ALLERGEN-INDUCED CHANGE IN AIRWAY RESPONSIVENESS TO DIRECT AND INDIRECT STIMULI IN MILD ATOPIC ASTHMATICS.

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
University of Saskatchewan,
Saskatoon

By
DANIEL OPPONG AMAKYE

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of the University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Physiology,
University of Saskatchewan,
Saskatoon, Saskatchewan, S7N 5E5.

ABSTRACT

Methacholine (MCh) and mannitol challenges are tests used to assess airway responsiveness. It has been shown that airway responsiveness to direct bronchoconstrictors like MCh tends to increase following exposure to allergen but the response to mannitol an indirect stimuli, is not known. Furthermore, the provocative concentration causing a 20% decrease in Forced Expiratory Volume in one second (FEV_1) for adenosine 5' monophosphate (AMP) correlates better to sputum eosinophilia than MCh PC_{20}. Hence, we hypothesized that airway responsiveness will be greater when measured with mannitol than MCh. We studied airway responsiveness to MCh and mannitol first at 3 hours and then later at 24 hours after allergen challenge. The 3-hour study yielded results contrary to our hypothesis therefore a twenty-four hour study was undertaken. Ten mild atopic asthmatics who had a positive MCh challenge and an allergic response to allergen extracts such as cat, horse, and house dust mite completed the 3-hour study. Eleven mild atopic asthmatics with the criteria above completed the 24-hour study. Both studies were non-blinded, randomized clinical trials. Airway responsiveness to MCh was quantitated by changes in PC_{20}. Airway responsiveness to mannitol was quantitated as PD_{15} in the 3-hour study and dose response ratio (DRR) in the 24-hour study. In both studies, the allergen challenges were separated by 14 days. Fractional exhaled nitric oxide measurements (F_ENO) were collected in both studies at varying time points to track airway inflammation. In the 3-hour study, the geometric mean MCh PC_{20} decreased significantly after allergen exposure from 0.88 mg/ml to 0.50 mg/ml ($p = 0.02$) indicating airway responsiveness to MCh increased. Conversely, the geometric mean mannitol PD_{15} increased significantly from 174 mg to 284 mg ($p = 0.02$) indicating a decrease in airway responsiveness to mannitol. In the 24-hour study, the geometric mean MCh PC_{20} again decreased significantly from 5.9 mg/ml to 2.2 mg/ml ($p = 0.01$) after allergen exposure. The mannitol DRR increased significantly from 63 mg/$\Delta\%FEV_1$ to 158 mg/$\Delta\%FEV_1$ ($p = 0.03$). F_ENO levels increased significantly in MCh arm but not mannitol arm. That is pre allergen challenge versus 24 hours after allergen challenge (for MCh arm: 26 ppb pre to 55 ppb post; for mannitol arm: 31 ppb pre to 39 ppb post). In conclusion, at three and twenty-four hours after allergen challenge, a time when the airways are more responsive to MCh, there is a significant decrease in airway responsiveness to mannitol.

ACKNOWLEDGEMENTS

First of all, the author will like to thank Dr. D.W.Cockcroft for taking him on as a student in his laboratory, becoming his supervisor in both his undergraduate honors project and graduate research. Secondly, the author will also like to thank Dr. Beth Davis for her immense support and contributions towards his learning in the asthma lab. Thirdly a special thanks to Dr. Gregory Peters for helping lay the original paper work foundation for this study, and Lexie Martin for her numerous laboratory assistance. The author also acknowledges Pharmaxis for supplying the mannitol kits. Finally the author appreciates all the friendly participants in the study, for sharing their precious time and lungs with the asthma lab. This work would not be possible without the combined efforts of all the wonderful people above. Once again the author appreciates you all.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF FORMULAE

LIST OF ABBREVIATIONS

AHR - Airway Hyperresponsiveness

AMP - Adenosine 5’ Monophosphate

APC - Antigen Presenting Cells

BHR - Bronchial Hyperreactivity

Cys-LTs - Cysteinyl-Leukotrienes

DC - Dendritic Cells

DP - *Dermatophagoides pteronyssinus*

EAR - Early Asthmatic Response

EIB – Exercise Induced Bronchoconstriction

EVH - Eucapnic Voluntary Hyperpnea

FcεRI - High Affinity IgE Receptor

F_ENO - Fractional Exhaled Nitric Oxide

GINA - Global Initiative for Asthma

GM-CSF - Granulocyte/Macrophage Colony-Stimulating Factor

HIB - Hyperventilation Induced Bronchoconstriction

IgE - Immunoglobulin E

IL - Interleukin

LAR - Late Asthmatic Response

LTC_4 - Leukotriene C4

LTD_4 - Leukotriene D4

LTE_4 - Leukotriene E4

PC_{20} - Provocative Concentration causing a 20% decrease in FEV_1

PD_{15} - Provocative Dose causing a 15% decrease in FEV_1

DRR – Dose Response Ratio

SD - Standard deviation of the mean

SE - Standard Error of the mean

TGF - Transforming Growth Factor-

T_H1 - T helper cell type 1

T_H2 - T helper cell type 2

VEGF - Vascular Endothelial Growth Factor

$VO_{2\ max}$ - Maximal Oxygen Consumption

WHO - World Health Organization

1. ASTHMA – AN OVERVIEW

1.1 Introduction

According to the Global Initiative for Asthma (GINA), asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness (AHR), leading to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing. A major characteristic of asthma is spontaneously reversible airflow obstruction or resolvable airway obstruction with treatment [Global Initiative for Asthma, 2014].

The complexity of asthma and the incomplete knowledge of asthma have caused an evolving standard for the classification of asthma into phenotypes and endotypes [Corren, 2013]. An example of a phenotype of asthma is eosinophilic asthma. The eosinophilic phenotype includes allergic asthma, aspirin sensitive asthma, and severe-late-onset hyper-eosinophilic asthma. Another asthma phenotype is poor steroid responsive asthma. This phenotype of asthma includes non-eosinophilic (neutrophilic) asthma, steroid-insensitive eosinophilic asthma, and airflow obstruction caused by obesity endotypes [Lötvall *et al*., 2011]. Asthma can be broadly classified into two main groups, namely, extrinsic (atopic) and intrinsic (non-atopic).Extrinsic asthma is triggered by allergens. In the extrinsic asthma, the immune system “overacts” in exerting a protective mechanism in response to harmless substances such as pollen. The causative mechanism for intrinsic asthma is unknown although extremes of emotions, contact with chemicals or exercises are known to cause intrinsic asthma. These agents stimulate the response of nerves in the airways culminating into the symptoms of asthma. Knowing the type or classification of asthma greatly influences the treatment.

The two main therapeutic effects sought after in asthma are the relaxation of airway smooth muscle (bronchodilation) and suppression of airway inflammation depending on disease severity [Rabe *et al.,* 2006]. Bronchodilators act through the sympathetic adrenergic neuroendocrine pathways, which cause a depletion of intracellular calcium and a decrease in contractile force leading to smooth muscle relaxation [Knox *et al.,* 1995]. Anti-inflammatory therapy targets the pathophysiological

mediators that contribute to the exacerbation of asthma inflammation. The anti-inflammatory therapies include antihistamines, lipooxygenase inhibitors, leukotriene antagonists [Cobanoglu *et al.,* 2013], inhaled corticosteroids and mast cell stabilizers.

Asthma is one of the most chronic diseases in the Canadian population. Asthma affects 2.4 million Canadians over the age of 12 (8.5 percent of the population) and other 490 000 children between the ages of 4 and 11 [Government of Canada, Statistics Canada, 2010; Government of Canada Public Health Agency of Canada, 2007]. According to doctors, fifty-three percent of Canadians with asthma poorly control the disease [Chapman *et al.,* 2008]. Asthma is more prevalent among First Nations, Inuit, and Metis communities than in the general Canadian population by a margin of 40 percent [Tait, 2008]. About 250 deaths from asthma are recorded yearly within the Canadian population [Rowe, 2010].Asthma and allergies affect the lives of many people, both in the low–income and high-income countries. WHO admits that asthma is a public health problem, however complete pathology of asthma is not known; as such, there is the need for research into allergic diseases especially asthma. Uncovering the group of population susceptible to a specific type of asthma can help track the causes, diagnosis and possible treatment of asthma. This can help the government to allocate resources and help fund researches specific for a population. For example if a specific pollen or chemical in the environment is causing an increase in a specific type of asthma, the appropriate steps will be taken to curtail the outbreak. The different types of asthma cannot be detected by a single test, hence the need to have more tests to help distinguish the different types of asthma and indicate the course of treatment for the asthma type. Allergic asthma happens to be a good model for elucidating the pathogenesis of asthma and producing new therapeutic strategies for asthma. For the purpose of this research study, more emphasis is put on allergic asthma.

1.2. Atopic Asthma

The American Academy of Allergy, Asthma and Immunology regards atopic asthma as the genetic tendency to develop allergic disease (asthma). An important feature of atopy is a heightened immune response to inhaled allergens. A cumulative

effect on the immune response to a sensitized allergen is the contraction, inflammation, and subsequent narrowing of the airways. These symptoms manifest as coughing, wheezing, and other asthmatic symptoms [American Academy of Allergy Asthma and Immunology, 2014]. An IgE response to allergenic proteins prompts the emergence of allergic airway inflammation [Platts-Mills, 2001]. The proteins cross link high affinity IgE receptors ($F\varepsilon RI$) on mast cells, which cause degranulation. Activation and degranulation of mast cells lead to the release of a variety of bronchoconstricting mediators such as histamines, leukotrienes (LTC_4), and pro-inflammatory prostaglandins.

1.2.1. Airway responses in allergen inhalation challenge

Allergen challenges are primarily employed in research regarding cellular and humoral mechanisms that surround the nature of allergen induced airway responses. Allergen inhalational challenges involve exposing the participant to an allergen. The participant is usually sensitized to the allergen before the laboratory exposure. The inhalation of allergen results in the subsequent activation of secretory pathways leading to the release of preformed and newly generated mediators of bronchoconstriction and vascular permeability [Gauvreau *et al.,* 2007]. Airway response is grouped according to the period that the symptoms of bronchoconstriction appear. The airway responses are categorized into early asthmatic response and late asthmatic response.

1.2.2. Early Asthmatic Response

The early asthmatic response describes an episode of bronchoconstriction occurring within 10 minutes of allergen exposure. EAR usually resolves spontaneously in 2 to 3 hours or sooner with treatment. EAR is the easiest allergen response to identify in a clinical setting due to its clinical symptoms occurring shortly after exposure to inhaled allergens. All allergic asthmatics have an EAR. EAR depends largely on the release of mediators from mast cells of the airways hence, EAR can be blocked by nedocromil, cromoglycate (mast cell stabilizers) and salbutamol (beta $_2$ agonists) [Cockcroft *et al*., 1987a].

1.2.3. Late Asthmatic Response

The late asthmatic response (LAR) describes an episode of recurrent bronchoconstriction, occurring between three and eight hours following allergen exposure. The LAR occurs in about half of people with a positive allergen challenge. LAR is closely associated with allergen-induced eosinophilic airway inflammation lasting up to several days [Pin *et al*., 1992]. In some severe cases, the LAR may not be fully abolished with just bronchodilators. This suggests that either cellular or non-cellular aspects of inflammation are also involved in the pathogenesis of asthma [Cockcroft *et al.,* 1987a]. LAR causes the influx and activation of inflammatory cells, particularly lymphocytes and eosinophils in the bronchial mucosa [Robinson *et al*., 1993]. As such, nedocromil, cromoglycate (mast cell stabilizers) and steroids [Cockcroft et al., 1987a] can also abolish the LAR. The figure below (figure 1.1) shows a sample of the early and late asthmatic responses.

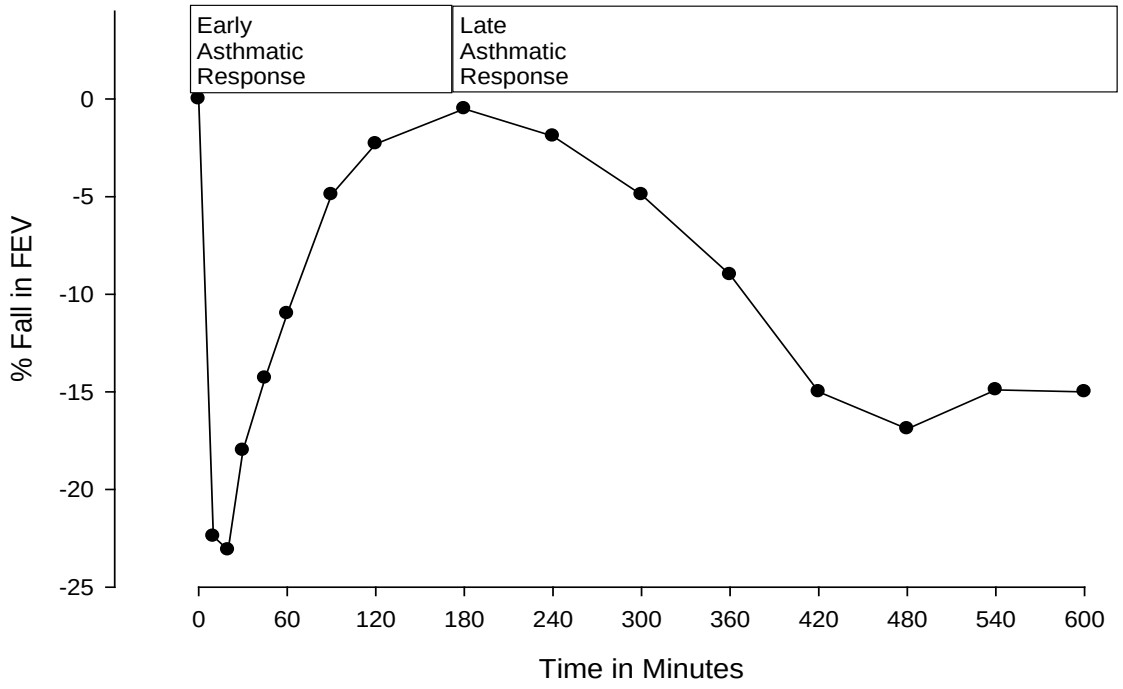

Figure 1.1. Graphical representation of the early and late asthmatic response assessed as percent decrease in FEV_1

1.2.4. Sequelae: Increased airway responsiveness and inflammation

According to the guidelines for diagnosis and management of asthma, airway inflammation and hyperresponsiveness are recognized as major characteristics of bronchial asthma [U S Department of Health and Human services, National Institutes of Health, National Heart, Lung and Blood Institute Expert Panel Report, 2007]. However, the relationship between airway inflammation, hyperresponsiveness, remodeling of the airway walls and their connection to airway smooth muscle in asthma is not clear [Holgate *et al.,* 2008]. A key defining characteristic of asthma is airway hyperresponsiveness (AHR) to direct acting stimuli like methacholine [Hargreave *et al.,* 1981].

Allergen exposure causes an increase in airway responsiveness consistent with the recruitment of inflammatory cells in the airways [Brusasco *et al.,* 1990]. This suggests that there may be a relationship between airway hyperresponsiveness and airway inflammation [Kirby *et al.,* 1987; Bradley *et al.,* 1991]. On the other hand, Crimi *et al.,* argue a dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma [Crimi *et al.,* 1998]. Allergic asthma is dependent on the presence of IgE antibodies specific to allergens in the lungs. Upon sensitization of an individual to a particular antigen, future encounters with the allergen cause the crosslinking of IgE bound to the high affinity IgE receptor FcεRI. The crosslinking of IgE stimulates the release of pre-formed mediators, and the newly generated mediators responsible for the early allergic response. A later reaction (late allergic response) may result from the subsequent release of cytokines and chemokines that recruit macrophages, eosinophils, and basophills [De Monchy *et al.*, 1985; Durham *et al.,* 1988; Macfarlane *et al.,* 2000].

Allergic asthma is widely believed to be triggered by T-helper type two (T_H2) lymphocytes of the immune system. The T_H2 cell pathway is initiated by the uptake of allergens by antigen presenting cells (APCs). The APCs present selected peptides to naïve T cells, by directing them in favor of T_H2 cell phenotype that mediates cytokine secretion [Hammad *et al.,* 2006]. Dendritic cells (DCs) are responsible for initiating and maintaining allergic T_H2 cell response to inhaled allergens in asthma [Hammad *et al.,* 2008]. T_H2 cells induce the survival and recruitment of eosinophils and mast cells. In

addition to the bronchial hyperreactivity, goblet cell hyperplasia, degranulation of mucus-secreting cells, micro-vascular remodeling, leakage, and chemo-attraction of inflammatory cells is also induced. These changes lead to potentiation of inflammation and contribute to some of the characteristics of asthma, including sputum production, airway narrowing, exacerbations and accelerated loss of lung function [Rose *et al.,* 1997, Fahy *et al.,* 1998]. These changes in bronchial hyperreactivity are via excitability of bronchial smooth muscle cells, in response to various nonspecific stimuli such as cold air or physical exercise [Hammad *et al.,* 2008]. T_H2 cell-mediated inflammation in asthmatic airways is suppressed by corticosteroids through the inhibition of expression of cytokines, chemokines, and adhesion molecules [Barnes *et al.,* 1998].

Bronchoconstriction in asthma can also happen via degranulation of mast cells and production of T_H2 cell associated cytokines. These processes by mast cells occur in the smooth muscle layer surrounding the airway wall [Brightling *et al.,* 2002]. B cells are key immunological cells that help in capturing and processing allergens [von Garnier *et al.,* 2007]. T cells on the other hand, help coordinate the up-regulation and expression of cytokines that include interleukin-3 (IL-3), IL-4, IL-5, IL-9, IL-13 and granulocyte/macrophage colony-stimulating factor (GM-CSF) [Cousins *et al.,* 2002]. These cytokines are involved in IgE synthesis of B-cell switching (IL-4 and IL-13), mast cell recruitment (IL-4, IL-9 and IL-13), eosinophil maturation (IL-3, IL-5 and GM-CSF) and basophils (IL-3 and IL-4). Basophils are also mediator-secreting effector cells of the allergic response [Holgate *et al.,* 2008]. The recent discovery of another subset of CD_4+ cells (regulatory T cells T_{REG}) has affected and driven research into allergic diseases. T_{REG} cells have been strongly implicated in the suppression of allergic responses [Bachetta *et al.,* 2007]. T_{REG} cells also control T_H2 cell responses in humans through cytokines IL-10, and transforming growth factor-β (TGF–β) [Larche *et al.,* 2007].

1.3. Bronchoprovocation with direct acting stimuli

Bronchoprovocation tests are done with the aim of causing bronchoconstriction via airway challenge tests with a stimulus. The direct acting stimuli provoke airway smooth muscle contraction by activating smooth muscle cell receptors. These kinds of stimuli include histamine, leukotrienes, and muscarinic agonists like methacholine. The

indirect stimulus involves intermediate steps leading to bronchoconstriction due to the release of mediators from inflammatory cells such as mast cells [Pauwels *et al*., 1988]. Unlike direct stimuli, indirect stimuli do not interact physically with airway smooth muscle receptors. Although both stimuli results in bronchoconstriction, they do so in different pathways.

1.3.1. Methacholine

Methacholine is a synthetic choline ester that acts as muscarinic receptor agonists in the parasympathetic nervous system. As a parasympathetic agent, methacholine reverses bronchodilation. In the case of an asthmatic airway, methacholine causes bronchoconstriction hence its use to diagnose bronchial hyperreactivity. The presence of a methyl group on methacholine makes it sensitive to muscarinic receptors as compared to nicotinic receptors hence it has little effect on nicotinic receptors and does not cross the blood brain barrier. It is resistant to acetylcholineterase hence it is broken down at a slower rate in the body. Adverse effects of methacholine are mostly cardiovascular that is bradycardia and hypotension, as such a preference for its use in the airways.

A 20% fall from a baseline FEV_1 at a methacholine concentration less than 16 mg/ml is considered a positive methacholine challenge [Crapo *et al.,* 2000]. The provocation concentration of methacholine causing a 20% fall in FEV_1 is termed methacholine PC_{20} (MCh PC_{20}). MCh PC_{20} is distributed in a normal log fashion in the population, with no sharp cut-point between normal participants without asthma and asthmatic participants. The methacholine challenge test involves a doubling methacholine concentration administered at a fixed five-minute interval. Within the five-minute interval, a known concentration of methacholine is administered followed by the measurement of FEV_1. The results are expressed as MCh PC_{20}. The methacholine test has a high negative predictive value; hence, a MCh PC_{20} greater than 16 mg/ml excludes asthma in some circumstances. Methacholine challenge testing is more useful in excluding a diagnosis of asthma rather than establishing the presence of asthma. This is because the methacholine challenge test has a greater negative predictive

power than positive predictive power [Crapo *et al.,* 2000]. Furthermore, methacholine tests are highly sensitive but not specific for asthma diagnosis.

1.4. Bronchoprovocation with indirect stimuli

Indirect stimuli include physical stimuli such as; exercise and cold air, chemical stimuli such as adenosine 5' monophosphate (AMP) and mannitol, inhaled particulate irritants and sensitizing stimuli such as allergens. Mannitol is an osmotic indirect stimulus. Indirect stimulus requires a relatively high dose of the stimulus to provoke bronchoconstriction when compared to direct stimuli. Natural occurring asthma involves exposure to indirect agents of bronchoconstriction; hence, indirect airway responsiveness is specific for asthma activity and inflammation. One could argue that a positive indirect challenge such as a positive exercise challenge can be used as a diagnosis or an inclusion criterion for asthma. Moreover, indirect challenges show a better correlation with airway eosinophils than direct challenges. Although both AMP PC_{20} and MCh PC_{20} correlate with airway eosinophils, it has been shown that AMP PC_{20} correlates better with airway inflammation level than MCh PC_{20} [van den Berge *et al.,* 2001a]. Indirect challenges have a high level of specificity and positive predictive value; hence, indirect challenges tend to complement direct challenges. Direct stimuli (methacholine) are better at ruling out asthma while indirect stimuli (mannitol, AMP, EIB) are better at predicting the presence of asthma. Since it is known that airway hyperresponsiveness to direct stimuli changes after allergen challenge, we will like to know what happens to indirect stimuli in a similar circumstance.

1.4.1. Exercise Induced Bronchoconstriction (EIB)

Exercise does not cause asthma: however, it is a frequent trigger. The lack of specific symptoms makes it difficult to diagnose EIB because the symptoms could be seen as a manifestation of vigorous exercise. Methacholine and mannitol are pharmacological agents used for the diagnosis of EIB. Methacholine challenge test have a lower sensitivity to EIB as compared to mannitol. EIB is closer to mimicking the asthma in a real case scenario as such attention is paid to how closer methacholine and mannitol

are at diagnosing EIB. Secondly, since mannitol is a newer diagnostic tool for asthma, researchers like to compare its performance with methacholine in different conditions that mimic asthma.

EIB describes the brief narrowing of the airways following participation in vigorous exercise. This condition is present in both asthma patients and non-asthma patients [Freed *et al.,* 2008]. Clinically, EIB is characterized by a post exercise decrease between 10% and 15% in forced expiratory volume in one second (FEV_1) of the pre-exercise FEV_1 [Anderson *et al.,* 2010]. Although spontaneous recovery of the FEV_1 occurs within 30 to 60 minutes following an EIB episode, half of the individuals become refractory to a repeated exercise stimulus within 4 hours [Freed *et al.,* 2008]. EIB is among the first symptoms to appear and the last symptoms to disappear with treatment [Porsbjerg *et al.,* 2005]. Scuba divers are among sport individuals whom the diagnosis of EIB is critical. The breathing of dry air from the oxygen tank during underwater or surface swimming tends to be a stimulus for EIB. Individuals with EIB who have a low aerobic fitness, have a high percentage of maximal oxygen consumption ($VO_{2\,max}$) when exercising, than aerobic fit individuals [Astrand *et al.,* 1970]. The onset and severity of EIB are related to exercise intensity [Carlsen *et al.,* 2000]; hence, improved fitness allows asthmatics to work at a lower VO_{2max} percentage to reduce EIB [Henriksen *et al.,* 1983]. It has been shown that between running (free-range and treadmill) and cycling, free-range running caused the most EIB [Anderson *et al.,* 1971]. Beta agonists could prevent EIB, via a direct effect on bronchial smooth muscle. In addition, the mast cell stabilizer such as sodium cromoglycate, which block the release of mediators from mast cells [Davies, 1968; Poppius *et al.,* 1970], are useful in reducing the severity of EIB when taken prior to exercise [Silverman *et al.,* 1972].

1.4.1.1. Mechanism of Exercise Induced Bronchoconstriction

Although the exact explanation for the mechanism of EIB has not been found yet, the crucial stimulus for EIB is heat loss or water loss from the airways during exercise. Factors used to determine the severity of EIB include pulmonary ventilation, water content of the airways and the temperature of inspired air. Deal and colleagues showed

a correlation between the severity of EIB and respiratory heat loss [Deal *et al.,* 1979a]. Deal *et al.,* placed emphasis on thermal load rather than the drying of the airways. Deal and colleagues concluded that the magnitude of EIB is directly proportional to thermal load on the airways. This confirms the importance of the temperature of the inspired air in EIB. The airway cooling and drying are considered to stimulate the release of inflammatory mediators such as prostaglandins [Finnerty *et al.* 1990], and leukotrienes [Reiss *et al.,* 1997]. Therefore, the control of the rate of water loss from the airways and the inspired water content is key to managing EIB.

1.4.1.2. Refractoriness in Exercise Induced Bronchoconstriction

A consequence of EIB following repeated exercise challenges is refractoriness. The airway response decreases as the interval between exercise challenges decrease [Edmunds *et al.,* 1978]. Cross refractoriness also exists between EIB and hyperventilation induced bronchoconstriction (HIB) [Bar-Yishay *et al.,* 1983; Ben Dov *et al.,* 1983]. Refractoriness to EIB and hyper-osmolar challenges is due to the release of inhibitory prostaglandins, whose effect persists for 30-60 minutes [Margolskee *et al.,* 1988; Mattoli *et al.,* 1978]. Manning *et al's* crossover challenges with exercise and leukotriene D4 (LTD4 with and without a prostaglandin synthetase inhibitor) found out that, refractoriness with all types of paired challenges were reduced by the prostaglandin inhibitor [Manning *et al.,* 1993]. This implicates LTD4 in EIB and the release of inhibitory prostaglandins in refractoriness to exercise.

1.4.1.3. Similarities between Exercise Induced Bronchoconstriction and Adenosine 5' Monophosphate (AMP)

In a study by Godfrey *et al.,* it was found that direct challenge by methacholine was able to distinguish both asthma and pediatric COPD from their controls with a sensitivity of 82% to 92%. However, the methacholine challenge test could not distinguish between asthma and pediatric COPD. Interestingly, both EIB and AMP distinguished asthma and pediatric COPD from their controls with a sensitivity and specificity of 85% to 90%. What's more, exercise and AMP were able to distinguish asthma from pediatric COPD with a sensitivity and specificity of 85% to 90% [Avital *et*

al., 1995; Godfrey *et al.,* 1991]. This suggests a similarity in the mechanisms of the EIB and AMP. Perhaps, shared intermediate pathways that involve the release of mediators of inflammation exist between AMP stimulation and EIB.

1.4.2. Eucapnic Voluntary Hyperpnea (EVH)

Eucapnic voluntary hyperpnea (EVH) is a recommended laboratory test used in the identification of indirect agents of AHR such as EIB [Rundell KW *et al.,* 2004]. The EVH test involves inhaling a dry gas mixture containing 4.9% to 5% carbon dioxide, 21% oxygen, and the remaining gas as nitrogen [Anderson*,* 2010]. EVH protocol requires the participant to hyperventilate the dry gas mixture for 6 minutes at 30 times FEV_1. The maximum level of ventilation achieved during exercise is 17-21 times the FEV1, which is below the ventilation achieved by voluntary hyperventilation (30 times the FEV_1). The high ventilation rate and the dry air result in a low rate of false negative test results for EIB. Although a United Kingdom study of the EVH test concluded that EVH could help identify the EIB in previously undiagnosed elite athletes, the clinical diagnosis of EIB was not confirmed by the test result [Dickinson *et al.,* 2011].

1.4.3. Adenosine 5’ Monophosphate

Adenosine is a potent bronchoconstrictor that stimulates the (non-osmotic) release of mediators from airway mast cells [Cushley *et al.,* 1985; Driver *et al.,* 1991]. It has been shown in mast cells derived from mouse bone marrow in tissue culture that, adenosine potentiates the release of preformed mediators, and not the newly generated mediators [Marquardt *et al.,* 1984]. Adenosine induces bronchoconstriction indirectly via stimulation of adenosine 2_B receptors on mast cells. This results in the release of mediators from mast cells [Phillips *et al.,* 1990; Peachell *et al.,* 1988; Polosa *et al.,* 1995]. Results from clinical studies have shown that, bronchial hyperreactivity to AMP depicts allergic airway wall inflammation more accurately than bronchial hyperreactivity to methacholine [van Velzen *et al.,* 1996; Oosterhoff *et al.,* 1993].It has been demonstrated that, AMP correlates with sputum eosinophilia in allergic rhinitis than methacholine correlating with sputum eosinophilia [Polosa *et al.,* 2000]. This supports the notion that AMP is a better marker for bronchial inflammation than methacholine.

Furthermore, AMP has been shown to be a more sensitive marker in identifying mild allergic airway inflammation. AMP has a positive correlation with the number of eosinophils in sputum and in peripheral blood. However, AMP is a less potent stimulus for bronchoconstriction than methacholine [Van den Berge *et al.,* 2001]. AMP and methacholine responsiveness are not correlated with each other. This suggests that each challenge represents a different path to bronchoconstriction.

1.4.4. Mannitol

Mannitol is a naturally occurring sugar alcohol [Anderson *et al.,* 1997] in fruits and vegetables. Mannitol causes bronchoconstriction when inhaled by some people with hyperresponsive airways especially some asthmatics. Mannitol is used as a pharmaceutical excipient, food additive, and bulk sweetener. Mannitol and exercise can also be used as separate tools in assessing bronchial hyperresponsiveness. Mannitol achieves bronchoconstriction by creating an osmotic condition via increasing the osmolarity of airway surface liquid. The tissue dryness or lack of moisture leads to the release of mediators like prostaglandins, leukotrienes, histamine from mast cells, and other inflammatory cells. Adverse effects of mannitol include headache, throat irritation, nausea, cough, rhinorrhea, dyspnea, chest discomfort, and wheezing in those with a positive test. Mannitol challenge tests are generally safe and well tolerated [Brannan *et al*., 2005].

1.4.4.1. Mannitol Challenge testing

The mannitol challenge was performed using a mannitol test kit named Aridol (Aridol; Pharmaxis Inc. French's Forest New South Wales, Australia). The mannitol kit consists of mannitol capsules and an inhaler device. The mannitol dry powder challenge involves the inhalation of increasing doses of mannitol dry powder up to a cumulative dose of 635 mg. FEV_1 is measured one minute after each dose of mannitol [Brannan *et al.,* 2005; Anderson *et al.,* 2009]. A 15% fall in FEV_1 from the baseline FEV_1 is considered a positive response to mannitol. Mannitol can indicate the presence of EIB in an individual. A positive response to mannitol is more likely in atopic patients; however, a positive response has also been recorded in non-atopic patients. The

provocative dose of mannitol causing the 15% fall in FEV_1 is termed PD_{15}. The PD_{15} serves as an index to assess an individual's sensitivity to mannitol. The response dose ratio (RDR) is another index used in expressing reactivity or rate of change of airway response to mannitol. The RDR is calculated by dividing the change in FEV_1 by the dose of mannitol that provoked the fall in FEV_1 [Brannan *et al.,* 2005]. Both the PD_{15} and RDR values have been shown to be indirect indices of the severity of EIB [Kersten *et al.,* 2009]. Alternatively, some investigators use a 10% decrease in FEV_1 of the patient's response to mannitol in comparison to the patient's response to EVH and exercise [Holzer *et al.,* 2003].

2. LITERATURE REVIEW

2.1. Allergen-induced increase in mediator release, airway inflammation and eosinophils

Atopic IgE mediated airway response to inhaled allergens induces the early asthmatic response, late asthmatic response, an increase in AHR, eosinophilia, and airway inflammation. The airway response to allergen challenge results in an increase in mediators of bronchoconstriction; hence, a subsequent direct or indirect challenge will lead to a more airway response than a direct or indirect challenge alone. It has been identified that there is an increase in bronchoalveolar eosinophils after allergen challenge. This increase occurs in participants with a dual asthmatic response (DAR) namely, the early and the late asthmatic response [de Monchy *et al.,* 1985]. The increase in airway responsiveness and eosinophils following allergen challenges are known to be inhibited by corticosteroids. This suggests that the airway inflammation seems to be the cause of LAR and increased airway hyperresponsiveness (AHR) [Cockcroft *et al.,* 1993]. The EAR is associated with the release of mediators of bronchoconstriction such as histamine [Keyzer *et al.,* 1984], leukotrienes [Manning *et al.,* 1990], and prostaglandins [Shephard *et al.,* 1985].

2.2. Allergen-induced increase in airway response to direct challenges: Methacholine and histamine challenges

Increase in non-allergic AHR is another feature of asthma. The degree of AHR has been shown to be a significant determinant in the airway response to allergen [Killian *et al.,* 1976]. This supports an earlier observation that, natural grass pollen exposure increases bronchial reactivity to inhaled histamine in grass pollen-allergic asthmatics [Altounyan 1964; Howell, 1977]. Cockcroft *et al.,* reported an increase in the airway response to both histamine and methacholine 7 hours and several days following allergen exposure [Cockcroft *et al.,* 1977]. Cockcroft *et al.,* measured non-allergic bronchial reactivity to inhaled histamine and methacholine, before and after allergen inhalation in thirteen participants. Although the allergen inhalation produced EAR (19%-40%), some of the participants also experienced an LAR. What's more, the non–allergic

bronchial reactivity persisted for up to seven days after allergen inhalation. Changes in bronchial reactivity to inhaled histamine and methacholine were examined eight hours after allergen inhalation. The PC_{20} for inhaled histamine was significantly reduced in seven participants, with the reduction reaching a maximum between eight and fifty-six hours post allergen inhalation . Similarly, the PC_{20} for methacholine was also reduced significantly in six participants with a maximum between eight and thirty-two hours. Although the increase in reactivity to histamine was greater than that of methacholine, the difference was not significant. It is worth mentioning that, the reduction in histamine and methacholine PC_{20} only happens in the LAR participants. LAR participants are participants with asthma that have 10% to 15% drop in FEV_1 between 3 and 5 hours after allergen exposure. This drop in FEV_1 follows an earlier drop 10 minutes and recovery after the participant is in contact with the allergen that he/she is susceptible to. It has been shown that the allergen-induced increase in bronchial reactivity to methacholine can be abrogated by corticosteroids [Lötvall *et al*., 2011].

2.3. Allergen-induced increase in airway response to indirect challenges (Exercise Induced Bronchoconstriction)

It has been well documented that LAR appears at three to ten hours following allergen exposure in some atopic asthmatics [Cartier *et al.,* 1982]. It has also been documented that LAR can appear following strenuous exercise [Bierman *et al.,* 1984; Speelberg *et al.,* 1991]. However, the prevalence of exercise-induced LAR is lower than allergen-induced LAR [Bierman *et al.,* 1984; Lee *et al.,* 1989], even though a similar pattern has been observed due to the release of similar mediators [Lee *et al.,* 1983]. Young *et al.,* have observed the occurrence of an LAR to exercise following allergen challenge [Young *et al.,* 1998]. However, one cannot distinguish between the allergen-induced LAR and the exercise-induced LAR. Although there are controversies surrounding the existence of exercise induced LAR, it has also been reported that there is an effect of exercise-induced LAR on allergen-Induced LAR. Koh *et al.* reported an increase in airway responsiveness to allergen twenty-four hours after exercise challenge [Koh *et al.,* 1994]. LAR to exercise may also occur in adult asthmatics following allergic LAR [Boulet *et al.,* 1992]. A key feature of LAR is inflammation; as

such, one can infer that, the influx of inflammatory cells, and hence the increase in mediators of inflammation culminates into the enhancement of bronchial responsiveness [Durham, 1991].

2.4. Allergen induced increase in the airway response to indirect challenges: Adenosine 5' Monophosphate

AMP is an indirect stimulus that provokes bronchoconstriction via mast cell degranulation and the release of pro-inflammatory mediators [Polosa *et al.,* 1995]. Mast cells are prominent sources of mediators of inflammation in atopic asthma; as such, a bronchial response to AMP can be deemed as a more direct marker of allergic inflammation, than when compared to direct challenges [Van den Berge *et al.,* 2001; Prieto *et al.,* 2002a]. There have been indications that in sensitized participants with atopic asthma or rhinitis, natural exposure to seasonal pollen elicits an increase in airway response to AMP [Prieto *et al.,* 2002b]. In a study by Lopez *et al.,* it was shown that AMP PC_{20} values were significantly lower in participants with pollen allergy during the pollen season. These participants included both healthy and patients with seasonal allergic rhinitis with or without mild asthma [Lopez *et al.,* 2012]. The decrease in AMP PC_{20} is consistent with increased airway sensitivity to AMP following exposure to pollen. This suggests that airway sensitivity to indirect bronchoconstrictors like AMP may be increased due to the presence of pro inflammatory stimuli, stemming from the allergen exposure.

2.5 Summary

Asthma is a heterogeneous airway phenomena characterized by spontaneous reversible airflow obstruction or with treatment. The two main features of asthma are chronic airway inflammation and airway hyperresponsiveness. These two features represent key symptoms that influence the choice of treatment for asthma. The diagnosis of asthma can be deduced from assessing airway functioning. Airway assessment can be achieved through bronchoprovocation challenges using stimuli such as methacholine, mannitol, exercise, cold air, histamine, hypertonic solution, and AMP.

The stimuli are classified into direct (methacholine and histamine) and indirect (mannitol, exercise, cold air, AMP and hypertonic solution) based on their site of action on the airways.

Allergen is a common trigger for asthma as such allergen inhalation is a very useful clinical and research tool for evaluating asthma. Allergen inhalation leads to crosslinking of allergen –specific IgE bound to IgE receptors on mast cells and basophils. This leads to release of mediators of bronchoconstriction. The timeframe of bronchoconstriction happening within three hours is termed early asthmatic response. A subsequent bronchoconstriction occurring between 3 and 8 hours is termed late asthmatic response.

It has been shown that asthmatics exposed to allergen have a different response to methacholine challenge than they did before allergen exposure. Our mandate is to investigate if this change in response happens with mannitol.

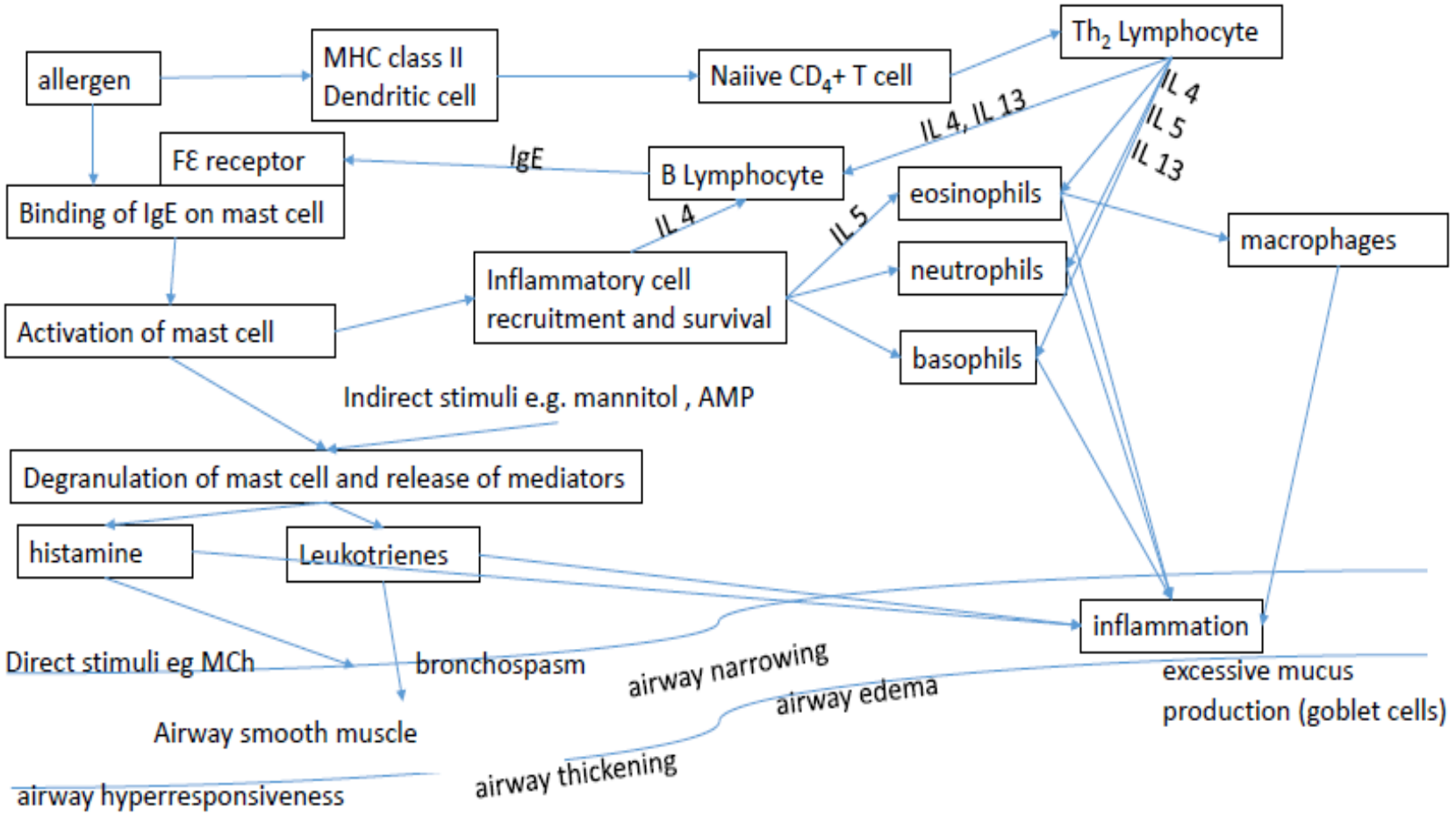

Figure 2.1. Overview of allergen, direct stimuli and indirect stimuli pathway to bronchoconstriction

3. ALLERGEN-INDUCED CHANGES IN AIRWAY RESPONSE TO METHACHOLINE AND MANNITOL THREE AND TWENTY FOUR HOURS AFTER ALLERGEN CHALLENGE

3.1. Rationale

It has been shown that the PC_{20} for AMP (indirect challenge stimulus similar to mannitol), correlates better with sputum eosinophilia (a measure airway inflammation) than methacholine PC_{20}. Although the increase in airway hyperresponsiveness after allergen challenge can be seen and measured at seven and twenty-four hours; it has been shown in as early as three hours. Although it has also been shown that there is an increased responsiveness to methacholine after allergen challenge, we do not know the responsiveness to mannitol after allergen challenge. However, we do know the presence of refractoriness of the airways to mannitol in repeated mannitol challenges and cross-refractoriness within the indirect stimuli causing bronchoconstriction. The occurrence of refractoriness causing a decrease in airway response to mannitol three hours post allergen challenge, calls for an extension of the post allergen's time point from three to twenty-four hours in a second study.

3.2. Background

Airway responsiveness to direct bronchoconstrictors such as methacholine has been shown to increase following allergen challenge, and has been observed primarily in those with a late asthmatic response. Indirect airway challenges, which include both an allergen challenge and a mannitol challenge, are expected to have a greater positive response than when an allergen challenge is done with a direct challenge like methacholine. Contrary to our expectations, the three-hour post allergen challenges resulted in a decreased airway response to mannitol; hence, a 24-hour post allergen challenge was aimed at ruling out refractoriness. Although F_ENO measurements were taken in the three-hour study, they were taken at the beginning of each visit to track inflammation at the start of the visits in the three-hour study. In addition to F_ENO measurements taken at the beginning of each visit in the twenty-four hour study, F_ENO

measurements were also made at the seven and twenty-four hour after allergen challenge to track airway inflammation.

3.3. Hypothesis

Since the PC_{20} for AMP correlates better with airway inflammation than methacholine $PC_{20,}$ we expect changes in airway responsiveness measured both at three and twenty-four hours post allergen challenge, to be greater when tested with mannitol than methacholine.

3.4. Objectives

Among the objectives we were examining in the study were as follows:

- The change in airway responsiveness at three and twenty-four hours after allergen challenge measured with mannitol.
- The change in airway responsiveness at three and twenty-four hours after allergen challenge measured with methacholine.

3.5. Study Design

The study was first designed to study the behavior of methacholine and mannitol challenges three hours after allergen challenge. However, the results from the three-hour study contradicted the hypothesis we proposed. We repeated the study but allowed twenty-four hours after allergen challenge before looking at the behavior of methacholine and mannitol. In the twenty-four hour study a slight change was made in taking F_ENO measurements as compared to the three-hour study

Both studies were phase III, single center, and open-label randomized studies. A screening methacholine challenge and an allergen skin test were run on the first visit. However, the screening methacholine challenge and the allergen skin test were not tested in individuals who had already successfully completed the three-hour study and were participating in the twenty-four-hour post allergen challenge. After the participant had passed all the screening tests, the participant was asked to come in twenty-four hours later for a F_ENO reading, and either a methacholine or mannitol challenge. After

24 hours, the participant was asked to come again for an additional F_ENO reading and an allergen challenge.

In the case of the three-hour study, the participant underwent another methacholine or mannitol challenge 3 hours after the allergen challenge, based on the challenge the participant did 24 hours earlier. If the participant did a methacholine challenge 24 hours earlier, the participant will repeat the methacholine challenge three hours after the allergen challenge. If the participant did a mannitol challenge 24 hours earlier, the participant will repeat the mannitol challenge three hours after the allergen challenge. Two weeks later, the methacholine challenge is replaced with the mannitol challenge or the mannitol challenge is replaced with the methacholine challenge. In both cases, the allergen challenge stays the same. If the first two visits were methacholine on visit-2 and allergen-methacholine on visit-3,then the next two visits were mannitol on visit-4 and allergen mannitol on visit-5 and vice versa. The schedule of assessment for the three-hour post allergen study is shown below.

Table 3.1. Schedule of assessments for the three-hour study.

Visit1 (Screening)	Visit 2	Visit 3	2 WEEKS WASHOUT	Visit 4	Visit 5
Read and sign consent form. Pregnancy test. Screening methacholine challenge. Allergen skin test.	Pre challenge F_ENO reading Methacholine or mannitol challenge	Pre allergen F_ENO reading Allergen challenge Methacholine or mannitol challenge		Pre challenge F_ENO reading Methacholine or mannitol challenge	Pre allergen F_ENO reading Allergen challenge Methacholine or mannitol challenge

In the 24-hour post allergen challenge, twenty-four hours following the start of the allergen challenge, the participant was required to perform another F_ENO test and

undergo another methacholine or mannitol challenge. If the participant did a methacholine challenge 24 hours earlier, the participant will repeat the methacholine challenge 24 hours after the allergen challenge. If the participant did a mannitol challenge 24 hours earlier, the participant will repeat the mannitol challenge 24 hours after the allergen challenge. After at least two weeks, the methacholine challenge was replaced with the mannitol challenge or the mannitol challenge was replaced with the methacholine challenge. In both cases, the allergen challenge stays the same. If the first three visits were methacholine on visit-2, allergen on visit-3 and methacholine on visit-4, then the next three visits were mannitol on visit-5, allergen on visit-6 and mannitol on visit-7. If the first three visits were mannitol on visit-2, allergen on visit-3 and mannitol on visit-4, then the next three visits were methacholine on visit-5, allergen on visit-6 and methacholine on visit-7. The second, third and fourth visits were separated from the fifth, sixth and seventh visits by at least a two week washout period.

Table 3.2. Schedule of assessments 24-hour post allergen study

Visit 1 (screening)	Visit 2	Visit 3	Visit 4	TWO WEEKS WASHOUT	Visit 5	Visit 6	Visit 7
Read and sign consent form. Pregnancy test. Screening methacholine challenge. Allergen skin test.	Pre challenge F_ENO reading. Methacholine or mannitol challenge.	Pre allergen F_ENO reading. Allergen challenge. Post allergen F_ENO.	Pre challenge F_ENO reading. Methacholine or mannitol challenge.		Pre challenge F_ENO reading. Methacholine or mannitol challenge.	Pre allergen F_ENO reading. Allergen challenge Post allergen F_ENO.	Pre challenge. F_ENO reading. Methacholine or mannitol challenge.

The order of the challenges that is methacholine/allergen /methacholine and mannitol/allergen/ mannitol) were randomized but not concealed. The study took place from September 2012 until the end of March 2014. The drugs used in the study included methacholine–powder for solution, and mannitol. The methacholine used for inhalation has been approved for testing in bronchoprovocation testing. The mannitol-capsules were punctured and inhaled by the inhaling device (osmohaler) in the mannitol kit. Mannitol is also a natural health product approved for use in bronchoprovocation testing in a number of countries, including Australia and the USA, but not yet in Canada

3.5.1. Primary Endpoints.

- Difference in PD_{15} of mannitol before allergen challenge and three hours after allergen challenge (Δlog PD_{15}).
- Difference in the dose response ratio (DRR) of mannitol before allergen challenge and twenty-four hours after allergen challenge).
- Difference in PC_{20} of methacholine before allergen challenge and three hours after allergen challenge (Δlog PC_{20}).
- Difference in PC_{20} of methacholine before allergen challenge and twenty-four hours after allergen challenge (Δlog PC_{20}).

3.5.2. Statistical Analysis.

Statistical analysis was performed with Microsoft Excel 2013 for Windows (Part of Microsoft Office Professional Plus 2013, Redmond WA, USA), Statistix (version 10 for Windows Tallahassee, Florida, USA), and Sigma plot (version 12.5 for Windows San Jose, California, USA). We compared bronchoprovocation data using a paired t-test. The level of significance was set at 0.05. Both studies were appropriately powered (99%) to detect a full concentration change in methacholine PC_{20}.

3.6. Methods.

3.6.1. Participants.

Ten atopic asthmatic participants aged 18-65 previously known to researchers or recruited from the University of Saskatchewan general population completed the three-hour post allergen study. Eleven participants also from the above group completed the twenty-four-hour post allergen study. The participants were provided with a University of Saskatchewan Biomedical Research Ethics Board approved consent form prior to participation in the study. Participants who participated in the three-hour post allergen study project were allowed and actively recruited into this project. These participants were not required to undergo screening methacholine and skin prick testing for allergies.

3.6.2. Exclusion criteria.

Participants were excluded from the study based on the following:

- The screening methacholine challenge resulted in a methacholine PC_{20} greater than 16mg/ml. Methacholine challenge is positive when there is a fall of about 20% in FEV_1 after administering about 32 mg/ml of methacholine. Hence more than 16mg/ml of methacholine without an FEV_1 of 20% will be considered as a negative methacholine challenge.
- Baseline FEV_1 of less than 70% predicted. A participant with baseline of less than 70% is not an advisable candidate for bronchoprovocation challenges especially challenges involving allergen could potentiate a fall in FEV_1 leading to breathing problems which could be fatal if not monitored properly.
- There were no clinically relevant positive allergies indicated with the allergen skin test. Allergen challenges can only be done in participants with a susceptibility to an allergen in the lab hence a positive allergen test is required.

In addition, participants could not have any requirement for controller medications such as inhaled glucocorticosteroids such as budesonide and fluticasone (alone or in

combination with long acting beta agonists). Participants should not have had any significant medical comorbidity, or any respiratory infection or allergen exposure within the 4 weeks of the study start date. Pregnant or lactating female participants were also excluded from the study. A participant was also excluded from the study if he or she was unable to stay off bronchodilators for an appropriate length of time.

3.6.3. Methacholine Challenge.

The procedures for each methacholine challenge were undertaken in accordance with the ATS guidelines from 1999 [Crapo *et al*., 2000].

1. Methacholine was prepared at the following concentrations (mg/ml): 0.03, 0.06, 0.125, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00, and 16.00.
2. Baseline spirometry was performed with a primary focus on FEV_1 and FVC (actual and predicted values).
3. By means of a Bennett-Twin jet nebulizer (calibrated to an output of 0.13 ml/min), via a face mask and with a nose clip on, the first concentration (diluent – normal saline) was administered for a period of 2 minutes during which the participant was asked to breathe normally.
4. The FEV_1 was measured at 30 and 90 seconds after the nebulization had ended.
5. The FEV_1 values were recorded and the lowest FEV_1 post methacholine inhalation compared to the lowest FEV_1 post diluent inhalation was assessed. A fall in FEV_1 of 20% was required.
6. Until the target FEV_1 was achieved, or a maximum concentration of methacholine was reached, steps 3 through step 5 were repeated for each concentration administered.
7. Participants were provided with bronchodilator (salbutamol) to reverse induced bronchoconstriction if necessary.

The methacholine PC_{20} was then calculated for each participant using the formula below.

Methacholine PC_{20} = antilog [log C1 + ((log C2- log C1)(20-R1)/(R2-R1)]…….. (3.1)

Where,

C1= second to last methacholine concentration.

C2= final concentration of methacholine resulting in ≥ 20% drop in FEV_1.

R1= percentage drop in FEV_1 after C1.

R2= percentage drop in FEV_1 after C2.

In the event where the participant's fall in FEV_1 was ≥ 17%, a single point extrapolation formula was used to theoretically obtain the methacholine PC_{20} [Jokic *et al*., 1998]. This formula is as follows.

Methacholine PC_{20} = [20/(current % fall in FEV_1)] * last concentration of methacholine

…….... (3.2)

3.6.4. Mannitol Challenge.

A standardized mannitol challenge protocol has been developed [Anderson *et al*., 1997] and mannitol was supplied by the manufacturer. Spirometry was performed before the challenge and the reproducibility of the resting baseline FEV_1 was established. The participant was seated comfortably and encouraged to maintain good posture to assist the effective delivery of mannitol to the lungs. The test was conducted as follows.

1. The participant was directed to breathe through the mouth with the help of an applied nose clip.
2. The 0 mg capsule was inserted into the inhalation device and was punctured by pressing buttons on the sides of the device once slowly.
3. The participant was asked to exhale completely before inhaling from the device using a controlled and rapid deep inhalation.
4. At the end of the deep inhalation, a 60-second timer was started with the participant holding his or her breath for 5 seconds before exhalation through the mouth.
5. After the 60 seconds had elapsed, the FEV_1 was measured in duplicate
6. Steps 2 through 5 were repeated following the dose steps in the table below until the patient had a positive response, or the total cumulative dose of 635 mg of mannitol had been administered.

7. Participants were provided with bronchodilator (salbutamol) to reverse induced bronchoconstriction if necessary.

Table 3.3. Dosage of the capsules of mannitol for the mannitol challenge.

Dose #	Dose (mg)	Cumulative Dose (mg)	Capsule / Dose (mg)
1	0	0	1x 0 mg
2	5	5	1x5mg
3	10	15	1x10mg
4	20	35	1x20mg
5	40	75	1x40mg
6	80	155	2x40mg
7	160	315	4x40mg
8	160	475	4x40mg
9	160	635	4x40mg

A positive response was achieved when the participant experienced a 15% fall in FEV_1 compared with the 0 mg dose. A mannitol PD_{15} was calculated for each participant twenty-four hours before and three hours after the allergen challenge using the formula below in the three-hour study.

mannitol PD_{15} = antilog [log D1+ (log D2-log D1)(15-R1)/(R2-R1)]……(3.3)

Where,

D1= second to last cumulative mannitol dose.

D2= final cumulative dose of mannitol resulting in a ≥ 15% drop in FEV_1.

R1= percentage drop in FEV_1 after D1.

R2= percentage drop in FEV_1 after D2.

In the event where the fall in FEV_1 was not quite 15% (e.g. 10-14%), a single point extrapolation was used to theoretically obtain the mannitol PD_{15}. This formula is as follows.

mannitol PD_{15} = [15/ % fall in FEV_1] cumulative dose of mannitol…… (3.4)

In the twenty-four hour study, mannitol responsiveness was assessed using a dose response ratio. The dose response ratio was calculated by dividing the cumulative dose of mannitol in mg by the percentage fall in FEV_1 at that dose.

3.6.5. Allergen Challenge.

The selected allergen was administered using a Wright nebulizer and two minutes of tidal breathing. The first concentration given ranged from three or four doubling concentrations below the predicted allergen PC_{20}. FEV_1 was measured ten minutes after each concentration of allergen was given until the targeted fall of 20% or more from the baseline was reached. In the three-hour post allergen study, lung function was monitored in the asthma lab at various time points over the next 3 hours after the allergen challenge. In the twenty-four hour post allergen study, lung function was monitored in the asthma lab at various time points over the next 7 hours after the allergen challenge. The allergen challenge was performed as follows:

1. Baseline spirometry was performed to determine the highest FEV_1 for comparison with the FEV_1 post allergen inhalation to determine the percent fall in FEV_1.
2. A target fall in FEV_1 was calculated at 80% of the participants' baseline FEV_1.
3. By means of a Wright nebulizer (calibrated to an output of 0.13 ml/min), and with the use of a nose clip, the first concentration of allergen was administered for a period of exactly two minutes. The first concentration was three or four concentrations below the predicted allergen PC_{20}. Participants were asked to breathe normally via a mouthpiece.
4. An FEV_1 was measured at ten minutes after the nebulization had ended. If the target fall in FEV_1 was not reached, the next concentration of allergen was administered.

After the FEV_1 had fallen by 20% or more, allergen administration was halted and the FEV_1 was measured at each of the following time points: 20, 30, 45, 60, 90,120, and 180 minutes in the three-hour post allergen study. FEV_1 was measured at 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 420 minutes in the twenty-four-hour post allergen study. Spirometric measurements were used to assess the development (or not) of the

late asthmatic response. Inhaled glucocorticosteroid (fluticasone propionate) was administered to some participants who underwent either methacholine or mannitol challenges three hours after allergen challenge.

3.6.6. Skin Prick Test and Skin Test Endpoint.

Each individual was skin-tested to determine and confirm his or her sensitivity to common aeroallergens. This involved the introduction of droplets of different allergens on the forearm, and pricking within the allergenic solutions to introduce the allergen to just below the skin. Introduction of the allergen elicited an allergic reaction in the form of a bright reddish bump on the forearm. 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 higher as necessary) of the chosen allergen were prepared and used to perform the skin test endpoint. The skin test endpoint was administered by introducing different concentrations of the sensitized droplets to the forearm. Pricks were made in the droplets of different concentrations to introduce the allergen to just below the skin. The skin test endpoint was defined as the minimum dilution of the allergen that produced a 2mm wheal in diameter or smaller. This was used in conjunction with the results of the screening methacholine challenge to determine the predicted allergen PC_{20} for the allergen inhalation challenge using the formula below [Cockcroft *et al*., 1987; Cockcroft *et al*., 2005].

Predicted allergen PC_{20} = antilog [0.68 * log (methacholinePC_{20} * skin test endpoint)]…….. …….…….. (3.5)

3.6.7. Fractional Exhaled Nitric Oxide Measurements (F_ENO).

F_ENO is a non-invasive tool used in assessing airway inflammation in allergic diseases like asthma. It is believed that during allergic airway inflammation there is an increase in eosinophil recruitment and an increase in nitric oxide in the airways.

F_ENO was measured using a chemiluminescence gas analyzer (Niox, Aerocrine Inc., New York, NY). Participants performed an inhalation to total lung capacity. This was followed by an exhalation with a constant flow rate of 50mL/sec via a

filter/mouthpiece. The procedure was performed in triplicate and continued until at least two measurements were reproducible within 10%. F_ENO measurements were performed before methacholine challenges, mannitol challenges, and pre allergen challenges in the three-hour post allergen challenge. In the twenty-four hour post allergen challenge, F_ENO measurements were performed before all methacholine, mannitol and allergen challenges. Additional measurements were made at 7 hours and 24 hours post allergen challenge.

For the three-hour study, the participants read and signed the consent form before they were allowed into the study. Screening for the study included testing negative for pregnancy in females. All participants in the study are required to pass a methacholine challenge and have an allergic reaction to at least one of the allergens in the lab. FENO measurement is done at the start of each visit after the subject has passed all screening requirements. On the second visit the participant underwent either methacholine or mannitol challenge In addition the allergen is titrated to figure out the concentration of allergen to cause a 20% fall in FEV_1 from the allergen skin test. The allergen challenge and methacholine or mannitol challenge is done on the next visit. A two-week washout period is allowed to prevent the effects of the previous allergen challenge from affecting the second allergen challenge. If the visit two challenge was methacholine, the fourth challenge is switched to mannitol or vice versa.

3.7. Results for three hour study

3.7.1. Participants.

Thirteen participants consented to take part in the study; however, ten atopic asthmatics aged between 21 and 36 completed testing successfully without any incidence of adverse effects. Three participants did not meet the entry criteria. The participants had a clinical diagnosis of mild a topic asthma at some point in their lives. Participants were asked to refrain from corticosteroids about four weeks before the start of the study and refrain from short acting bronchodilators 6 hours prior to each visit. The mean age was 26 years ± 5.7 S.D. The mean height and weight of the participants was 171 cm ± 11.2 S.D. and 69 kg ± 17.8 S.D. respectively. The mean baseline FEV_1 was 3.46L ± 0.65 S.D.

The participants had a positive methacholine challenge and had an allergic response to some of the allergens such as cat, horse, and house dust mite *dermatophagoides pteronyssinus* (DP). Table 3.4 below shows the participant's demographics for the 3-hour post allergen challenges.

3.7.2. Methacholine challenge

The geometric mean methacholine PC_{20} before allergen challenge was 0.88 mg/ml ± 0.10 S.E. The geometric mean methacholine PC_{20} after allergen challenge was 0.50 mg/ml ± 0.10 S.E. The decrease in MCh PC_{20} was significant ($p = 0.02$). Table 3.5 shows the raw data for the methacholine challenge. In addition, Figure 3.1 shows the various participants' methacholine PC_{20} before and after allergen challenge.

3.7.3. Mannitol challenge

The geometric mean mannitol PD_{15} before allergen challenge was 174mg ± 0.16 S.E. The geometric mean mannitol PD_{15} after allergen challenge was 284mg ± 0.18 S.E. There was a significant increase in mannitol PD_{15} after allergen challenge ($p=0.02$). Table 3.5 shows the raw data for the mannitol challenge. In addition, Figure 3.2 shows the individual mannitol PD_{15} before and after allergen challenge.

Table 3.4. The demographics of participants in the three-hour study.

Participant	Sex	Age (years)	Baseline FEV_1 (L)	Baseline FEV_1 (%predicted)	Height (cm)	Weight (kilograms)	Allergen information		
							Allergen	Skin test end point	Final Concentration Inhaled
1	F	22	3.41	100	165	68	*HDM-DP	1:4096	1:256
2	M	36	3.69	86	178	82	Cat	1:4096	1:32
3	F	21	3.16	85	173	73	Cat	1:1024	1:64
4	F	21	3.52	96	173	66	Cat	1:512	1:16
5	M	25	3.99	83	183	103	Cat	1:1024	1:16
6	F	26	3.00	89	165	45	HDM-DP	1:128	1:1024
7	F	24	3.06	102	155	54	Horse	1:2048	1:32
8	F	36	2.69	93	157	53	HDM-DP	1:512	1:256
9	F	21	3.10	89	168	57	Cat	1:256	1:32
10	M	27	4.96	97	191	86	Cat	1:256	1:2
Mean ± SD		26 ± 5.7	3.46 ± 0.65	92 ± 6.6	171 ± 11.2	69 ± 17.8			

* House dust mite dermatophagoides *pteronyssinus*

Table 3.5. The raw and logged PC_{20} and PD_{15} data for Methacholine and Mannitol challenges in the three-hour study.

	PC_{20}(mg/ml)		PD_{15}(mg)		Log PC_{20}		Log PD_{15}	
Participant	Methacholine Pre Allergen	Methacholine Post Allergen	Mannitol Pre Allergen	Mannitol Post Allergen	Methacholine Pre Allergen	Methacholine Post Allergen	Mannitol Pre Allergen	Mannitol Post Allergen
1	1.80	0.25	168	206	0.2553	-0.6021	2.2253	2.3139
2	0.70	0.36	755	1905	-0.1549	-0.4437	2.8779	3.2799
3	0.72	0.70	145	196	-0.1427	-0.1549	2.1614	2.2923
4	0.94	0.36	202	275	-0.0269	-0.4437	2.3054	2.4393
5	0.53	0.45	130	194	-0.2757	-0.3468	2.1139	2.2878
6	0.91	0.59	28	75	-0.0410	-0.2292	1.4472	1.8751
7	1.51	1.44	1047	2646	0.1790	0.1584	3.0199	3.4226
8	0.27	0.28	128	78	-0.5686	-0.5528	2.1072	1.8921
9	0.54	0.25	82	189	-0.2676	-0.6021	1.9138	2.2765
10	3.10	1.60	N	N	0.4914	0.2041	N	N
				Geometric mean±SE	0.88±0.10	0.50±0.10	174±0.16	284±0.18

N - Negative test for mannitol

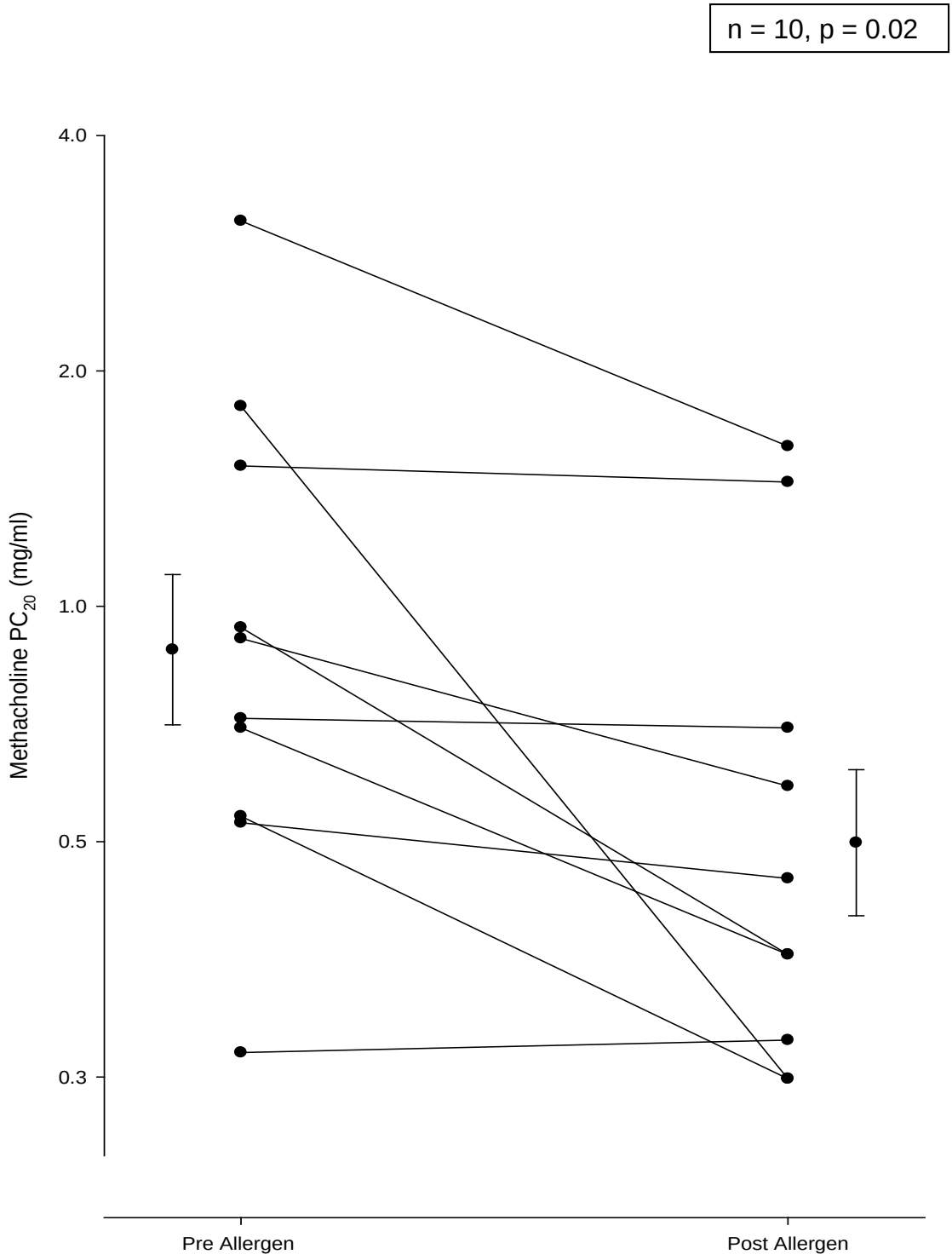

Figure 3.1. Graph of methacholine PC_{20} before and after allergen challenge in the three-hour study. The vertical axis is a log scale. Individual data points are geometric means ± S.E.

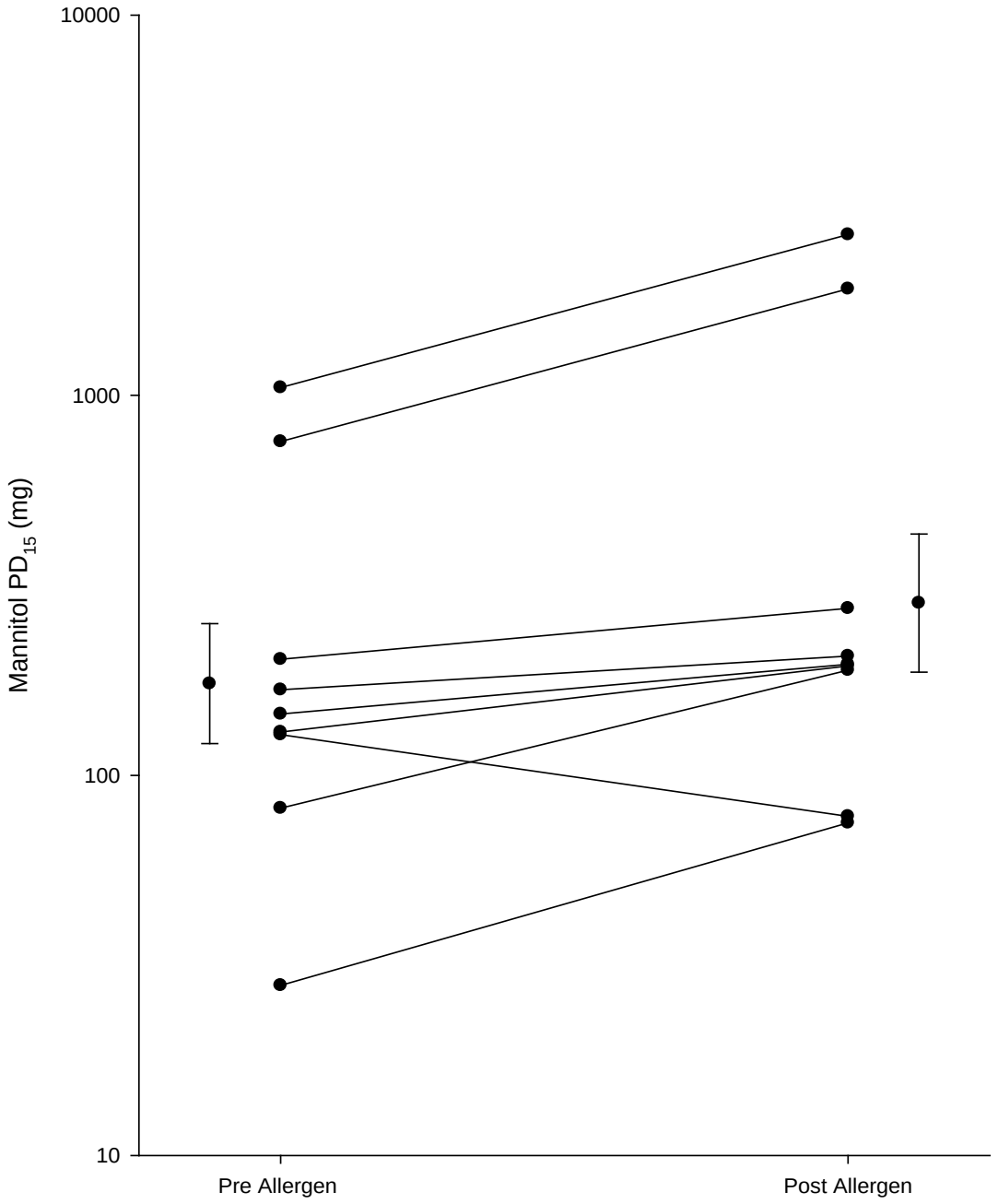

Figure 3. 2. Graph of mannitol PD_{15} before and after allergen challenge in the three-hour study. The vertical axis is a log scale. Individual data points are geometric means ± S.E.

3.7.4. Baseline F_ENO

The difference between pre and post allergen F_ENO was not significant during methacholine (p = 0.85) or mannitol (p = 0.42) challenges. Table 3.6 shows all F_ENO data.

Table 3.6. The baseline F_ENO of all participants in the three-hour study

	Baseline F_ENO (ppb)				Log baseline F_ENO			
	Methacholine Arm		Mannitol Arm		Methacholine Arm		Mannitol Arm	
Participant	Day1	*Day2	Day1	Day 2	Day 1	Day 2	Day 1	Day 2
1	41	40	23	21	1.61	1.60	1.36	1.32
2	57	150	25	25	1.76	2.18	1.40	1.40
3	57	48	56	61	1.76	1. 68	1.75	1.79
4	82	61	99	76	1.91	1.79	2.00	1.88
5	92	91	79	87	1.96	1.96	1.90	1.94
6	21	19	26	26	1.32	1.28	1.41	1.41
7	39	38	35	40	1.59	1.58	1.54	1.60
8	61	58	68	98	1.79	1.76	1.83	1.99
9	39	38	35	40	1.59	1.58	1.54	1.60
10	111	105	50	49	2.05	2.02	1.70	1.69
Geometric mean ± SE					54±1.2	55±1.2	44±1.2	46±1.2

*Day 2 measurements were taken before the allergen challenge

3.7.5. Baseline FEV_1.

There were no significant differences between baseline FEV_1 values for either the methacholine arm or the mannitol arm (p=0.42 and p=0.42). Raw data are shown in Table 3.7 below.

.

Table 3.7. The baseline FEV_1 of participants in the three-hour study.

	Baseline FEV_1 (L)			
	Methacholine Arm		Mannitol Arm	
Participant	Pre	Post	Pre	Post
1	3.39	3.33	3.31	3.25
2	3.53	3.73	3.73	3.54
3	2.99	3.04	2.75	2.91
4	4.41	3.33	3.48	3.24
5	3.31	3.51	3.75	3.42
6	2.84	2.77	2.78	2.66
7	2.99	3.05	3.02	3.00
8	2.77	2.48	2.43	2.43
9	3.10	3.05	2.86	3.10
10	4.72	4.77	4.87	4.95
mean±SE	3.41±0.21	3.31±0.20	3.30±0.22	3.25±0.22

3.7.6. Allergen Challenge

There was no significant difference between the two allergen challenges. Results from the allergen challenges were grouped into methacholine arm in table 3.8 and mannitol arm in table 3.9. Figure 3.3 shows the similarities between the allergen challenges on the mannitol and methacholine arm.

Table 3.8. Percentage FEV_1 fall following allergen challenge in the methacholine arm in the three-hour study.

Participants	$\%\Delta FEV_1$ @ 10m	$\%\Delta FEV_1$ @ 20m	$\%\Delta FEV_1$ @ 30m	$\%\Delta FEV_1$ @ 45m	$\%\Delta FEV_1$ @ 60m	$\%\Delta FEV_1$ @ 90m	$\%\Delta FEV_1$ @ 120m
1	55.2	51.7	48.3	42.9	21.9	14.7	7.2
2	16.4	18.2	17.2	13.1	8.6	4.8	4.8
3	19.7	13.8	19.4	8.6	7.2	0.1	5.6
4	20.4	19.2	15.3	8.7	7.5	1.2	0.9
5	27.1	14.5	22.2	8.5	6.6	0.5	2.6
6	19.1	15.9	26.4	23.1	20.2	16.6	5.1
7	15.7	29.2	20.7	9.8	14.1	1.0	0.0
8	29.0	25.0	22.6	16.1	11.3	12.5	4.8
9	35.7	30.8	19.3	21.3	20.0	17.4	3.0
10	37.7	35.0	34.8	29.8	18.7	14.3	8.6
mean±SE	27.6±3.9	25.3±3.7	24.6±3.1	18.2±3.6	13.6±1.9	8.31±2.3	4.26±0.8

Table 3.9 Percentage FEV_1 fall following allergen challenge in the mannitol arm in the three-hour study..

Participant	%ΔFEV_1 @10m	%ΔFEV_1 @20m	%ΔFEV_1 @30m	%ΔFEV_1 @45m	%ΔFEV_1 @60m	%ΔFEV_1 @90m	%ΔFEV_1 @120m
1	44.3	17.8	11.7	4.0	5.6	0.3	0.3
2	22.6	38.1	35.6	26.8	19.5	0.8	5.9
3	16.8	24.1	18.6	17.9	16.2	3.9	0.0
4	23.8	24.1	21.3	12.3	7.7	3.7	0.1
5	19.6	26.0	15.5	10.8	6.4	2.0	1.0
6	19.5	21.1	21.8	16.9	7.1	7.9	0.8
7	22.7	27.7	11.3	7.3	4.0	2.3	4.0
8	26.7	23.9	24.7	17.7	17.7	15.6	6.7
9	21.6	20.0	18.4	15.8	12.9	5.2	2.9
10	21.8	21.6	19.6	12.7	11.3	3.6	5.7
mean+SE	23.9±2.4	24.4±1.8	19.9±2.2	14.2±2.0	10.8±1.7	4.5±1.4	2.7±0.8

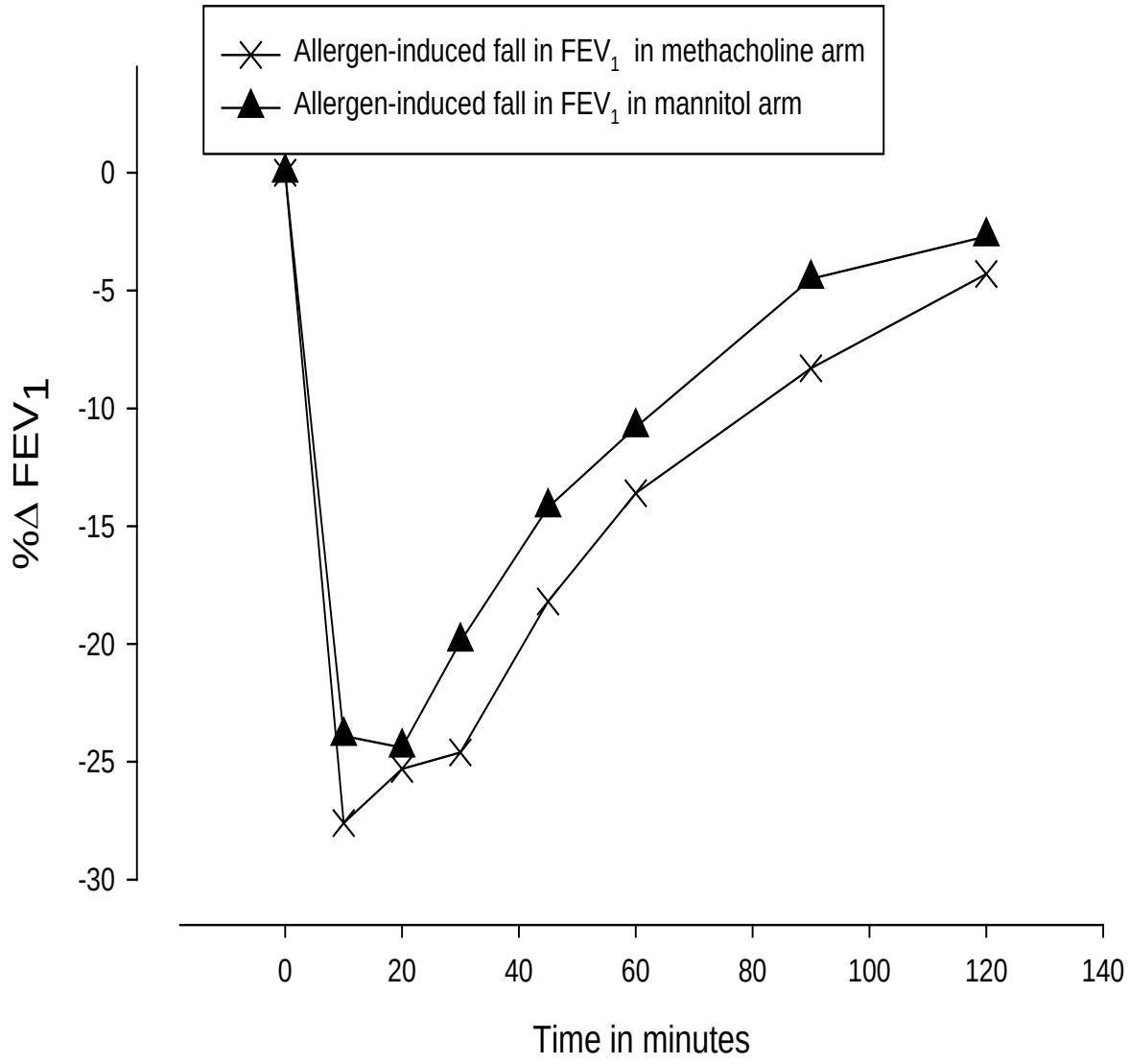

Figure 3.3. Mean changes in FEV_1 following allergen challenge in the methacholine and mannitol arm in the three-hour study

3.8. Discussion for three-hour study.

Airway responsiveness to methacholine has been shown to increase following allergen challenge. However, we do not know airway responsiveness to mannitol following allergen challenge. We have shown in the 3-hour study that the airway responsiveness to methacholine was increased by almost one doubling concentration. Conversely, airway responsiveness to mannitol was reduced. The change in airway responsiveness

to methacholine was expected. This confirms the observation by Cockcroft *et al.,* that allergen-induced challenge produces an increase in airway responsiveness to direct stimuli [Cockcroft *et al.,* 1987a; Amakye *et al*., 2013]. Subsequent studies attributed the increase in airway response and inflammation to LAR following allergen challenge [Gauvreau *et al.,* 1996]. The time frame however, in which participants experience increased airway responsiveness to methacholine following allergen challenge has been documented as early as 3 hours [Durham *et al*.,1988]. Although allergen-induced airway eosinophilia and allergen-induced airway responsiveness seem to occur at similar time points, they are not associated through a cause and effect relationship [Leckie *et al.,* 2000].

Indirect challenges, including allergen and mannitol, which are associated with airway hyperresponsiveness are also associated with airway inflammation [Van den Berge *et al.,* 2001;Van Velzen *et al.,* 1996]. Even though both methacholine and mannitol are bronchoprovocation challenges, airway hyperresponsiveness is more sensitive to methacholine than mannitol and mannitol is associated more with airway inflammation than methacholine Allergen challenges are also associated with airway inflammation. Airway inflammation is directly correlated with F_ENO measurements. Increased production of nitric oxide and the recruitment of eosinophils occur together and are key characteristic features of inflammation in asthma. Moreover, the high values of F_ENO (≥20 ppb) suggest underlying inflammation. As such, the high F_ENO values should correlate with a positive mannitol test. This was evident in about 90% of participants having a positive mannitol response. Even though there were no significant differences in F_E NO between all the baseline F_ENO visits, the participants who had ≥ 35ppb, had no significant differences in F_ENO across all the baseline F_ENO visits. Participants with a likely eosinophilic inflammation confirmed by a positive mannitol test did not have a significant change in baseline F_ENO across all the visits in our study. This could mean that the participants had a stable amount of inflammation. A similar amount of ongoing inflammation could also be supported by figure 3.3 showing that the allergen challenges on both the methacholine and mannitol arms were similar.

Our original hypothesis was that the change in airway responsiveness to mannitol might increase more than that of methacholine; however, our results showed otherwise.

Another phenomena associated with indirect challenges that need to be taken into account are refractoriness and cross refractoriness. Refractoriness has been documented in hyperventilation induced asthma [Rakotosihanaka *et al*., 1986], repeated challenges of adenosine 5' monophosphate challenge, [Daxun *et al*., 1989] and repeated challenges with mannitol [Suh *et al*., 2011]. Refractoriness has also been documented in hypertonic airway challenges and exercise induced bronchoconstriction [Belcher *et al.,* 1987]. The common ground that all these challenges share is that they are all indirect stimuli to bronchoconstriction. Although the exact mechanism of refractoriness is unknown, it has been reported that there is cross-refractoriness between EIB (osmolar indirect stimuli) and leukotriene D_4 (LTD_4) [Manning *et al*., 1993]. There is a potential for cross refractoriness between indirect stimuli, which could also help explain cross refractoriness between allergen challenges and mannitol challenges, if they shared a common pathway. It is also worth mentioning that, refractoriness may not have been caused by depletion of mast cells, but rather, desensitization of leukotriene receptors and the release of inhibitory prostaglandins. [Manning *et al*., 1993; Larsson *et al*., 2011; Larsson *et al*., 2013].

Allergen induced inflammation can be seen in people with a late asthmatic response. Another possibility why the study failed to support our hypothesis was that, we did not select for atopic asthmatics with an LAR. The increase in airway responsiveness to methacholine (direct challenges) following allergen challenge is present in almost all atopic asthmatics irrespective of having an LAR or not. Traditionally, people with an LAR also have an EAR; hence, the interchangeable use of LAR and DAR. Nevertheless, people with an EAR do not necessarily have an LAR. Furthermore, the time allowed post allergen might not have been enough to see the LAR which is usually maximal about 7 hours post allergen. We looked however at 3 hours post allergen, which is a timepoint at which the airway responsiveness to direct stimuli has been previously shown to significantly increase.

In the three-hour study, there were no significant differences between all the baseline F_ENO measurements across all visits. This could mean that even though inflammation could be occurring three hours after the allergen challenge, the three hours might not be enough to see a surge in the amount of eosinophils indicative of underlying

inflammation. One could assert that three hours might not be enough to observe a change in inflammation. This assertion was also supported by the fact that the entire baselines FEV_1 across all the visits were not significantly different from each other. In an instance where the F_ENO values did not accurately capture the amount of inflammation, the baseline FEV_1 values across all the visits show that, there were no significant changes in the airway diameter.

It will be necessary that we study the allergen-induced increase in both direct and indirect challenges at twenty-four hours post allergen inhalation. Although the three hour duration after allergen challenge has been enough to observe an increase in responsiveness to methacholine, the three hour time point has not be established as long enough to see an increase in responsiveness to indirect stimuli. Moreover, an indirect stimuli like mannitol might need more time than three hours to mount an expected increase in airway response. Indirect stimuli do not attach directly to airway smooth muscles rather, they prompt the release of subsequent mediators from mast cells and basophils to cause bronchoconstriction. This whole process might need more than three hours to see its full effect. In addition, we believe twenty-four hours post allergen challenge will be enough to see the LAR, and how it will affect the indirect challenges. Furthermore, the participants in the future study should have an LAR and the measurement of F_ENO should be made at critical time-points in order to follow the changes in ongoing inflammation during the study. A future study should include more asthma subjects as a smaller sample size might not accurately capture the asthma population in the geographic area where the research is conducted. Asthma is a heterogeneous disease therefore it will also be beneficial to group asthmatic subjects into specific populations in research study for example late asthmatic responders, EIB subjects, AHR subjects. Having a categorized research subjects can help clarify the possible interactions and between the stimuli and the participants for example allergen induced change in airway responsiveness to mannitol in late asthmatic responders, EIB subjects and e.t.c.

3.9. Results for twenty-four-hour study.

3.9.1. Participants.

All participants were provided with, and signed, a University of Saskatchewan Biomedical Research Ethics Board consent form before commencing the study. Thirteen participants (male or female) screened for the study; however only eleven participants aged between 19 and 37 completed testing successfully without any incidence of adverse effects. The participants had an allergic response to at least one of the allergens used in the lab, which mainly included cat, house dust mite, and grass. The participants had a clinical diagnosis of mild atopic asthma at some point in their lives. Participants were asked to refrain from corticosteroids about four weeks before the start of the study, and refrain from short acting bronchodilators 6 hours before any study visit. The mean baseline FEV_1 of all participants at the start of the study was 3.53L ± 0.25 SD and greater than 70% predicted.

Table 3.10. The demographics of all participants in the twenty-four hour study.

Participant	Sex	Age(years)	Baseline FEV_1 (L)	Baseline FEV_1 (% predicted)	Height (cm)	Weight (kg)	Allergen	Skin Test Endpoint	Concentration Inhaled
1	M	28	4.71	96	185	85	Cat	1:128	*1:4
2	M	37	3.54	82	178	86	Cat	1:128	1:64
3	F	27	2.74	88	160	47	**HDM-DP	1:2048	1:64
4	M	29	4.57	99	180	81	Grass	1:4096	1:64
5	F	23	3.19	93	168	87	***HDM-DF	1:8192	*1:64
6	F	30	2.24	77	160	82	Grass	1:16384	1:1024
7	F	22	4.04	101	180	61	HDM-DF	1:256	1:256
8	M	21	4.57	93	183	80	Cat	1:256	*1:8
9	F	26	3.01	101	155	54	Cat	1:2048	1:16
10	F	19	3.06	97	157	54	Cat	1:512	1:8
11	F	22	3.13	89	168	61	Cat	1:128	1:16
Mean		26±1.6	3.53±0.25	92.4±2.3	170±3.4	71±4.6			

**Allergen concentration was not completely inhaled for two minutes due 20% fall in FEV_1 from the baseline for safety reasons.*

*** House dust mite dermatophagoides pteronyssinus*

****House dust mite dermatophagoides farinae*

3.9.2. Allergen challenge

Figure 3.4 shows the changes in FEV_1 for both the methacholine arm and mannitol arm up to 7 hours following allergen exposure. The raw data is presented in Tables 3.11 and 3.12 for methacholine and mannitol respectively. There were no significant differences ($p > 0.4$) in mean fall in FEV_1 at any time point between the methacholine and mannitol arms.

3.9.3. Methacholine Challenge

The geometric mean methacholine PC_{20} before allergen was 5.9 mg/ml ± 0.23 SE The geometric mean methacholine PC_{20} after allergen was 2.2 mg/ml ± 0.19 SE. The decrease was statistically significant ($p= 0.01$). Figure 3.5.

3.9.4. Mannitol challenge

The geometric mean dose response ratio for mannitol before allergen was 63 mg /$\Delta\%$ FEV_1 ± 0.15 SE. The geometric mean dose response ratio for mannitol after allergen was 158 mg /$\Delta\%FEV_1$ ± 0.19 SE. The increase was statistically significant ($p = 0.03$). Figure 3.6 shows the individual changes in the dose response ratio before and after allergen challenge. All data, including log transformations are shown in the table 3.13.

Table 3.11. Percentage fall in FEV_1 in the methacholine arm following allergen inhalation in the 24-hour post allergen study.

Participant	%ΔFEV$_1$ @ 10m	%ΔFEV$_1$ @ 20m	%ΔFEV$_1$ @ 30m	%ΔFEV$_1$ @ 45m	%ΔFEV$_1$ @ 60m	%ΔFEV$_1$ @ 90m	%ΔFEV$_1$ @ 120m	%ΔFEV$_1$ @ 180m	%ΔFEV$_1$ @ 240m	%ΔFEV$_1$ @ 300m	%ΔFEV$_1$ @ 360m	%ΔFEV$_1$ @ 420m
1	17.5	18.9	20.7	16.3	12.2	4	1.3	-4.7	0.0	1.3	10.0	8.2
2	30.2	37.6	37.1	35.3	28.7	21.8	13.2	5.7	8.0	5.5	10.1	14.1
3	14.2	22.4	22.4	19.7	16.3	4.7	13.2	9.8	14.2	3.1	-0.7	1.0
4	17.3	19.5	23.0	15.8	8.3	6.8	3.9	1.3	5.5	7.9	14.4	17.3
5	26.5	15.6	13.7	4.0	1.6	-0.9	-0.6	0.6	2.2	6.9	9.7	10.9
6	28.3	35.8	24.7	14.0	11.2	0.9	-8.4	-8.4	-12.1	-7.0	-1.9	6.0
7	29.8	23.6	19.6	16.5	19.9	11.5	7.1	2.6	3.7	-0.5	0.0	3.1
8	21.4	22.5	17.9	14.2	11.4	6.3	4.8	2.8	0.2	1.5	3.1	3.5
9	21.7	25.7	27.6	11.8	9.2	8.6	5.3	3.3	2.9	8.2	6.3	8.6
10	28.1	23.7	18.4	6.4	3	-0.3	-2	-3.3	-5.7	-6.4	-6.0	-5.7
11	7.80	13.3	4.90	2.90	-0.3	-2.9	-1.9	-2.3	1.6	-1.9	-2.6	-1.6
Mean ±SD	22.1 ± 6.9	23.5 ± 7.1	20.9 ± 7.7	14.3 ± 8.5	11.0 ± 8.0	5.5 ± 6.6	3.3 ± 2.0	0.7 ± 1.5	1.9 ± 2.1	1.7 ± 1.6	3.9 ± 2.0	5.9 ± 2.1

Table 3.12. Percentages fall in FEV_1 in the mannitol arm following allergen inhalation in the 24-hour study.

Participant	%ΔFEV_1 @ 10m	%ΔFEV_1 @ 20m	%ΔFEV_1 @ 30m	%ΔFEV_1 @ 45m	%ΔFEV_1 @ 60m	%ΔFEV_1 @ 90m	%ΔFEV_1 @ 120m	%ΔFEV_1 @ 180m	%ΔFEV_1 @ 240m	%ΔFEV_1 @ 300m	%ΔFEV_1 @ 360m	%ΔFEV_1 @ 420m
1	13.5	12.0	10.7	5.9	2.6	-1.3	-2.4	-4.9	-2.4	-3.8	-2.4	-1.3
2	22.2	22.2	22.5	18.4	9.3	2.6	0.6	2.3	0.0	4.7	4.9	3.5
3	20.4	14.2	18.3	18.7	6.6	6.9	5.5	5.2	4.2	4.5	4.8	3.5
4	38.0	37.3	27.0	22.9	20.2	14.9	10.11	12.3	18.2	27.9	27.9	24.7
5	32.3	11.1	12.0	8.9	3.8	-0.6	-0.9	-1.3	3.5	4.7	9.8	6.6
6	6.2	10.6	5.3	9.3	-1.8	-8.8	-8.8	-10.6	-11.1	-6.2	-3.5	7.5
7	11.8	34.5	39.0	14.4	5.5	4.3	5.8	5.3	4.8	12.8	3.8	2.0
8	25.5	21.1	17.0	16.3	8.3	4.6	1.3	0.0	0.4	0.4	2.2	4.6
9	17.0	15.0	11.7	6.7	2.3	0.0	-2.0	-3.3	0.3	1.7	2.3	3.7
10	20.9	19.7	17.2	11.1	10.8	4.9	4.3	0.9	0.6	5.5	5.8	7.1
11	27.7	34.2	29.5	20.5	12.7	4.8	4.8	-3.4	-3.8	-1.0	-3.4	-2.7
Mean±SE	21.4 ± 2.8	21.1 ± 3.0	19.1 ± 2.9	13.9 ± 1.8	7.3 ± 1.8	2.9 ± 1.8	1.7 ± 1.6	0.2 ± 1.8	1.3 ± 2.2	4.7 ± 2.8	4.7 ± 2.6	5.4 ± 2.2

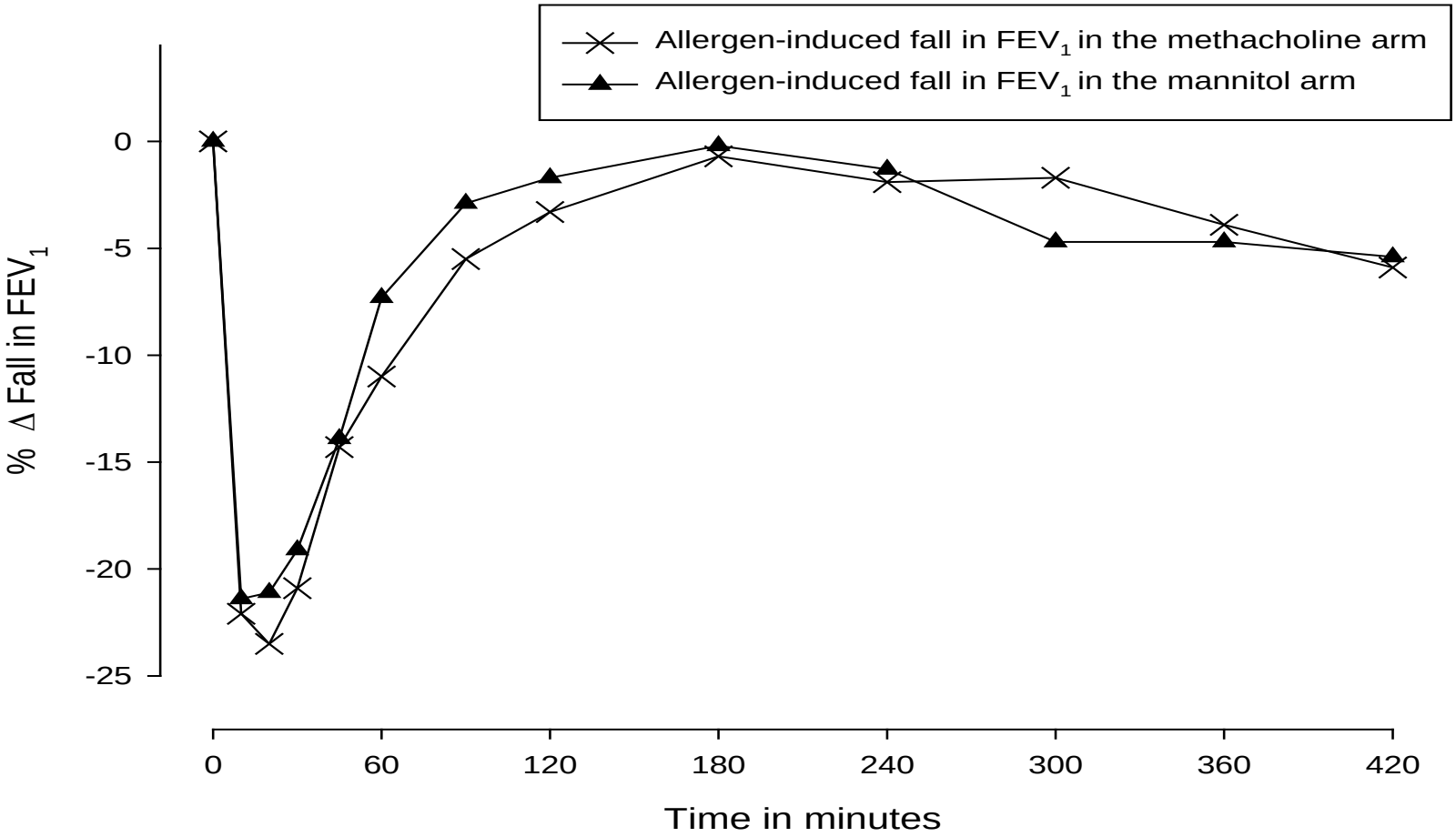

Figure 3.4. Allergen-induced FEV_1 fall in all the participants grouped into the methacholine and mannitol arm in the 24-hour study.

Table 3.13. Changes in PC_{20} in methacholine and dose response ratio in mannitol before and after allergen challenge in the 24-hour study.

Participant	MCh PC_{20}		Man Dose response				Methacholine		Mannitol	
	PRE ALLERGEN	POST ALLERGEN	PRE ALLERGEN	% FEV_1 FALL	POST ALLERGEN	% FEV_1 FALL	Log MCh pre	Log MCh post	Log DR pre	Log DR post
1	5.5	3.0	635	3.0	635	13.2	0.7404	0.4771	2.3010	1.6778
2	1.1	0.3	315	22.2	315	15.5	0.0414	-0.5376	1.1549	1.3098
3	1.2	1.0	635	5.2	635	4.7	0.0792	0.0170	2.0969	2.1549
4	73.0	27	635	1.8	635	1.1	1.8633	1.4314	2.5229	3.0000
5	1.6	2.6	155	19.4	635	21.5	0.2041	0.4150	0.9031	1.4685
6	0.2	0.3	475	18.6	635	10.6	-0.6197	-0.6021	1.4089	1.7696
7	14.2	5.3	635	12.6	635	0.8	1.7559	0.9294	1.6990	3.0000
8	57.0	8.5	635	16.4	635	7.7	1.1847	-0.2757	1.5850	1.9208
9	15.3	0.53	635	13.0	635	3.4	1.1523	0.7243	1.6990	2.3010
10	12.6	8.0	635	9.1	635	0.7	1.1004	0.8633	1.8539	3.0000
11	9.9	1.9	635	5.1	635	4.0	0.9956	0.2788	2.0969	2.2219
Geometric mean ± SE							5.9±0.20	2.2±0.23	63±0.15	158±0.18

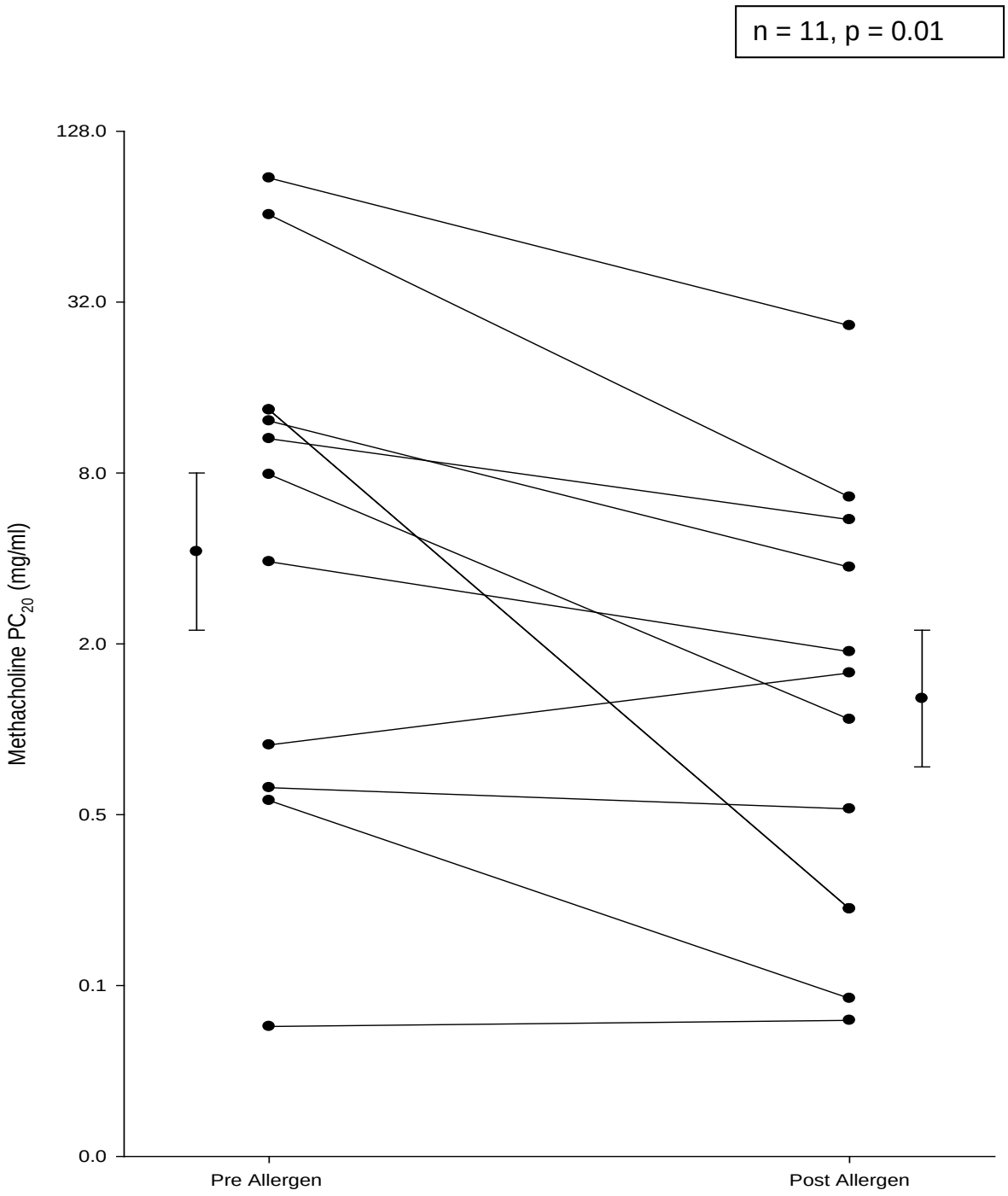

Figure 3.5. A graph showing the decrease in methacholine PC_{20} before and after the allergen challenge in the twenty-four hour study. The vertical axis is a log scale. Individual data points are geometric means ± S.E.

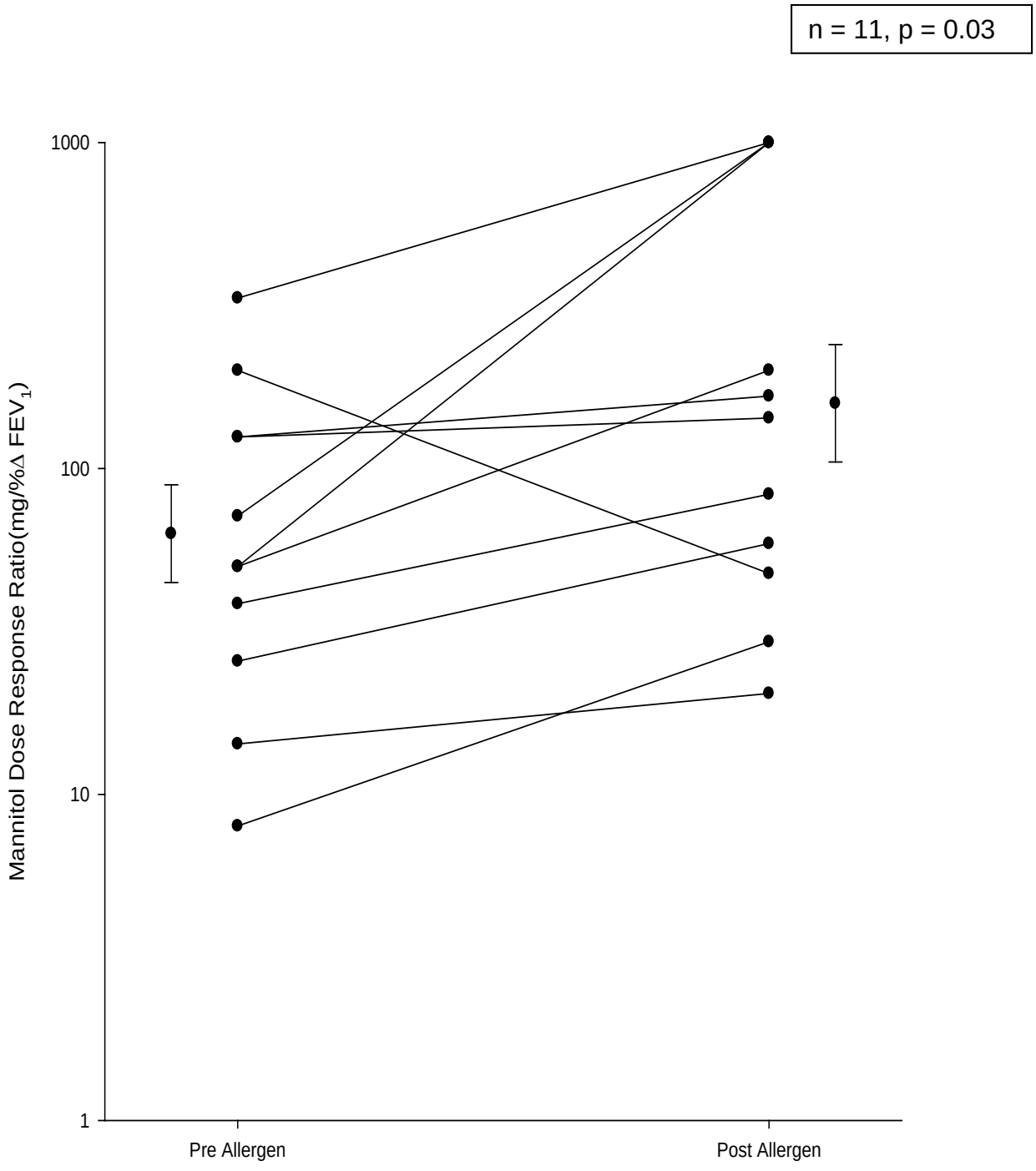

Figure 3.6. A graph showing the mannitol dose response slope before and after allergen challenge in the 24-hour study. The vertical axis is a dose in mg per percent change in FEV_1 scale. Individual data points are geometric means ± S.E.

3.9.4. Baseline FEV_1.

Baseline lung function was assessed at each visit using the highest of reproducible spirometric maneuvers. Twenty-four hours after the allergen challenge on the methacholine arm, the baseline FEV_1 was significantly lower than both the twenty-four hour pre allergen FEV_1 and the immediate pre allergen FEV_1 ($p < 0.05$). Conversely, there were no differences between baseline FEV_1 values during mannitol allergen mannitol testing ($p > 0.05$). Table 3.14 shows all the baselines FEV_1 (L) raw data

Table 3.14. Baseline FEV_1 (L) raw data

	Methacholine			Mannitol		
Participant	24H-Pre allergen	Pre Allergen	Post Allergen	24H-Pre allergen	Pre Allergen	Post Allergen
1	4.57	4.49	4.44	4.71	4.68	4.8
2	3.54	3.48	3.23	3.52	3.42	3.43
3	2.77	2.95	2.67	2.74	2.89	2.77
4	4.58	4.57	3.85	4.54	4.45	3.57
5	3.13	3.21	3.05	3.19	3.16	3.02
6	2.24	2.15	1.62	2.49	2.26	3.49
7	3.89	3.82	3.51	3.97	3.97	3.80
8	4.63	4.57	4.22	4.46	4.59	4.58
9	3.01	3.04	2.74	2.95	3.00	2.94
10	3.16	2.99	3.00	3.05	3.25	2.86
11	3.08	3.08	3.01	2.96	2.92	3.00
Mean ± SE	3.51±0.24	3.50±0.24	3.21±0.23	3.51±0.24	3.51±0.24	3.48±0.21

3.9.6. Fractional Exhaled Nitric Oxide in methacholine arm.

Geometric mean F_ENO levels across methacholine triad testing were 27, 26, 37, and 55 ppb. There was a significant difference after both the 7-hour post allergen FENO level and 24-hour post allergen FENO level ($p < 0.05$) Table 3.15 shows the F_ENO measurements on methacholine arm in all the participants. Figure 3.7 shows the F_ENO measurements on methacholine arm in all participants.

Table 3.15. Fractional Exhaled Nitric Oxide measurements on methacholine arm

Participant number	24-hour Pre Allergen	Pre Allergen	7 hour Post Allergen	24 hour Post Allergen
1	58	54	56	90
2	47	52	47	63
3	19	19	*EF	*EF
4	21	20	21	76
5	44	47	57	108
6	34	30	28	29
7	9	9.7	*EF	*EF
8	72	65	86	96
9	19	19	21	32
10	12	9	*EF	54
11	21	19	22	21
Geometric Mean (ppb) ±SE	27 ± 0.09	26 ± 0.09	37 ± 0.09	55 ± 0.10

* *Equipment failure*.

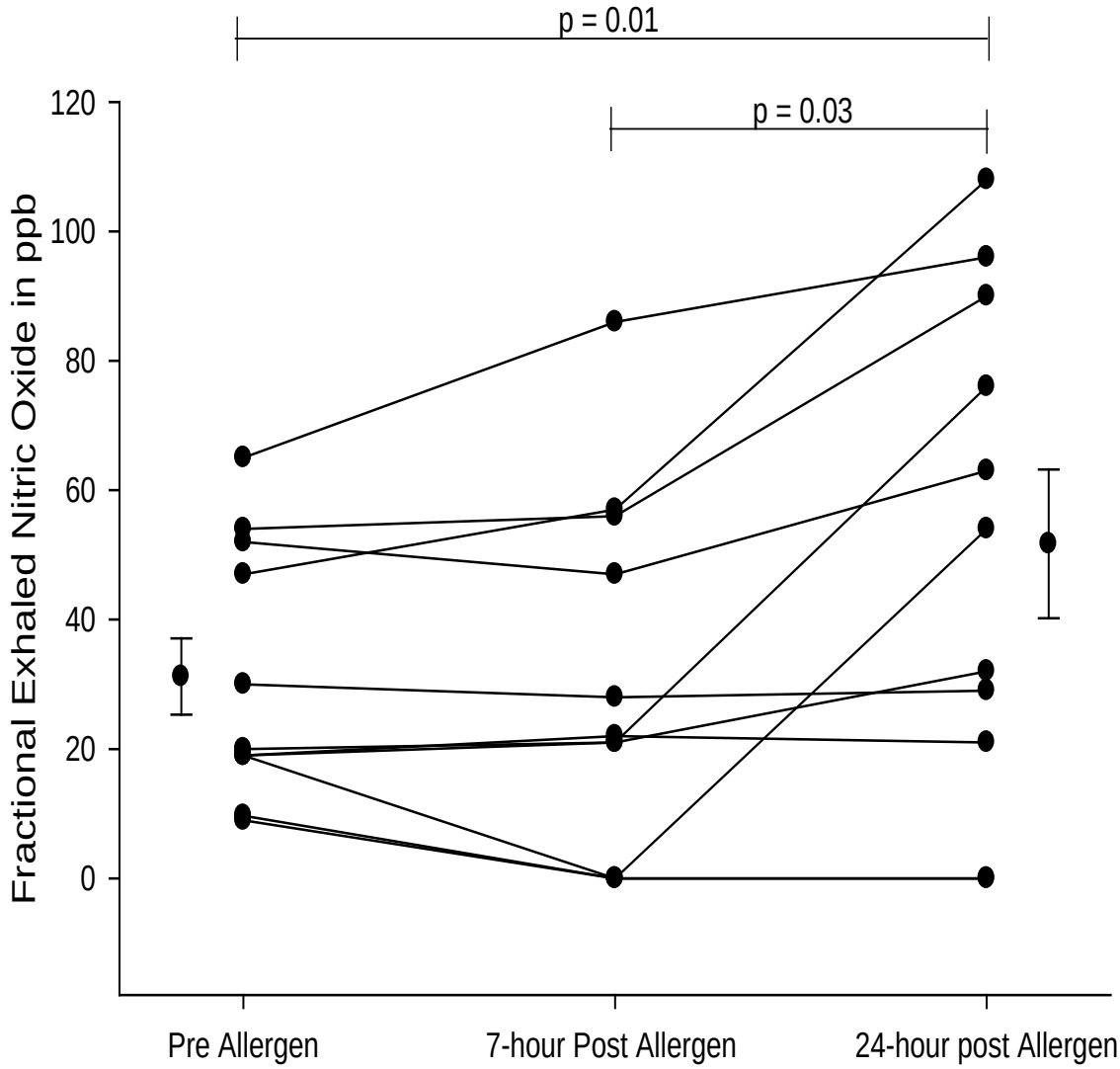

Figure 3.7 Nitric oxide measurement on methacholine arm before allergen challenge, 7-hour and 24-hour time points after allergen challenge in the 24 hour study.

3.9.7. Fractional Exhaled Nitric Oxide in mannitol arm.

Geometric mean F_ENO across the mannitol arm were 29, 31, 39 and 39 ppb. There was no significant difference after both the 7 hour post allergen F_ENO level and 24 hour post allergen F_ENO level ($p = 0.05$).

Table 3.16 shows the F_ENO measurements on mannitol arm in all the participants. Figure 3.8 shows the F_ENO measurement on mannitol arm in all participants.

Table 3. 16. Fractional Exhaled Nitric Oxide measurements on mannitol arm

Participant	24-hour Pre Allergen	Pre Allergen	7 hour Post Allergen	24 hour Post Allergen
1	24	29	39	63
2	37	47	51	70
3	13	10	17	21
4	106	62	68	57
5	53	67	61	96
6	16	22	17	15
7	20	21	24	21
8	58	61	78	93
9	33	40	42	40
10	32	*EF	*EF	28
11	8	13	*EF	20
Geometric Mean(ppb)±SE	29±0.10	31±0.09	39±0.08	39±0.09

* *Equipment failure*

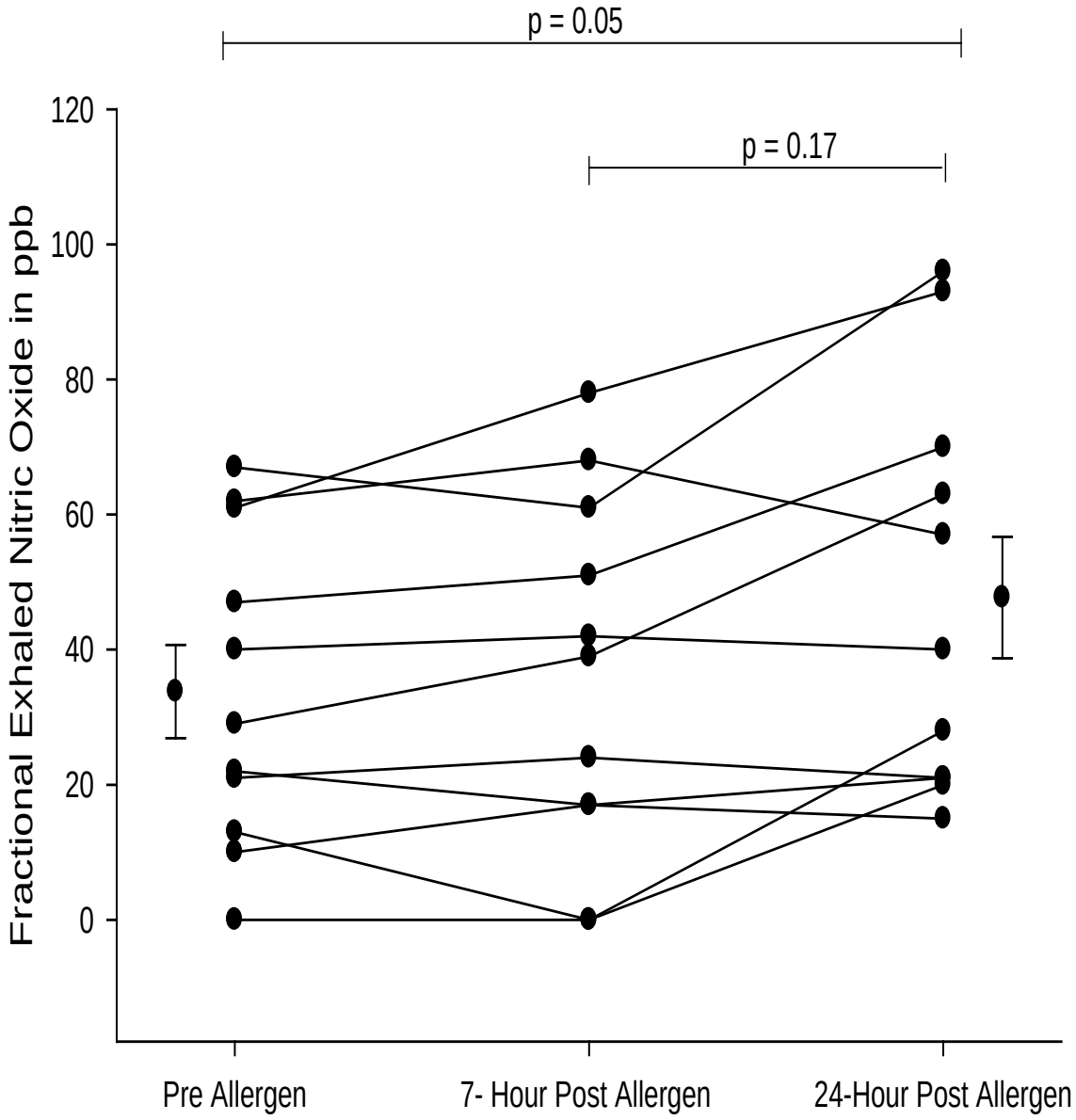

Figure 3.8. Nitric oxide measurements on mannitol arm before allergen challenge, 7-hour and 24-hour post allergen time points in the 24 hour study.

3.10. Discussion for 24-hour study.

In the 24-hour study, we again found an increase in airway response to methacholine and a decrease in airway response to mannitol. The increase in airway responsiveness to methacholine was associated with an increase in F_ENO. However, F_ENO did not increase after allergen in the mannitol arm. According to the literature, the

increase in airway responsiveness to methacholine tends to occur in those with an LAR [Cockcroft *et al*., 1977; Cartier *et al*., 1982]. In our study, the majority of the participants did not have an LAR (i.e. a 15% drop in FEV_1).Only one person had a fall in FEV_1 of more than 15% in the three to seven hours after allergen challenge. This documents a dissociation between LAR and an increase in hyperresponsiveness to methacholine. It is possible that if airway inflammation increased and an LAR was present, that airway responsiveness to mannitol would occur. We had hoped for a large percentage of LAR responders in our sample but unfortunately only one participant met LAR criteria. This participant did not respond to mannitol even in the presence of an LAR.

One potential limitation was not requiring a positive response to mannitol at study entry. We had anticipated that participants who were initially negative with mannitol would convert to a positive mannitol challenge. This however was not the case and as a result, we calculated a dose response ratio (cumulative dose [635mg]/ $\%\Delta FEV_1$) instead of a mannitol PD_{15}.

Sputum eosinophilia tends to increase after allergen challenge, which correlates with LAR [De Monchy *et al.,* 1985].Additionally, airway responsiveness to indirect stimuli, correlates with airway inflammation [Van den Berge *et al*., 2001]. We assessed airway inflammation by measuring F_ENO. We found that allergen challenge increased airway inflammation in the methacholine arm and this was associated with an increase in airway responsiveness to methacholine.

The increase in allergen–induced airway inflammation in the mannitol arm was borderline not significant. However if the participants had an LAR and a positive mannitol response, one could speculate that the increase in allergen-induced airway inflammation would be significant. This suggests airway response to mannitol may have increased if the extent of airway inflammation was greater. It may be worth mentioning that, the relationship between allergen–induced airway eosinophilia and allergen–induced airway responsiveness is not clear-cut in terms of what causes or proceeds the other [Leckie *et al*., 2000].

In order to help explain the decrease in airway response to mannitol, we turned our focus on refractoriness and cross-refractoriness which are well-recognized phenomena, when dealing with indirect stimuli challenges [Schoeffel *et al*., 1980;

Rakotosihanaka *et al*., 1986; Daxun *et al*., 1989; Suh *et al*., 2011;Belcher *et al*., 1987]. Although the specifics of the mechanism of refractoriness is also unclear, it has been established that, repeated inhalation of mannitol within 90 minutes induces refractoriness, at the airway smooth muscle's responsiveness to mediators of bronchoconstriction, rather than the depletion of mast cell mediators [Larsson *et al*., 2011] . A later time point (i.e. 24 hours) should be enough time for the mast cell to manufacture and repackage mediators of inflammation.

Moving on from the amount of mediator release, another possible explanation is airway receptor desensitization of the mast cell at the site of the airway smooth muscle. [Kern *et al*., 1986]. Among the potential receptors that are desensitized to the mediators are leukotriene receptors in the airway smooth muscle. This is evident from desensitization to aspirin challenge in aspirin-intolerant asthma, associated with decreased expression of leukotriene receptors [Sousa *et al*., 2002]. Again, 24-hour post allergen time should allow the airway smooth muscle cell to recover from sensitization. It has been established that following repeated exercise within 2 hours, about half of asthmatics will have less than half of the initial response. This is observed with yet another unclear mechanism of bronchoconstriction via indirect stimuli, which primarily affects the osmolarity of the airways, similar to mannitol.

Another reason is the protective response of mast cell mediators [Larsson *et al.,* 2011] that accounts for the decrease in airway responsiveness to mannitol following allergen exposure. Since the release of mediators has been confirmed by an increase in nitric oxide measurements, one can assume that different mediators that offer protection to the airway smooth muscle in response to an allergen and subsequent mannitol challenges are also released. These "airway smooth muscle protection mediators" result in the less responsiveness of the airway smooth muscle 3-hour post allergen and 24-hour post allergen. Moreover, the activation of cysteinyl-leukotrienes receptors cause a dose dependent secondary release of prostaglandins and other cyclooxygenase products in the lungs [Dahlen, 1983; Omini *et al.,* 1981]. Among these prostaglandins are the bronchoprotective PGE_2. This is evident in the fact that premedication with cyclooxygenase inhibitors such as indomethacin attenuates the refractoriness following exercise challenge [O'Byrne *et al*., 1986].

According to Larsson *et al*., not only is an increase in mast cell mediator release, but also a more sustained elevation of mediator excretion from refractory participants within 90 minutes of repeated mannitol inhalation. This was more pronounced for LTE_4 mediators. Cross-refractoriness exists between EIB, which is an osmolar indirect stimulus and $LTD_{4,}$ when both challenges are done one hour apart. [Manning *et al*., 1993].The refractoriness to LTD_4 was abolished by the cyclooxygenase inhibitor, proving the involvement of leukotrienes and prostaglandins in refractoriness to repeated exercise challenge in interdependent pathways.

In conclusion, airway responsiveness to methacholine increased twenty-four hours after allergen challenge however airway response to mannitol was decreased. Even though there was enough time between the allergen challenge and the mannitol challenge to rule out refractoriness, the response to mannitol was similar to that of the three-hour study. This opens up questions about the mechanisms of indirect stimuli; airway inflammation, allergen challenge and how it differs from that of direct stimuli.

4. GENERAL DISCUSSION AND FUTURE RESEARCH

Increase in methacholine responsiveness has been documented as early as three hours however, methacholine responsiveness is routinely measured at twenty-four hours post allergen. [Cockcroft *et al*., 1977]. Not much is known about allergen-induced change in airway responsiveness to mannitol. We looked at the change in mannitol responsiveness at three hours and twenty-four hours post allergen. In the three-hour post allergen study, we found that, there was an increase in airway responsiveness to methacholine (i.e. a decrease in PC_{20}) Airway responsiveness to mannitol however, decreased (i.e. an increase in PD_{15}) at 3 hours post allergen which was the complete opposite of what we hypothesized. A potential explanation was refractoriness, a phenomenon associated with indirect stimuli (e.g. EIB). This led us to extend the time between allergen challenge and mannitol challenge from three hours to twenty-four hours. Again, however methacholine responsiveness increased and mannitol responsiveness decreased twenty-four hours post allergen challenge.

We expected a greater response to mannitol than methacholine because airway responsiveness to indirect stimuli correlates better with airway inflammation than the direct stimuli. Both an increase in AHR to methacholine and an increase in airway inflammation are associated with allergen exposure. We assessed changes in airway inflammation in the twenty-four hour study by measuring F_ENO. We found that F_ENO increased significantly in the methacholine arm but not significantly in the mannitol arm.

We believe our hypotheses were valid and our methodologies to test our hypotheses were sound. We assumed however that people with a negative mannitol challenge would potentially shift to positive following allergen challenge. This did not occur and the absence of mannitol responsiveness at study entry could be a limitation. Notably however, airway responsiveness to mannitol was significantly decreased and not just absent.

Twenty four hour post allergen challenge measurements and the utility of the allergen challenge model is usually employed for assessing mechanisms of asthma and novel drug treatments for asthma in dual responders. Ideally, the majority of our participants in the twenty-four hour study would have included late responders. This however was not the case. There was only one participant who had a fall in FEV_1 of

greater than or equal to 15% in the three to seven hours post allergen. Interestingly, this participant did have an increase in airway responsiveness to methacholine but not to mannitol. It is hard to draw conclusions from one out of eleven participants however; we should acknowledge the data generated by this individual.

With respect to airway inflammation, we measured baseline airway inflammation in the three-hour study prior to testing at each visit. The increased F_ENO values are indicative of underlying airway inflammation, which suggested all participants should have tested positive for mannitol.

In the 24-hour study, there was an increase in F_ENO after allergen challenge in the methacholine arm but not in the mannitol arm. This suggests that twenty-four hours is enough to observe the recruitment of inflammatory cells and therefore an increase in airway inflammation, which supports the potential for an increase in airway responsiveness to mannitol. There was a significant increase between the F_ENO levels at 7 and 24 hours in the methacholine. However, there were no significant difference between the seven and 24-hour time points of F_ENO in the mannitol arm. The increase in FENO at 24 hours in the methacholine arm is consistent with the literature for both increased airway inflammation and subsequent increase in airway responsiveness to methacholine. Interestingly, airway inflammation in the mannitol arm did not increase and there was an associated decrease in airway responsiveness to mannitol.

Our hypotheses were driven by the observation of Van den Berge *et al.* that, the AMP PC_{20} was more closely related to airway inflammation than methacholine PC_{20} [Van den Berge *et al.,* 2001]. AMP is a known indirect stimulus of airway hyperresponsiveness; as such, we made the jump from AMP to mannitol. The assumption was due to the fact that they both are indirect stimuli, and they possibly share a similar pathway with the various indirect stimuli of bronchoconstriction namely allergen, EIB, AMP, and mannitol. The sharing of a common pathway was unveiled mostly in the studying of refractoriness across indirect stimuli [Schoeffel *et al.,* 1980; Rakotosihanaka *et al.,* 1986; Daxun *et al.,* 1989; Suh *et al.,* 2011; Belcher *et al.,* 1987]. Since we associate greater airway inflammation with indirect challenges (that is AMP) [Van den Berge *et al.,* 2001] by way of LAR, we generalized that airway responsiveness

to mannitol challenge test post allergen should be higher than methacholine challenge test post allergen.

The nature of the 3-hour post allergen study did not accommodate the detection of late asthmatic responders, due to the administering of either the mannitol or the methacholine challenge at the 3-hour post allergen time, and controlling the bronchoconstriction with medication at the end of the study. There was not enough time to detect the LAR, which is mostly associated with inflammation. However, the 24-hour post allergen study took this into consideration and waited for the LAR before administering either the mannitol or the methacholine challenge. The 24-hour post allergen study also resulted in a negative mannitol challenge. Moreover, the dose response ratio of the mannitol increased twenty-four hours after allergen challenge. Even though F_ENO measurements increased, the dose response ratio of mannitol increased instead of decreasing. The LAR has been seen mostly in indirect challenges. Allergen challenge is classified as an indirect stimulus. Allergen-induced LAR is a phenomenon well understood; however, the emergence of exercise-induced LAR is a rare but possible phenomenon considered under heavy criticism. There seems to be an emergence of indirect stimuli induced LAR. The interaction of these LAR's is also another possible explanation as to why mannitol response decreased following allergen challenge. Even though there are heavy criticisms surrounding the presence of indirect stimuli's LAR aside from allergen induced LARs, future research is called for into the mechanisms of LARs, and the different LARs interactions among indirect stimuli.

At this point, we can stand by our hypothesis and attribute the decrease in airway response to mannitol to a lack of LAR in participants, refractoriness, cross refractoriness, absence of airway inflammation and desensitization of leukotriene receptors. We can also refute our hypothesis and pin it on the unknown pathway that indirect stimuli 'supposedly' share. The various indirect stimuli might not necessarily act in the same manner whenever they are thrown together in asthma challenges. It might also not be wise to substitute the behavior of one indirect stimulus for another indirect stimulus although; all indirect stimuli seem to have a better correlation with airway inflammation than their direct counterparts do. This calls for further research on the degree of correlation between all the various indirect stimuli and airway inflammation.

Research questions should be aimed at finding a possible interaction between allergen challenges and mannitol or AMP. Allergen is classified as an indirect challenge hence future research should be aimed at any possible interference between the allergen and indirect stimuli. This could be further investigated by looking at allergen induced late response, exercised induced late response and mannitol induced late response. In the case of establishing the existence of a late response with each of the indirect stimuli, one should look at the type of asthma subjects. Moreover, one should also keep a curious eye on people who are not consistent to a type of bronchoprovocation stimulus. These participants usually test positive for a stimulus and later on (in a year) test negative for that same stimulus. Research efforts should be geared toward finding out why there are inconsistencies in response to bronchoprovocation stimulus occurring in research participants along the years. Shedding light on these could help answer research questions about why there is an increase and a decrease in response to direct and indirect stimulus respectively. Further research is also needed on the impacts of allergen challenges on the various indirect stimuli namely EIB, AMP and mannitol. The supposed pathway that indirect stimuli share should be uncovered at least in terms of what is common to all indirect stimulus if not all. Knowing their pathways can help scientists conduct research to eliminate possible refractoriness and cross-refractoriness in further studies.

Although the term asthma is sometimes used loosely to describe EIB or EIA, AHR, allergen induced bronchoconstriction. Research efforts should be made at classifying these categories. The results of allergen induced increase in airway response to mannitol in EIB participants might not be the same as that in subjects with AHR. As such, the research criteria for participants should be aimed at selecting individuals with AHR only or EIB only or EIA only and not a participant with both. This classification should help researchers answer questions on the behavior of bronchoprovocation stimuli in a specific group of research participants.

4. REFERENCES

Altounyan R.E.C. 1964. Variation of Drug Action on Airway Obstruction in Man. Thorax. 19(5):406-415

Amakye D. O., Davis B.E., Martin A.L., Peters G. Cockcroft D.W.2013. Refractoriness to inhaled mannitol 3 hours after allergen challenge. Annals of Allergy, Asthma and Immunology 111; 182-184

American Academy of Allergy, Asthma and Immunology. 2014. http://www.aaaai.org/conditions-and-treatments/conditions-a-to-z-search/Atopy.aspx

Anderson S.D. 2010. Exercise-Induced Bronchoconstriction in the 21st century J Am Osteopath Assoc 111 (1): S3-S10

Anderson S.D., Brannan J., Spring J., *et al*. 1997. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. Am J Respir Crit Care Med.156: 758-65

Anderson S.D., Brannan J., Spring J., Spalding N., Rodwell L.T., Chan K., Gonda I., Walsh A., Clark A.R. 1997. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. Am J Respir Crit Care Med.156:758-65.

Anderson S.D., Connolly N.M., Godfrey S.1971 Comparison of bronchoconstriction induced by cycling and running Thorax, 26:396–401

Anderson S.D., Charlton B., Weiler J.M., Nichols S., Spector S.L., Pearlman D.S., A305 Study Group. 2009. Comparison of mannitol and methacholine to predict exercise-induced bronchoconstriction and a clinical diagnosis of asthma. Respir Res.10:4.

Astrand P.O., Rodahl K. 1986 Textbook of work physiology, 3rd Ed. McGraw Hill, New York

Avital A., Springer C., Bar-Yishay E. *et al*. 1995. Adenosine, methacholine, and exercise challenges in children with asthma or paediatric chronic obstructive pulmonary disease Thorax. 50:511–516

Bacchetta, R., Gambineri, E. & Roncarolo, M. G. 2007. Role of regulatory T cells and FOXP3 in human diseases. J. Allergy Clin. Immunol. 120:227–235

Barnes, P. J., Chung, K. F. & Page, C. P.1998. Inflammatory mediators of asthma: an update. Pharmacol. Rev. 50:515–596.

Bar-Yishay E., Ben Dov I., Godfrey S.1983.Refractory period after hyperventilation-induced asthma Am Rev Respir Dis, 127:572–574

Belcher N.G., Rees P.J., Clark T.J., Lee T.H.1987. A comparison of the refractory periods induced by hypertonic airway challenge and exercise in bronchial asthma. Am Rev Respir 135:740-751

Ben Dov I., Gur I, Bar-Yishay E., *et al*. 1983. Refractory period following induced asthma: contributions of exercise and isocapnic hyperventilation. Thorax, 38:849–853

Bierman C.W., Spiro S.G., Petheram I. 1984. Characterization of the late response in exercise induced asthma J Allergy Clin Immunol. 74:701-6

Boulet L.P., Corbeil F., Turcotte H.1992. Influence of a single antigenic challenge on the pattern of airway response to exercise in asthma. Ann Allergy. 68(4):363-70

Bradley L. B., Azzawi M., Jacobson B., Assoufi J. V., Collins A. M., Irani L,. Schwartz B.,

Durham S. R., Jeffery P. K., and Kay A. B. 1991. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. J. Allergy Clin. Immunol. 88:661–674.

Brannan JD, Anderson SD, Perry CP, Freed-Martens R, Lassig AR, Charlton B, Aridol Study Group.2005. The safety and efficacy of inhaled dry powder mannitol as a bronchial provocation test for airway hyperresponsiveness: a phase 3 comparison study with hypertonic (4.5%) saline. Respir Res.6:144.

Brightling, C. E. *et al*. 2002. Mast-cell infiltration of airway smooth muscle in asthma. N. Engl. J. Med. 346:1699–1705

Brusasco V., Crimi E., Gianiorio P., Lantero S., and Rossi G. A.1990. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. J. Appl. Physiol. 69:2209–2214.

Carlsen K.H., Engh G., Mork M.2000.Exercise-induced bronchoconstriction depends on exercise load Respir Med, 94:750–755

Cartier A., Thomson N.C., Frith P.A., Roberts R., Hargreave F.E. 1982. Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and change in airway caliber. J Allergy Clin Immunol 70:170-77.

Chapman K. R., Boulet L. P., Rea R. M., Franssen E.2008. Suboptimal asthma control: prevalence, detection and consequences in general practice. European Respiratory Journal. 31(2):320–325.

Cobanoglu B., Toskala E., Ural A., Cingi C. 2013. Role of leukotriene antagonists and antihistamines in the treatment of allergic rhinitis. Curr Allergy Asthma Rep 13:203-8.

Cockcroft D.W., McParland C.P., O'Byrne P.M., *et al*. 1993. Beclomethasone given after the early asthmatic response inhibits the late response and the increased methacholine responsiveness and cromolyn does not. J Allergy Clin Immunol. 91:1163-8.

Cockcroft D.W., Ruffin R.E., Dolovich J., Hargreave F.E.1977. Allergen-induced increase in non-allergic bronchial reactivity. Clin Allergy.7:503-13.

Cockcroft *et al*. 1987. Prediciton of Airway Responsiveness to Allergen from Skin Sensitivity and Airway Responsiveness to Histamine. Am Rev Respir Dis 13 5:264-267.

Cockcroft *et al*. 2005 'The Link between Allergen Skin Test Sensitivity, Airway Responsiveness and Airway Response to Allergen' Allergy 60:56-59.

Cockcroft, D. W., Hargreave, F. E., O'Byrne, P. M. & Boulet, L. P. 2007. Understanding allergic asthma from allergen inhalation tests. Can. Respir. J. 14:414–418

Cockcroft, D.W., Murdock K.Y.1987a. Comparative effects of inhaled salbutamol, sodium cromoglycate, and beclamethasone dipropionate on allergen-induced early asthmatic response, late asthmatic responses, and increased bronchial responsiveness to histamine. J Allergy Clin Immunol. 79:734-736.

Corren J. 2013. Asthma Phenotypes and Endotypes: An evolving Paradigm for classification. Discov Med 15(83):243-249

Cousins D.J., Lee T.H., Staynov D.Z. 2002 Cytokine expression during human Th1/Th2 Cell differentiation: direct evidence for coordinated expression of Th2 cytokines. J Immunol. 169(5):2498-506

Crapo R.O., Casaburi R., Coates A.L., *et al*. 2000.Gulidelines for Methacholine and Exercise Challenge Testing-1999.Am J Respir Crit are Med.161:309-329

Crimi E., Spanevello A., Neri M., Ind P.W., Rossi G.A., Brusasco V.1998. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. Am J Respir Crit Care Med 157(1):4-9

Cushley M.J., Holgate S.T.1985. Adenosine-induced bronchoconstriction in asthma: role of mast cell-mediator release. Allergy, Clin Immunol.75:272-8.

Dahlén S.E., Hansson G., Hedqvist P., Björck T., Granström E., Dahlén B.1983. Allergen challenge of lung tissue elicits bronchial contraction that correlates with the release of leukotrienes C4 D4 and E4 Proc Natl Acad Sci U S A. 80(6):1712-6

Davies S.E. 1968. The effect of disodium cromoglycate on exercise-induced asthma Br Med J, 3:593–594

Daxun Z., Rafferty P., Richards R., Summerell S., Holgate S.T.1989. Airway refractoriness to adenosine 5'-monophosphate after repeated inhalation. J Allergy Clin Immunol. 83(1):152-8

de Monchy J.G.R., Kauffman H.F., Venge P., *et al*. 1985.Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am Rev Respir Dis 131:373-6.

de Monchy J.G.R., Kauffman H.F., Venge P., Koeter G.H., Jansen H.M., Sleuter Deal E.C., McFadden E.R., Ingram R.H. *et al*. 1979**.** Esophageal temperature during exercise in asthmatic and non-asthmatic subjects J Appl Physiol, 46 .pp. 484–490

Deal E.C., McFadden E.R., Ingram R.H. *et al*. 1979a**.** Role of respiratory heat exchange in production of exercise-induced asthma J Appl Physiol. 46:467–475

Dickinson J., McConnell A., Whyte G.2011. Diagnosis of exercise-induced bronchoconstriction: eucapnic voluntary hyperpnea challenges, identify previously

undiagnosed elite athletes with exercise-induced bronchoconstriction [Published online ahead of print July 20, 2010]. Br J Sports Med.45 (14):1126-1131

Driver A.G., Kukoly C.A., Metzger W.J., Mustafa S.J. 1991. Bronchial challenge with adenosine causes the release of serum neutrophil chemotactic factor in asthma. Am Rev Respir Dis.143:1002-7.

Durham S.R., Craddock C.F., Cookson W.O., Benson M.K. 1988. Increases in airway responsiveness to histamine precede allergen-induced late asthmatic responses. J Allergy Clin Immunol 82:764-70.

Durham S.R. 1991. The significance of late response in asthma Clin Exp Allergy 21:3-7

Edmunds A.T., Tooley M., Godfrey S.1978.The refractory period after exercise-induced asthma: its duration and relation to the severity of exercise Am Rev Respir Dis117:247–254

Fahy J.V.1998. Airway mucus and the mucociliary system. Middleton, E Reed, CE Ellis, EF*et al* eds.Allergy principles and practice 5th ed.520-531

Finnerty J.P, Holgate S.T 1990. Evidence for the roles of histamines and prostaglandins as mediators in exercised induced asthma: the inhibitory effect of terfenabine and flurbiprofen alone and in combination Eur Respir J. 3:540-547

Freed A.N., Anderson S.D. 2008. Exercise-induced bronchoconstriction: human models. In: Kay AB, Bousquet J, Holt PG, Kaplan AP, eds. Allergy and Allergic Diseases. Oxford, England: Blackwell Scientific Publications. 1:808-822.

Gauvreau G.M., Doctor J., Watson R.M., Jordana M. O'Byrne P.M.1996. Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. AM J Respir Crit Carte Med 154:1267-1271.

Gauvreau G.M., Evans M.Y., 2007. Allergen inhalation challenge: a human model of asthma exacerbation. Contrib Microbiol.14:21-32.

Global Initiative for Asthma. 2014. http://www.ginasthma.org

Godfrey S., Springer C. Noviski N. *et al*. 1991.Exercise but not methacholine differentiates asthma from chronic lung disease in children Thorax. 46:488–492

Government of Canada, Public Health Agency of Canada. 2007. Life and Breath: Respiratory Disease in Canada. November 21.

Government of Canada, Statistics Canada. 2010. Canadian community health survey (CCHS)—annual component. Ottawa: Statistics Canada.

Hahn A.G., Nogrady S.G., Tumilty D.M., Lawrence S.R., Morton A.R.1984. Histamine reactivity during the refractory period after exercise induced asthma. Thorax.39:919-923

Hammad H., Lambrecht B. N.2008.Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nature Rev. Immunol. 8:193–204.

Hammad H., Lambrecht B. N.2006. Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation.J. Allergy Clin. Immunol. 118:331–336.

Hargreave F.E., Ryan G., Thomson N.C., *et al*.1981. Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance. J Allergy Clin Immunol 68:347-55

Henriksen J.M., Nielsen T.T.1983. Effect of physical training on exercise-induced bronchoconstriction Acta Paediatr Scand. 72:31–36

Holgate TS, Polosa R. 2008. Treatment strategies for allergy and asthma Nature Reviews Immunology 8:218-230

Holzer K, Anderson S.D., Chan H.K., Douglass J. 2003. Mannitol as a challenge test to identify exercise-induced bronchoconstriction in elite athletes. Am J Respir Crit Care Med.167 (4): 534-547.

Howell, J.B.L. 1971. Asthma: a clinical view, identification of Asthma (cd. R. Porter and J. Birch), p. 151. Churchill Livingstone, Edinburgh and London

Jokic R, Davis EE, Cockcroft DW.1998. Extrapolation of methacholine PC_{20}. Chest.103:1796-1797

Kern R., Smith L.J., Patterson R., Krell R.D., Bernstein P.R. 1986. Characterization of the airway response to inhaled leukotriene D_4 in normal subjects. Am Rev Respir Dis 133: 1127–1132.

Kersten E.T., Driessen J.M., van der Berg J.D., Thio B.J. 2009. Mannitol and exercise challenge tests in asthmatic children. Pediatr Pulmonol.44(7): 655-661.

Keyzer J.J., Kauffman H.F., de Monchy J.G., Keyzer-Udding J.J., de Vries K. 1984. Urinary W-methylhistamine during early and late allergen-induced bronchial obstructive reaction.J Allergy Clin Immunol. 74:240-245.

Kirby J. G., Hargreave F. E., Gleich G. J., and O'Byrne P. M. 1987. Bronchoalveolar cell profiles of asthmatic and non-asthmatic subjects. Am. Rev. Respir. Dis. 136:379–383.

Knox A.J., Tattersfield A.E. 1995. Airway smooth muscle relaxation Thorax.50(8): 894–901

Koh Y.Y., Lim H.S., Min K.U.1994.Airway responsiveness to allergen is increased 24 hours after exercise challenge J allergy Clin Immunol. 9: 507-16

Larche, M.2007. Regulatory T cells in allergy and asthma. Chest 132:1007–1014.

Larsson J., Perry C.P., Anderson S.D., Brannan J.D., Dahlén S.E., Dahlén B.2011. The occurrence of refractoriness and mast cell mediator release following mannitol-induced bronchoconstriction. J Appl Physiol.110:1029-1035.

Larsson J., Anderson S.D., Dahlén S.E., Dahlén B.2013.Refractoriness to exercise challenge: a review of the mechanisms old and new [published online March 29, 2013]. Immunol Allergy Clin N Am. doi: 10.1016/j.iac.2013.02.004.

Leckie M.J., ten Brinke A., Khan J., *et al*. 2000.Effects of an interleuken-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. Lancet 356:2144-2148.

Lee T.H., Nagakura T., Papageorgiou N. *et al*. 1983. Exercised–induced late asthmatic reactions with neutrophil chemotactic activity N Eng J Med. 308:1502-5

Lee T.H., O'Hickey S.P. 1989 Exercised induced asthma and late phase reaction Eur Respir J. 2;195-7

Lopez V., Prieto L., Perez-Frances C., Barato D., Marin J. 2012. Natural pollen exposure increases the response plateau to adenosine 5' monophosphate and bronchial but not alveolar nitric oxide in sensitized subjects Respiration. 83(3):225-32.

Lötvall J., Akdis C.A., Bacharier L.B., Bjermer L., Casale T.B., Custovic A., Lemanske R.F. Jr., Wardlaw A.J., Wenzel S.E., Greenberger P.A.2011. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol. 127(2):355-60

Lötvall J., Palmqvist M., Arvidsson P. 2011.Comparing the effects of two inhaled glucocorticosteroids on allergen-induced bronchoconstriction and markers of systemic effects, a randomized cross-over double-blind study. Clinical and Translational Allergy, 1:12

Macfarlane A.J., Kon O.M., Smith S.J., Zeibecoglou K., Khan L.N., Barata L.T., McEuen A.R., Buckley M.G., Walls A.F., Meng Q.,Humbert M., Barnes N.C., Robinson D.S., Ying S., Kay A.B.2000.Basophils, eosinophils, and mast cells in atopic and non-atopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol. 105(1):99-107

Manning P.J., Rokach J., Malo J.L., Ethier D., Cartier A., Girard Y., Charleson S., O'Byrne P.M. 1990. Urinary leukotriene E4 levels during early and late asthmatic responses. J Allergy Clin Immunol. 86: 211-20

Manning P.J., Watson R.M., O'Byrne P.M .1993.Exercise-induced refractoriness in asthmatic subjects involves leukotriene and prostaglandin interdependent mechanisms Am Rev Respir Dis, 148:950–954

Margolskee D.J., Bigby B.G., Boushey H.A. 1988. Indomethacin blocks airway tolerance to repetitive exercise but not to eucapnic hyperpnea in asthmatic subjects. Am Rev Respir Dis 137(4):842-6

Marquardt D.L., Gruber H.E., Wasserman S.I.1984.Adenosine Release from stimulated mast cells Proc Natl Acad Sci USA 81(19):6192-6

Mattoli S., Foresi A., Corbo G.M. *et al*. 1987.The effect of indomethacin on the refractory period occurring after the inhalation of ultrasonically nebulised distilled water J Allergy Clin Immunol, 79: 678–683

O'Byrne P.M., Jones G.L.1986. The effect of indomethacin on exercise-induced bronchoconstriction and refractoriness after exercise. Am Rev Respir Dis.134(1):69-72.

Omini C., Folco G.C., Viganò T., Rossoni G., Brunelli G., Berti F.1981.Leukotriene C4 induces generation of PGI2 and TXA2 in guinea pig in vivo Pharmacol Res Commun.13(7):633-40

Oosterhoff Y., Koeter G.H., de Monchy J.G.1993. Circadian variation in airway responsiveness to methacholine, propranolol, and AMP in atopic asthmatic subjects. Am Rev Respir Dis.147:512–517.

Pauwels R., Joos G., Van Der Straeten M. 1988. Bronchial hyperresponsiveness is not bronchial hyperresponsiveness is not bronchial asthma. Clin Allergy.18:317-321.

Peachell P.T., Columbo M., Kagey-Sobotka A., Lichtenstein L.M., Marone G. 1988. Adenosine potentiates mediator release from human lung mast cells. Am Rev Respir Dis.136:1143–1151.

Phillips G.D., Ng W.H., Church M.K., Holgate S.T.1990. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5_-monophosphate, and allergen in atopic non-asthmatic subjects. Am Rev Respir Dis.141:9–13.

Pin I., Freitag A.P., O'Byrne P.M., *et al*. 1992. Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. Am Rev Respir Dis.145:1265-1269.

Platts-Mills T.A. 2001. The role of immunoglobulin E in allergy and asthma Am J Respir Crit Care Med. Oct 15; 164 (8 Pt 2):S1-5.

Polosa R., Ciamarra I., Mangano G., Prosperini G., Pistorio M.P., Vancheri C., Crimi N. 2000.Bronchial hyperresponsiveness and airway inflammation markers in nonasthmatics with allergic rhinitis. Eur Respir J15:30–35

Polosa R., Ng WH., Crimi N., Vancheri C., Holgate S.T., Church M.K., Mistretta A. 1995 Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma. Am J Respir Crit Care Med.151: 624–629.

Poppius H.,Muittari A., Kreus K.E. *et al*. 1970.Exercise induced asthma and disodium cromoglycate Br Med J.4:337–339

Porsbjerg C., von Linstow M.L., Ulrik C.S., Nepper-Christensen S.C., Backer V. 2005.Outcome in adulthood of asymptomatic airway hyperresponsiveness to histamine and exercise-induced bronchospasm in childhood. Ann Allergy Asthma Immunol. 95(2):137-142.

Prieto L., Gutierrez V., Torres V., Uixera S., Marin J. 2002b.Concentrations of exhaled nitric oxide in asthmatics and subjects with allergic rhinitis sensitized to the same pollen allergen. Clin Exp Allergy, 32:1728–1733

Prieto L., Uixera S., Gutierrez V., Bruno L. 2002a.Modifications of airway responsiveness to adenosine 5’-monophosphate and exhaled nitric oxide concentrations after the pollen season in subjects with pollen-induced rhinitis Chest, 122:940–947

Rabe K.F., Atienza T., Magyar P., Larsson P., Jorup C., Lalloo U.G. 2006 Effect of budesonide in combination with formoterol for reliever therapy in asthma exacerbations: a randomised controlled, double-blind study. Lancet. 368:744-753

Rakotosihanaka F., Melaman F., d'Athis P., Florentin D., Dessanges J.F., Lockhart A.1986. Refractoriness after hyperventilation-induced asthma. Bull Eur Physiopathol Respir.22:581-587

Reiss T. F., Hill J. B., Harman E., Zhang J., Tanaka W. K., Bronsky E., Guerreiro D., Hendeles L.1997. Increased urinary excretion of LTE, after exercise and attenuation of exercise-induced bronchospasms by montelukast. a cysteinyl leukotriene receptor antagonist. Thorax 52(12):1030-5.

Robinson D., *et al*. 1993. Activation of CD4+ T cell, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol. 92:313-324.

Rose M.C., Gendler S.J. 1997. Airway mucin genes and gene products. Rogers, DF Lethem, DI eds. Airway mucus: basic mechanisms and clinical perspectives Birkhauser Verlag. Boston, MA:

Rowe, B., *et al*. 2010. Admissions to Canadian hospitals for acute asthma: A prospective, multicentre study. Canadian Respiratory Journal, 17(1), 25-30

Rundell K.W., Anderson S.D., Spiering B.A., Judelson D.A.2004. Field exercise vs laboratory eucapnic voluntary hyperventilation to identify airway hyperresponsiveness in elite cold weather athletes. Chest. 125(3):909-915.

Schatz M., Carmargo C.A. Jr.2003. The relationship of sex to asthma prevalence, health care utilization and medications in a large managed care organization. Ann Allergy Asthma Immunol 91:553-8.

Schoeffel R.E., Anderson S.D., Gillam I., Lindsay D.A.1980. Multiple exercise and histamine challenge in asthmatic patients. Thorax. 35:164-170.

Shephard E.G., Malan L., Macfarlane C.M., Mouton W.,Joubert J.R. 1985. Lung function and plasma levels of thromboxane B2, 6-ketoprostaglandin F and B-thromboglobulin in antigen-induced asthma before and after indomethacin pretreatment. Br J Clin Pharm, 1985, 19, 459-470

Silverman M., Andrea T. 1972.Time course of effect of disodium cromoglycate on exercise-induced asthma Arch Dis Child. 47:419–422

Sousa A.R., Parikh A.,Scadding G., Corrigan C.J., Lee T.H., 2002.Leukotriene- receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis N Engl J Med 347(19):1493-9

Speelberg B., Panis E.A.H., Bijl D. *et al*. 1991. Late asthmatic responses after exercise challenge are responsible J Allergy Clin Immunol . 87:1128-37

Suh D.I., Lee J.K., Kim J.T., Koh Y.Y. 2011. Airway refractoriness to inhaled mannitol after repeated challenge. Pediatr Pulmonol.46:1007-1014.

Tait, H. 2008. In Statistics Canada, Statistics Canada and Canadian Electronic Library (Firm) (Eds.), Aboriginal peoples survey, 2006: Inuit health and social conditions. Ottawa, Ont.: Statistics Canada.

Tantisira K.G., Colvin R., Tonascia J., *et al*.2008. Airway responsiveness in mild to moderate childhood asthma: sex influences on the natural history. Am J Respir Crit Care Med 178:325-3.

U S Department of Health and Human services, National Institutes of Health, National Heart, Lung and Blood Institute .2007. Expert Panel Report: Guidelines for the Diagnosis and Management of Asthma – Update on Selected Topics 2002. http://www.nhlbi.nih.gov/guidelines/archives/epr-2_upd/index.htm

van den Berge M., Kerstjens H.A., Meijer R.J., de Reus D.M., Koeter G.H., Kauffman H.F., Postma D.S. 2001. Corticosteroid-induced improvement in the PC20 of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC20 of methacholine. Am. J. respir Crit Care Med 164(7):1127-32

van den Berge M., Meijer R.J., Kerstjens H.A., de Reus D.M., Koeter G.H., Kauffman H.F., Postma D.S. 2001a. PC20 adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC20 methacholine. Am J Respir Crit Care Med 163:1546-50

van Velzen E, van den Bos JW, Benckhuijsen JA, van Essel T, de Bruijn R, Aalbers R. 1996.Effect of allergen avoidance at high altitude on direct and indirect bronchial hyperresponsiveness and markers of inflammation in children with allergic asthma. Thorax 51:582–584.

von Garnier, C. *et al*. 2007.Allergic airways disease develops after an increase in allergen capture and processing in the airway mucosa. J. Immunol. 179:5748–5759.

World Health Organization .2013. Asthma Fact sheet Number 307. http://www.who.int/mediacentre/factsheets/fs307/en/

Young Y. K, Jin H. J., Seon M. J., Chang K. K., Kyung U. M. 1998. The occurrence of Late Asthmatic Response to Exercise after Allergen Challenge. Annals of Allergy asthma & Immunol. 81(4):366-372

5. APPENDICES

5.1. APPENDIX A: Certificates for study.

5.1.1. Certificate of approval U of S Biomedical Ethics

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	10-227

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Ellis Hall
Respiratory Medicine
103 Hospital Drive
Saskatoon SK S7N 0W8

SUB-INVESTIGATOR(S)
Beth Davis

STUDENT RESEARCHER(S)
Greg Peters

FUNDER(S)
UNFUNDED

TITLE
Protocol IIS-A-524: Allergen Induced Increase in Indirect Non-Allergic Bronchial Reactivity

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
13-Dec-2010	27-Jun-2011	Study Protocol IIS-A-524 version 1.2 (20-June-2011) Revised Researcher's Summary (29-June-2011) Participant Information and Consent Form version 1.3 (27-June-2011)	26-Jun-2012

Delegated Review: ☐ Full Board Meeting: ☒ Date of Full Board Meeting: 13-Dec-2010

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. 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 Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been 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).

Please send all correspondence to:
Research Ethics Office
University of Saskatchewan
Box 5000 RPO University
1607 – 110 Gymnasium Place
Saskatoon, SK Canada S7N 4J8

Health Canada / Santé Canada

Health Products and Food Branch / Direction générale des produits de santé et des aliments

Notice of Authorization

Company Code 29036
File No. 178367
Submission No. 178367

April 17, 2012

Dr. Donald Cockcroft
University of Saskatchewan
University Hospital, 103 Hospital Drive
Saskatoon, SK
S7N 0W8

Dear Dr. Cockcroft:

Re: CLINICAL TRIAL APPLICATION for Aridol
(Protocol,Number IIS-A-524) *Natural Health Products Regulations* Section: 67

The Natural Health Products Directorate, Bureau of Clinical Trials and Health Sciences, is pleased to inform you that the information and material provided to support the above Clinical Trial Application, have been assessed and we have no objection to your proposed study. Please consider this as your notice of authorization to sell or import this natural health product for the purposes of this clinical trial in Canada.

I would remind you of the necessity of complying with the *Natural Health Products Regulations*, Part 4, in the sale of this product for clinical testing. In addition, the Regulations (Part 4) impose responsibilities, including commencement notice, record keeping and reaction reporting, on those conducting clinical trials. Please ensure that all systems are compliant in order to meet these responsibilities.

To notify NHPD in an expedited manner in the case of serious adverse reactions and/or serious unexpected adverse reactions, please fax your report(s) to the following number: 613-946-0174.

You are also reminded that all clinical trials should be conducted in compliance with the Health Canada Guidance for Industry: Good Clinical Practice: Consolidated Guideline ICH Topic E6.

Should you have any questions concerning this letter, please contact the submission coordinator, nhpd-cta.dec-dpsn@hc-sc.gc.ca.

Yours sincerely

Adam Gibson
Senior Executive Director, Bureau of Product Review and Assessment
Natural Health Products Directorate
2936 Baseline Rd. (A.L. 3302C), Ottawa, ON K1A 0K9

Canada