individuals from participating in an already very demanding study.

We wanted to study the effects of ibuprofen on an allergen challenge, and to test this we gave one dose of 400 mg one hour prior to testing. This time frame was chosen because the drug has a time of maximal activity (Tmax) of 0.6 – 1.9 hours. However, we had no way of ensuring that our subjects took the drug at the appropriate time, if it had its maximal effects during the challenge, or if they took the drug at all. Although each subject brought their empty pill container to the lab, it may have been more prudent to have them come in a half an hour early, take the medication, then begin the challenge. Another check would have been to measure ibuprofen levels in the blood. Prior to testing we usually had the individuals rest for 15-20 minutes, do their baseline spirometry (5 – 10 minutes), then begin the allergen challenge. Inhalations generally took 30 – 60 minutes, so there would have been ample time for the drug to take effect.

In addition, it may have been interesting to place test subjects on continual doses of ibuprofen for the first day of their challenge, as well as perhaps during their second. The recommended maximal daily dose is 3200 mg (69), so we could have easily given a continual dose of 400 mg every 6 – 8 hours, and participants would have remained well under this amount. By doing so we would be able to better control the variance in kinetics of an individual's metabolism on the drug, plus get a more complete understanding of the effects of NSAIDs on the allergen challenge. It may have allowed us to gain a greater appreciation of the effects of COX inhibitors on both the early and late response, as well as refractoriness.

One of the subjects (#11) did not complete the study because their baseline spirometry was too low before their scheduled last challenge. This individual was a dual-responder, and on the first challenge day of the second triad (day 5), they had a drop in FEV1 of 19% after an inhalation,

then after 10 minutes of waiting had a FEV1 of 15%. We proceeded to give them one more doubling concentration of allergen so they would have had the same amount as the day before (for standardization purposes). The result was a significant drop in their FEV1, as well as worsening of their asthmatic symptoms. They followed the standard course of having an EAR, returning to baseline, and then having a late response. The next day their baseline lung function was below 70% and we decided not to give them any more allergen as they were complaining of chest tightness and fatigue. Perhaps this last dose was not necessary, and by not giving it to them not only would they have avoided physical discomfort, but also may have completed testing. Given that they were so close to 20%, and that we described our results in unit/ml based on their FEV1 and concentrations administered, it would have been reasonable to stop administering allergen.

Our primary measure of the effect of allergen inhalation in an allergic asthmatic was by interpreting pulmonary lung function. Given that this was a clinical trial it may have been prudent to also receive subjective feedback from individuals about how they felt throughout the challenges. We could have used a symptom severity scale (such as a numeric rating scale) which assessed chest tightness, shortness of breath, and fatigue in order to get an appreciation about each participant's experience. This finding would be especially useful in future studies where allergen challenges are repeated in a short time frame, as we would have an awareness of the discomfort we are putting our test subjects through, and could decide whether it is warranted.

Finally, standardization is extremely important in conducting scientific experiments. In our study, for safety reasons, we were not able to administer the same amount of allergen to each

participant across all challenges, which means some results require cautious interpretation. To obtain the most accurate and comparable results it would have been ideal to have each participant inhale the same total dose of allergen across all four challenges. This is standard procedure for studies that look for an LAR. This did not happen because some had 20% falls in FEV1 at lower concentrations than were administered during the first challenge. We tried to control for this initially by starting at the same concentration (even if the subject had to inhale numerous doubling concentrations) as we hypothesized that the inflammatory response would begin at these lower doses (as a reminder we started 3-4 concentrations lower based on the predicted allergen PC20 equation, and in some instances we had to do give individuals as much as 8 doubling concentrations). However, over half of our test subjects were not able to consistently inhale the same dose of allergen. The ramifications include questioning the validity of many of the findings of the study; including the effects of ibuprofen on the LAR, mediator results, and airway hyper-responsiveness to MCh.

4.3) Future Research Considerations

4.3.1) Non-steroidal Anti-inflammatory Drugs and Allergen Challenges

NSAIDs are one of the most commonly used over the counter medications. Our findings demonstrated that their use diminishes both the early and late asthmatic response when performing allergen challenges. This is significant because of the prevalence of NSAID use and that most studies which assess new medications to treat asthmatics use the allergen challenge model. Based on the results of this study it would be advisable that test subjects refrain from

NSAID use while involved in these experiments as it would confound the results. This could result in new therapeutics appearing more beneficial than they actually are in reality.

4.3.2) Using Allergen Challenge to Determine Phenotype and Treatment

Asthma researchers are currently reconsidering the way they categorize the disease. Traditionally it was classified as intrinsic and atopic/extrinsic, or by symptom severity, frequency, and medication use. However, with the advances made in molecular techniques and standardization of the allergen challenge model over the past few decade's researchers now understand a great deal more about asthma.

Although there is still discrepancy among asthma researchers on its classification, some of the more current common categories in the literature include; early onset allergic, late onset eosinophilic, obesity related, exercise induced, and neutrophilic (15, 70, 71). Determining an individual's phenotype could have clinical applications.

For example, early onset allergic asthma is the most well studied and predominant form, and is what most fits into the current algorithm of asthma treatment guidelines (72). Conversely, neutrophil predominant asthma has been associated with developing more frequent sudden attacks, treatment resistant, and the development of chronic disease (73). Obesity related asthma has also been found to be difficult to manage (74). Currently medications are being developed to target these specific groups based on their phenotypes.

The allergen challenge model currently is one of the most commonly used tools to investigate novel therapeutics in the laboratory setting. However, it may have clinical implications in the future. Individuals who have severe symptoms, or are treatment refractive, may benefit from

having an allergen challenge and assessing biologic samples to see which mediators are predominant. It may help the clinician, themselves, and their families make sense of their condition, as they would gain a greater appreciation of the underlying mechanisms causing their disease. In addition, it could be useful in guiding their treatment.

4.3.3) Using PC15 - 20 Equation to Assess the Late Asthmatic Response

Currently there are different ways about how to accurately describe and quantify the LAR. One of the main issues arises from a lack of understanding of the exact relationship between the dose of the allergen, the underlying mechanisms and the response, although it is widely conceived that all three parameters are inter-related. In addition, an issue we had in our study was that we could not measure the LAR by the usual method, area under the curve and maximum fall in FEV1, because of the differing allergen concentrations participants were exposed to on the different allergen challenge days.

The EAR following an allergen challenge is calculated based on the fall in FEV1 and concentrations of allergen inhaled. We calculated this using the following equation:

PC/PD20 = antilog [logC1 + (log C2 – logC1) x (20 – R1)]/(R2-R1);

where C1 refers to 2nd to last concentration given, C2 is last concentration given, R1 equals percent fall in FEV1 after C1 and R2 is % fall in FEV1 after C2. This equation is used because rarely will an individual fall 20% exactly.

Given that the EAR is caused by the dose of allergen given, it makes sense that we could calculate and describe the LAR in a similar fashion. Both responses are the result of exposure to

an atopic substance (this is especially true in the laboratory setting where the environment is controlled). Even in "real life situations", individuals who are dual responders get a late exacerbation because of the initial contact with an allergen. Since it is this exposure which causes both the early and late responses it seems intuitive that we could describe them mathematically in the same way. A potential means of proving this mathematically would involve testing a large amount of individuals, and comparing the results derived from the equation with those found by plotting the variables graphically. This hypothesis has future research implications, as it could be used for other experiments where test subjects are not able to receive the same total amount of allergen.

4.3.4) Future Research Projects

There are two future studies which could logistically spawn from this project. The first would be a mediator quantification study. By performing one we would be able to gain an appreciation of what is happening at the cellular level. The mediators which should be assessed include the pro-inflammatory mediators/cytokines, PGD2, LTB-E4, IL-4, IL-5, IL-13, INF-gamma, histamine, mast cells, and the anti-inflammatory prostaglandins E2 and I2. This could be performed as we have urine and plasma samples frozen in the lab from this study.

Another investigation which may prove fruitful would be to do a repeated allergen challenge study on isolated early responders on the same day. Perhaps the issue is that 24 hours is too long, and that we missed the refractory period. This study would most likely involve performing an allergen challenge, waiting for at least an hour after recovery (would have to err on the side of caution as allergen challenges have not to my knowledge been performed in such close

proximity), challenge them again, and follow their lung function. In this experiment tissue samples should once again be collected and mediators quantified with the hopes that we can determine the pathophysiology underlying the refractory response (if one were too develop).

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APPENDICES

APPENDIX A – APPLICATION FOR ETHICS APPROVAL

For administrative use only	
File Number:	Date received:

Application for Biomedical Research Ethics Review

PART 1: IDENTIFICATION		
1.1	**Project Title** Assessment of Repeated Allergen Challenge and the Effects of Ibuprofen on the Inflammatory Process Protocol Number (if applicable):	
1.2	**Principal Investigator** Full Name: Dr. Donald Cockcroft Mailing Address: Division of Respirology, Critical Care and Sleep Medicine, University of Saskatchewan, 546 Ellis Hall, 103 Hospital Drive, Saskatoon, Saskatchewan, S7N 0W8 Email: dwc614@mail.usask.ca Phone: 306-844-1446 NSID number (U of S faculty only):	
1.3	**University/Institutional Affiliation of Principal Investigator** Position: Professor Department: College of Medicine Division: Respiratory Medicine	
1.4	**Project Personnel (including graduates/post graduates/residents)**	
	Full Name: Dr. Beth Davis Project Position/Role: Research Supervisor/Co-investigator University/Institutional Affiliation: U of S Email: beth.davis@usask.ca Phone: 306-844-1444	Full Name: Shawn Nomani Project Position/Role: Master's Student University/Institutional Affiliation: U of S Email: syn023@mail.usask.ca Phone: 306-
	Full Name:	Full Name:

	Project Position/Role: University/Institutional Affiliation: Email: Phone:	Project Position/Role: University/Institutional Affiliation: Email: Phone:
	If this is a student/graduate/resident project, please provide the following information:	
	a) Student Name: Shawn Nomani	b) Supervisor Name: Dr. Donald Cockcroft
1.5	**Primary Contact Person for Correspondence (if different than Section 1.2)** Full Name: Shawn Nomani Mailing Address: Room 346 Ellis Hall, University of Saskatchewan, 103 Hospital Drive, Saskatoon, Saskatchewan, S7N 0W8 Email: syn023@mail.usask.ca Phone: 306-844-1443	
1.6	**Research Site(s) where project will be carried out:** Room 346 Ellis Hall, University of Saskatchewan	
1.7	**Proposed Project Period:** From (MM/DD/YY) - 12/15/14 - To (MM/DD/YY) – 09/30/15	
	Specify any time considerations the REB should be aware of (e.g. short enrolment period): If possible we would like to begin this study in mid December, and would appreciate feedback about our application at your earliest convenience. We anticipate that most of our participants will be students at the U of S, and it would be easier to run a few of the experiments while they are on break as it is very time consuming.	
1.8	**Has this project applied for/received ethical approval from any other Saskatchewan REB?** ☐ Yes ■ ***No*** If yes, specify where:	
	Has this project applied for/received ethical approval from another Research Ethics Board outside of Saskatchewan? ☐ Yes ■ ***No*** If yes, specify where (if known):	
1.9	**Do you consider this project to involve:** ■ ***Minimal Risk*** ☐ More than Minimal Risk	
1.10	**Provide name of funding source:**	
	Source of Funds: ☐ Industry ☐ Not-for-Profit Foundation ☐ Tri-Council Grant ☐ Grant-in-aid ☐ National Institute of Health (NIH) ☐ Cooperative Group (NCIC, COG, RTOG) ☐ Internally funded	
	Status of Funds: ☐ Awarded ☐ Pending	
1.11	**Name of Sponsor if different from above funding source:**	

PART 2: REGULATORY REQUIREMENTS

2.1 **If the project involves an investigational drug, natural product, medical device or marketed drug/device being used outside of the approved indication, check whether or not the No Objection Letter (NOL) or the Investigational Testing Authorization (for devices) has been obtained from the appropriate Health Canada regulatory agency.** GN 2.1

■ ***N/A – Proceed to Question 2.2***

	Yes	Pending	N/A
Therapeutic Products Directorate (TPD)	☐	☐	☐
Natural Health Products Directorate (NHPD)	☐	☐	☐
Biologics and Genetics Therapies Directorate (BGTD)	☐	☐	☐
Investigational Testing Authorization (ITA)	☐	☐	☐

Date of approval (MM/DD/YY):

Please forward the NOL and/or ITA to the Research Ethics Office when available.

2.2 **Is there a requirement for this research to comply with United States (OHRP/FDA) regulations for research ethics?**

☐ Yes ■ ***No***

2.3 **Clinical trials are required to be registered with clinicaltrials.gov.** Please submit confirmation of registration when available.

2.4 **Peer Review**

For research with *more than minimal risk*, the REB must be satisfied about both the value and the scientific validity of the project. Under some circumstances and depending on the level of risk, the REB may request that a peer review be conducted as a condition of approval. Research that poses minimal risk will not usually require peer review.

Has this research proposal received any independent scientific review? ☐ Yes (please attach) ■ ***No*** ☐ Not applicable

2.5 According to Good Clinical Practices Section 3.1.2, the Principal Investigator should submit a current curriculum vitae (CV) providing evidence of qualifications to conduct the project. If a CV has not been submitted within last 5 years, please attach.

Is the PI's CV attached? ☐ Yes ☐ Not applicable -is on file

PART 3: BRIEF OVERVIEW OF RESEARCH PROJECT (two page maximum)

3.1 **Research Question/Hypothesis**

Specify the research question(s) being evaluated in the project.

Is there a decrease in airway hyper-responsiveness when an allergen challenge is done on consecutive days, and what effect would ibuprofen have on this?

3.2	**Academic Validity** Provide evidence (scientific literature, pilot projects, etc.) that the scientific reasoning and design of the project are sufficiently sound to meet the objectives of this project. Recent studies conducted in this department have shown that there is a refractory period after indirect challenges, however, the mechanism of why this occurs eludes us. Allergen challenge is similar to indirect broncho-provocation testing, but few tests have been conducted which show; a) what happens when back-to-back allergen testing is done, b) the effects of NSAIDs on these tests, and c) what are the measures of different biomarkers of asthma during repeated trials. Current literature has shown that repeat testing is safe, and there have been no reported contraindications to ibuprofen use in those with mild extrinsic asthma. A list of abstracts will be attached to the end of this application.
3.3	**Research Design/Methods** Provide a description of research design (e.g. parallel group or cross-over design) and methods to be used. Include a justification for the use of a placebo, if applicable. Please note that if the analysis or the interpretation of the research results refers to Aboriginal people, language, culture or history as a primary focus of the project, consultation with the appropriate community is required. Please outline the process to be followed. Randomized double blind placebo controlled study. A placebo will be used to see if ibuprofen has an effect on multiple allergen challenges. It will be double blind to eliminate investigator bias.
3.4	**Statistical Analysis** Include a summary of the primary and secondary end-points/outcomes, the planned sample size (with justification) and planned statistical and interim analyses. The planned sample size will be 10 – 15 individuals, and we will be comparing the placebo and ibuprofen challenges using paired t-tests. We may also choose to do ANOVA regression.
3.5	**Potential Significance/Justification** Explain the significance of the project in order to support the ethical tenet that the proposed research has value (i.e., what are the anticipated public and scientific benefits of the project?). To gain a greater understanding into inflammatory process underlying allergen challenges in those who have mild atopic asthma. In the future it could contribute to how we test and diagnose asthma, as well as help determine possible mechanisms which can be targeted by therapeutics.

PART 4: PARTICIPANT RECRUITMENT	
4.1	**How many participants will be enrolled in the project:** Globally? ***Locally - 10 - 15***
4.2	**Describe who will be selected (target population) and the criteria for their inclusion.** Ages 18 – 70, mild asthma, has a positive skin prick test to allergen, shows obstructive lung disease upon methacholine challenge
4.3	**Describe who will be excluded from participation.** Recent respiratory infection, suffers from uncontrolled/moderate to severe asthma, hypersensitivity to ibuprofen, bleeding diathesis, recent UTI, uro-genital issues, severe skin reaction to allergen testing, have a serious co-morbid condition, pregnant or lactating, recent vaccination with live attenuated virus, undergoing allergy specific immunotherapy.

4.4	**Provide a detailed description of the method of recruitment.** a) How will prospective participants be identified? ***Advertisements at the U of S and recruitment through the Respiratory Clinic at RUH.*** b) Who will contact prospective participants? ***Shawn Nomani and Dr. Beth Davis*** c) How will this be done? (Ensure that any letters of initial contact or other recruitment materials are attached to this submission (e.g. advertisements, flyers, verbal or telephone script, etc.). ***Via telephone and email.***

PART 5: CONSENT PROCESS

5.1	**Describe the consent process.** a) Who will ask for consent? ***Dr. Donald Cockroft, Dr. Beth Davis, and Shawn Nomani*** ***b)*** Where, and under what circumstances? ***In the respiratory lab during the initial screening visit.*** c) Describe any situation in which the renewal of consent for this research might be appropriate and how this would take place (e.g. Participant turns 18 or emergency situation). ***There should be no need for renewal of consent.***
5.2	**How long will the participant have to decide whether or not to participate? (**If less than twenty-four hours, provide an explanation). ***3 weeks prior to the end of the study date, or if we reach the maximum amount of test subjects.***
5.3	**Will all participants be able to consent on their own behalf?** ■ ***Yes*** ☐ No If No, explain why: a) If a participant is unable to consent, who will consent on his/her behalf? b) Will the participant be able to assent to participate? ☐ Yes ☐ No If yes, explain how assent will be sought:
5.4	**If monetary compensation or reimbursements for expenses will be offered to the participants please provide the details.** A $1600 honorarium will be provided which was calculated based on the type and amount of testing that a participant will undergo.
5.5	**Describe your plans for providing project results to the participant?** Up-to-date results will be provided by the PI or one of the sub-investigators at any time when requested by a participant, or after the all the data has been collected and interpreted.

PART 6: PROCEDURES AND RISKS

6.1	**Identify those procedures that are different from the current standard of care (i.e. unique to the research project).** There will be no differences from current standard procedures.
6.2	**What are the known risks associated with the procedures outlined in Section 6.1? Also include any risks associated with the placebo or wash out periods, if applicable.** Minor side effects include; headaches, light

	headedness, throat irritation, and skin irritation. Also, there is always a small risk that an individual will have an asthmatic response.
6.3	**What strategies will be put in place to minimize and/or manage the potential risk(s) to participants and other affected individuals?** Ventolin will be on hand which is a short-acting rescue inhaler commonly used for those with asthma. Also, all testing will be conducted in Ellis Hall which is close the Department of Respiratory Medicine and ER at RUH, and will be under the supervision of a trained medical personnel.
6.4	**For double blind projects, describe the provisions made to break the code in an emergency situation [24 hour availability], and indicate who has the code.** If it is clearly articulated in the clinical protocol, it is acceptable to append the information or provide the protocol page reference. ☐ N/A, not a double blind project There are no foreseeable reasons why a code will be required, or need to be broke due to an emergency situation.

PART 7: DATA SECURITY AND STORAGE

The *Saskatchewan Health Information Protection Act (HIPA)* requires an assessment of the risks to privacy and how the risks will be minimized. Accessing existing patient information, such as Health Records, requires consent of the individual which must be addressed in the consent form.

7.1	**Indicate from which sources personal and health information data will be collected:** ■ ***Participant data collected prospectively for the purpose of this project (e.g. case report form)*** ☐ Family physician record ☐ Heath Region – please specify Region, Site & Dept. if applicable: ☐ SK Ministry of Health ☐ SK Cancer Agency ☐ Other – please specify: ☐ Not applicable (No personal or health information to be collected). Proceed to Section 8.
7.2	**How will the confidentiality of participants and their health information be protected?** Password encrypted electronic files, and all paper documents will be locked in a filing cabinet in the Respiratory Lab in Room 346 Ellis Hall, U of S. Only the PI or one of the other researchers will have access to these.
7.3	**Describe the storage arrangements and final disposition of the project data collected.** Computer file which will be deleted once the project is completed, and paper files which will be locked in the filing cabinet in the Respiratory Lab for 5 years, as per University of Saskatchewan ethics recommendations.
7.4	**List the project personnel who have access to any identifiable personal health information and who will have access to any list that links participant names to their project ID number, consent form, enrolment log, etc.** Dr. Donald Cockcroft, Dr. Beth Davis, Shawn Nomani
7.5	**Check all applicable boxes below to provide an assessment of the potential privacy risks and the safeguards/solutions that you will put in place to mitigate the risks**.

Potential Privacy Risks	**Possible Safeguards/Solutions** (check all that you will use)
■ Unauthorized external or internal access to identifying	■ Project personnel screening/agreements

	information through active use or transmission	☐ Access authorization procedures ☐ Designated systems administrator ■ Passwords/screen timeouts ☐ System access audits/disclosure logs ■ Secure mail/transport ■ Firewall/virus protect ☐ Encrypted transmission
	☐ Identification through publication or release	☐ Aggregation levels ☐ Alternate identifiers
	☐ Identification through data-matching	☐ Use of non-linkable elements or identifiers
	☐ Loss of data control outside jurisdiction	☐ Confidentiality and security agreements for out-of-province recipients or storage providers

PART 8: CONFLICT OF INTEREST

8.0 **Is there any real or perceived conflict of interest (any personal or financial interest in the conduct or outcome of this project)? Will any of the researcher(s), members of the research team and/or their immediate family members:**

- Receive personal benefits in connection with this project over and above the direct costs of conducting the project, such as remuneration or employment?
  ☐ Yes ■ ***No***
- Receive significant payments of other sorts from the sponsor such as grants, compensation in the form of equipment or supplies or retainers for ongoing consultation and honoraria?
  ☐ Yes ■ ***No***
- Have a non-financial relationship with a sponsor (such as unpaid consultant, board membership, advisor or other non-financial interest?
  ☐ Yes ■ ***No***
- Have any direct involvement with the sponsor such as stock ownership, stock options or board membership?
  ☐ Yes ■ ***No***
- Hold patents, trademarks, copyrights, licensing agreements or intellectual property rights linked in any way to this project or the sponsor?
  ☐ Yes ■ ***No***
- Have any other relationship, financial or non-financial, that if not disclosed, could be construed as a conflict of interest?
  ☐ Yes ■ ***No***

  If yes, please describe the personal benefits or relationship.

PART 9: DECLARATION BY PRINCIPAL INVESTIGATOR

(OR SUPERVISOR FOR STUDENT PROJECTS)

Project Title:

Assessment of Repeated Allergen Challenge and the Effects of Ibuprofen on the Inflammatory Process

- I confirm that the information provided in this application is complete and correct.
- I accept responsibility for the ethical conduct of this project and for the protection of the rights and welfare of the human participants who are directly or indirectly involved in this project.
- I will comply with all policies and guidelines of the University and Health Region/affiliated institutions where this project will be conducted, as well as with all applicable federal and provincial laws regarding the protection of human participants in research.
- I will ensure that project personnel are qualified, appropriately trained and will adhere to the provisions of the REB-approved application.
- I will ensure that any significant changes to the project, including the proposed method, consent process or recruitment procedures, will be reported to the Research Ethics Board for consideration in advance of its implementation.
- I will ensure that a status report will be submitted to the Research Ethics Board for consideration within one month of the current expiry date each year the project remains open, and upon project completion.
- If personal health information is requested, I assure that it is the minimum necessary to meet the research objective and will not be reused or disclosed to any parties other than those described in the REB-approved application, except as required by law.
- I confirm that adequate resources to protect participants (i.e., personnel, funding, time, equipment and space) are in place *before* implementing the research project, and that the research will *stop* if adequate resources become unavailable.
- I understand that if the contract or grant related to this research project is being reviewed by the University or Health Region, a copy of the ethics application inclusive of the consent document(s), may be forwarded to the person responsible for the review of the contract or grant.
- I understand that if the project involves Health Region resources or facilities, a copy of the ethics application may be forwarded to the Health Region research coordinator to facilitate operational approval.

_____	_____	_____
Signature of Principal Investigator	Printed Name of Principal Investigator	Date (MM/DD/YY)

Department Head (*or supervisor for student projects***:** The signature/approval of the Department/Administrative Unit acknowledges that he/she is aware of and supports the research activity described in the proposal.

_____	_____	_____
Signature of Department Head	Printed Name of Department Head	Date (MM/DD/YY)

PART 11: ATTACHMENTS

Provide a full and accurate listing of all documents submitted with this application.

All projects requiring the use of RQHR resources must complete this section.

Document	Included?	Comments
Certificate of Approval from another REB	☐ Yes ☐ N/A	
Peer Review reports	☐ Yes ☐ N/A	
Participant Consent Form	☐ Yes ☐ N/A	
Control Participant Consent Form	☐ Yes ☐ N/A	
Assent Form	☐ Yes ☐ N/A	
Tissue/Blood Banking Consent Form	☐ Yes ☐ N/A	
Letter of Initial Contact	☐ Yes ☐ N/A	
Advertisement to Recruit Participants	☐ Yes ☐ N/A	
Questionnaires, tests, interview scripts, etc.	☐ Yes ☐ N/A	
Other- please specify:	☐ Yes ☐ N/A	
Other- please specify:	☐ Yes ☐ N/A	

APPENDIX B – CONSENT FORM (SYN MSc/ 2014/15 December 12, 2014 missing in footer)

PARTICIPANT INFORMATION SHEET AND CONSENT FORM

STUDY TITLE: Assessment of Repeated Allergen Challenge and the Effects of Ibuprofen on the Inflammatory Process

PRINCIPAL INVESTIGATOR: Dr. Donald W. Cockcroft MD FRCP
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
Room 546 Ellis Hall, 103 Hospital Drive
Saskatoon, SK, Canada
S7N 0W8
1-306-844-1446

SUBINVESTIGATORS: Dr. Beth Davis, PhD and Dr. Shawn Nomani, MD

DEPARTMENT: Division of Respirology, Critical Care and Sleep Medicine,
College of Medicine, University of Saskatchewan

LOCATION OF STUDY: Room 346, Ellis Hall
University of Saskatchewan
1-306-844-1443

24 HOUR EMERGENCY CONTACT

In case of emergency or last minute scheduling changes feel free to phone or text message Shawn Nomani at xxxxxxxxxxxx, or email him at xxxxxxxxxxxxxx.

INTRODUCTION

Thank you for considering being a part of this study. You have been asked to consider participating in this study because you have been diagnosed with atopic (allergic) asthma. Your participation is entirely voluntary and you will be free to withdraw whenever you wish. Please take your time to review what will be required of you, and feel free to ask questions to ensure you fully understand what we will be doing, as well as the tasks we will ask of you. The

principal investigator and sub-investigators will be happy to answer any questions you may have. You may also discuss the study and your potential participation with friends, family, and/or your personal healthcare provider.

This study is being conducted by Dr. Cockcroft, Dr. Davis and the student researcher, Dr. Shawn Nomani. There are no external funding sources. The researchers and the University of Saskatchewan are not being paid to conduct this study.

Ten to fifteen participants will be required to complete the study.

PURPOSE OF STUDY

It has been found that the response of the airway to certain stimuli decreases when tests using these stimuli are performed at short time intervals. For example, in individuals that have exercise induced bronchoconstriction, the response to a second exercise test is less than the response to the first test when the tests are performed relatively close together (e.g. within an hour of each other). This is commonly referred to as a refractory period. A refractory period means a time frame when an individual's airways are less responsive to triggers of bronchoconstriction. Basically, the body is doing something to protect your lungs from another reaction. What we will be assessing in this study is the effects of performing back-to-back (24 hours) allergen challenges in those with atopic (allergic) asthma.

In addition, we will also be assessing what effect, if any, ibuprofen ingestion may have on allergen challenge tests. Ibuprofen has been shown to be safe to consume in those with asthma. Although the physiological mechanism which underlies the refractory period is unknown, we hypothesize that the body overproduces protective substances (mediators) which inhibit inflammation. One of these compounds is prostaglandin E2 (PGE2), which has been shown to be increased when individuals are exposed to repeat indirect challenges (e.g. exercise). Ibuprofen is a commonly used NSAID which inhibits the production of prostaglandins, including PGE2. Therefore, we will be able to see what effect, if any, ibuprofen will have on individuals who undergo an allergen challenge test, and hopefully gain insight into what causes this protection.

TYPE OF STUDY

This is a randomized double blind placebo controlled crossover study. It is randomized because participants will be randomly assigned to receive either the ibuprofen or placebo treatment first. It is a crossover study meaning you will receive and undergo testing after both treatments (i.e. active and placebo). It is double blind because neither the investigators nor the participants will know which treatment is being administered. A placebo arm is required so that the response following an active treatment (i.e response after ibuprofen) can be compared to the response when no treatment was administered. A placebo looks identical to the study drug but contains no active ingredient. There is no increased risk associated with receiving placebo prior to the allergen challenge. The response will likely be similar to what might happen to you

in real life if you were exposed to an allergen that triggers your asthma (e.g. cat), except that the exposure is controlled and emergency measures are readily available if necessary.

TRIAL DESIGN

Participating in this study will require 7 visits to the asthma research lab and span a minimum of 20 days (13 of which are "downtime"). The duration of each visit varies. The screening visit and visits 1 and 4 will require 1-2 hours. Visits 2, 3, 5 and 6 will require about 8 hours. During the initial screening visit we will review the study design, obtain your consent, gather pertinent medical and demographic information (e.g. height, weight) perform a baseline methacholine challenge test, and do a skin prick test (i.e. determine your eligibility). If you meet the inclusion criteria a schedule for the remaining visits will be organized.

After screening, you will be required to come to the lab for 2 sets of 3 consecutive days testing ("a triad"). A minimum of 13 days between each triad will be required. During Visit 1 we will have you come to the lab in the afternoon and we will perform a methacholine challenge test, collect sputum, blood and urine, and assess exhaled nitric oxide levels. We will also perform another skin prick test (SPT), the skin titration endpoint (STE), in which we will place doubling dilutions of a single allergen (chosen from the SPT results done during screening) to determine which dilution produces a small bump on your arm (2mm wheal or smaller). This will help us determine a safe starting dilution for the allergen challenge. As noted above, you can expect to be in the lab for approximately 1 – 2 hours on this day.

Visits 2 and 3 will be full days, and we will require you to come to the lab in the morning (between 7 – 8 am ideally) and undergo testing for approximately 8 - 9 hours. One hour before arriving to the lab we will require you to take a pill (provided to you) which will be either 400mg ibuprofen or placebo. We will also ask you to refrain from eating breakfast until we have completed the allergen challenge and collected some additional breathing measurements (about 2 hours). At the end of the day, after all the allergen challenge data has been collected we will assess exhaled nitric oxide levels, perform a methacholine challenge test, and collect sputum, blood and urine.

Visit 4, 5 and 6 will be identical to visits 1, 2 and 3 except that no STE test will be performed at visit 4.

Schematic Representation of Trial

Screening	Visit 1	Visit 2	Visit 3	13 day minimum washout	Visit 4	Visit 5	Visit 6
Consent Mch SPT	FeNO Mch STE sputum blood urine	AC (7h) FeNO Mch sputum blood urine	AC (7h) FeNO Mch sputum blood urine		FeNO Mch sputum blood urine	AC (7h) FeNO Mch sputum blood urine	AC (7h) FeNO Mch sputum blood urine

Mch – methacholine challenge test, SPT – skin prick test, FeNO – exhaled nitric oxide,
AC – allergen challenge test; STE – skin test endpoint

SAMPLES TO BE COLLECTED

During this study we will be collecting various biological samples including sputum (airway secretions), blood, and urine. Your samples will be collected and kept frozen in the lab until processing. We will be using these samples for assessing the types of cells and mediators/products of inflammation that may change after allergen exposure or in response to the ibuprofen. The specific proteins and cells which we will be assessing have not yet been determined but will relate to inflammation and asthma. We would also like to store your samples for future asthma related research. This future research will only use your samples to assess cells and mediators involved in allergic asthma and airway inflammation. This future research will NOT include genetic testing. You can indicate whether or not this is okay with you by checking the appropriate box on the signature page of this document.

TESTING PROCEDURES

Skin Prick Test and Skin Titration Endpoint (STE):

The skin prick test will be conducted once at the screening visit. This will involve small droplets of common allergens (animals, pollens, etc.) being placed on your forearm. A small scratch within the droplet will be performed which will determine if you have an allergy to that particular allergen. If so, a small bump similar to a mosquito bite will appear and will likely be red and itchy. From the skin prick test we will choose an allergen to use for the allergen challenge. At Visit 1, doubling dilutions of this single allergen will be prepared and these dilutions will also be placed on your forearm (in duplicate), scratched and observed for reactions.

Spirometry:

Breathing tests will be conducted at all visits. You will blow into a machine which you will hold in your hand. The machine has a mouthpiece attached to it which you will place in your mouth, inhale and exhale through while wearing nose clips. The rate of air which you exhale goes through a machine and is measured by a software program which will display the results on a computer screen.

Methacholine Challenge Test:

Methacholine is a commonly used agent in asthma testing that can cause constriction of bronchiole smooth muscles and a narrowing of the airways. The methacholine challenge involves breathing maneuvers as described above. In addition, you will be required to inhale a substance called methacholine. You will be inhaling increasing concentrations of methacholine by placing a mask over your nose and mouth. The mask is attached to an aerosol generating piece of equipment which functions to provide an inhalable solution of the methacholine. You will inhale the methacholine by breathing normally for two minutes. Any constriction that may result will be monitored by the breathing maneuvers. When and if a certain level of airway constriction occurs (20% decrease in the amount of air you can forcefully exhale), the test will be stopped. You may also stop the test at any time for any reason.

Allergen Challenge Test:

The allergen challenge also involves a number of breathing tests and the inhalation of an allergen identified by the skin prick test. The allergen is inhaled via mouthpiece and with nose clips on, over two minutes of tidal (normal) breathing. Ten minutes after an inhalation, spirometry is performed to assess the level of bronchoconstriction. The inhalation of allergen is stopped when the target decrease (20%) in the amount of air you can forcefully exhale is reached. Your lung function is then measured at each of the following time points: 20, 30, 45, 60, 90 and 120, 180, 240, 300, 360 and 420 minutes after the allergen challenge to assess the development of a late asthmatic response.

Exhaled Nitric Oxide:

Exhaled nitric oxide (FeNO) will be measured using a chemiluminescence gas analyzer; a small machine which you hold in your hands. You will perform an inhalation to total lung capacity followed by an exhalation with a constant flow rate of 50 mL/sec via a filter/mouthpiece. The procedure will be performed in triplicate and will continue until at least 2 measurements are reproducible within 10%. FeNO will be collected at each visit except the screening visit. FeNO is an indicator of airway inflammation. By measuring FeNO we can monitor how levels of airway inflammation are changing and whether or not ibuprofen has an effect on this.

Sputum Induction/Collection:

This is a procedure that is used to help you produce sputum (i.e. mucus/airway secretions) from your lungs. You will be required to inhale salty water (i.e. hypertonic saline) of different concentrations (3%, 4%, and 5%) each for 7 minutes and then, after blowing your nose and rinsing your mouth, you will try to produce a mucus sample that you will spit into a cup. We then process and analyze the sample to obtain information about inflammation in your lungs.

BENEFITS

If you choose to participate in this study, there are no direct benefits to you. However, it is hoped that the information gathered in this study can be used in the future to further research and help those who suffer from allergic asthma.

POSSIBLE RISKS

Skin Prick Testing:

Skin prick tests are usually well tolerated. Local itch and swelling may occur and will normally subside within 1 to 2 hours. Severe reactions may be treated with an oral antihistamine, topical corticosteroid cream, and/or an ice pack.

Spirometry:

Performing spirometry may cause you to cough, experience chest tightness and shortness of breath, or feel light-headed. While these symptoms may be uncomfortable, there is no safety concern. Resting between spirometry maneuvers, administering a bronchodilator or not performing spirometry are possible solutions. Trained medical personnel will be on hand to deal with any concerns.

Methacholine and allergen challenges:

Methacholine and allergen inhalation challenges are done to induce your asthma. If you are an allergic asthmatic these tests will very likely result in symptoms of asthma such as shortness of breath, cough, chest tightness and wheezing similar to what you would experience in the real world. Rarely, symptoms may include headache, throat irritation or light headedness. Responses to allergen can be severe. Both challenge tests are performed according to highly standardized procedures by extensively trained and experienced personnel. Should the need arise, immediate medical care will be available.

Sputum Induction

Sputum induction may also cause cough, shortness of breath/wheezing and/or narrowing of the airways. If your experience symptoms and these are bothersome, the test can be stopped and the discomfort reversed with a bronchodilator (e.g. Ventolin®).

Blood Draws

Blood draws can be associated with discomfort where the needle is inserted, bruising, swelling, and rarely, a local infection at the site of the needle poke.

Ibuprofen risks

Ingesting ibuprofen once, as a single 400mg dose is associated with minimal risk. High dose, chronic use of ibuprofen however can be associated with serious side effects. We can discuss this with you further and/or provide you with the product insert if you would like additional information.

Reproductive risks

The reproductive risks of methacholine and allergen challenges are unknown. If you are a sexually active woman capable of becoming pregnant (sexually mature woman who has not undergone a hysterectomy or who has not been post-menopausal for 24 consecutive months), you must use a medically approved effective method of birth control, or you must not have sexual intercourse that could result in pregnancy during your participation in this study.

If you become pregnant during the study you will be withdrawn from the study. Women who are pregnant or breastfeeding are not eligible to participate in this study.

VOLUNTARY WITHDRAWL

Your participation in this research is voluntary. You may withdraw from this study at any time. You do not have to provide a reason. There will be no penalty or loss of benefits if you choose to withdraw. Your current or future medical care or academic status will not be affected.

If you choose to enter the study and then decide to withdraw later, all data collected about you during your enrolment will be retained for analysis.

In addition, you may be withdrawn from the study by study staff because of reasons not known at the present or for reasons including the possibility that staying in the study could be harmful to you or you require a medication that you are not allowed to use while participating in the study.

COSTS ASSOCIATED WITH THE STUDY

There will be no charges incurred to you for any research related procedures. An honorarium of $ will be provided to cover your time and out-of-pocket expenses such as travel, parking, and meals. We will require your Social Insurance Number (SIN) which will be forwarded to financial

services at the University of Saskatchewan for taxation audit purposes. Please be aware that you will receive a T4 relating to your honorarium. If you decide to withdraw early from this study, your compensation will be proportional to your time in the study.

RESEARCH RELATED INJURY

In the case of a medical emergency related to the study, you should seek immediate care, and notify the study doctor at your earliest convenience. Feel free to consult your own personal medical staff about your participation in this study, and seek their advice if you want an outside opinion. Any necessary medical treatment will be made available to you at no cost. During testing we will have rescue inhalers and trained medical professionals on site. By signing this document you do not waive any of your legal rights.

CONFIDENTIALITY

In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. The study data will be stored securely (in a locked cabinet contained within a locked office under the supervision of the PI) by the study team for a minimum of 5 years after the final results are published. Research records and medical records identifying you may be inspected in the presence of the Principal Investigator and/or the Research Ethics Board for monitoring purposes. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.

STUDY RESULTS

Study results will be available after all testing in all participants has been completed and the data analyzed. As mentioned above, we hope to publish the data in a scientific journal. Once this has been done, a copy of the paper can be provided to you. Indicate your interest in obtaining study results (or not) by checking the appropriate box on the signature page of this form.

CONTACT INFORMATION IF YOU HAVE ANY QUESTIONS

We encourage you to ask any questions at any time about this study and your participation in it. Dr. Donald Cockcroft and Dr. Beth Davis can be reached at 306-844-1444. Dr. Shawn Nomani can be reached via email at xxxxxxxxxxxxxxxxxxxx.

The chair of the University of Saskatchewan Ethics Research Board can be telephoned at 306—966-4053. You are encouraged to contact them if you have any concerns about your rights as a

research participant, complaints about how you have been treated by the investigators or any negative experiences you had while being part of the study.

The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Biomedical Research Ethics Board.

CONSENT TO PARTICIPATE

Study Title: Assessment of Repeated Allergen Challenge and the Effects of Ibuprofen on the Inflammatory Process

- I have thoroughly read the information in this consent form, and understand what will be required of me.
- I understand the purpose, procedures, and potential risks.
- I was given sufficient time to decide if I wanted to participate in this study, without any coercion.
- I understand that I will give blood, sputum, and urine for testing purposes, described in this form.
- I had the opportunity to ask questions and have received satisfactory answers.
- I understand that I am free to withdraw from this study at any time for any reason, and I will not be required to provide my rationale and it will not affect my future care or academic status.
- I give permission for the researchers to use all information collected during this study, and acknowledge that it will be used for publication purposes in a de-identified manner.
- I understand that by signing this document I do not waive any of my legal rights.
- I understand I will be given a signed and dated copy of this consent form.

I have read the information pertaining to the use of my blood, urine and sputum samples for future allergic asthma research.

□ Yes, you may store biological samples for future research on allergic asthma.

□ No, you may not store my biological samples for future research on allergic asthma.

□ I am interested in the study results. Please contact me when the results are available.

□ I am not interested in the study results.

I agree to participate in this study:

_____

Printed name of participant: Signature Date

_____

Printed name of person obtaining consent: Signature Date

APPENDIX C – CERTIFICATE OF APPROVAL

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	14-283

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
University of Saskatchewan

Saskatoon SK

SUB-INVESTIGATOR(S)
Beth Davis

STUDENT RESEARCHER(S)
Shawn Nomani

FUNDER(S)
INTERNAL FUNDING

TITLE
Assessment of Repeated Allergen Challenge and the Effects of Ibuprofen on the Inflammatory Process

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
03-Dec-2014	15-Dec-2014	Research project as outlined in the revised Application for Biomedical Research Ethics Review and the study protocol Revised Participant Information Sheet and Consent Form (12-Dec-2014) PAWS Recruitment Poster	14-Dec-2015

Delegated Review ☐ Full Board Meeting ☒

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The Bio-REB considered the requirements of section 29 under the Health Information Protection Act (HIPA) and is satisfied that this study meets the privacy considerations outlined therein. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://www.usask.ca/research/ethics_review/.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	14-283

approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research projects involving human subjects under section 29 of The Health Information Protection Act (HIPA).

[redacted]

Ildiko Badea, Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

APPENDIX D – PUBLICATION INFORMATION

This study was conducted with the intent of publishing the results. A manuscript was drafted and has been accepted for publication. Publication information is as follows:

Allergy, Asthma & Clinical Immunology.2016, 12:24

DOI:10.1186/s13223-016-0127-z

URL:http://www.aacijournal.com/content/12/1/24