BRONCHOPROTECTION PROVIDED BY LONG-ACTING MUSCARINIC ANTAGONISTS AND ULTRA LONG-ACTING β_2 AGONISTS AGAINST METHACHOLINE-INDUCED BRONCHOCONSTRICTION IN MILD ASTHMATICS

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ABSTRACT

Background: Although asthma treatments are extensively studied for their physiological effects in animal models and their safety and efficacy in humans, their effects on standard testing methods and their physiological benefits in asthmatics are often unknown. The result is a gap in knowledge on newer therapies such as long-acting muscarinic antagonists (LAMAs) and ultra-long acting β_2 agonists (uLABAs); standardization guidelines for methacholine challenge testing (MCT), a common research and clinical technique, remain uninformed as to an appropriate abstinence period from these drug classes prior to testing. In addition, the mechanisms through which these drugs improve airway hyperresponsiveness, an important factor in asthma, remain unknown. These mechanisms can be elucidated through methacholine dose-response curves, which illustrate airway hyperresponsiveness in terms of airway sensitivity (position), reactivity (slope), and maximal airway narrowing response.

Methods: Two single dose, double blind, double dummy, randomized clinical trials were performed; the first crossover study examined the duration and degree of bronchoprotection provided by two LAMAs, tiotropium and glycopyrronium, against methacholine-induced bronchoconstriction in mild asthmatics; the second study had a three-way crossover design for investigating the effects of glycopyrronium (LAMA) and indacaterol (uLABA), alone and in combination, on the methacholine dose-response curve of mild asthmatics. The first study entailed baseline MCT, treatment administration, and repeat MCT at 1, 24, 48, 72, 96, and 168 hours for each treatment, while the second study used a similar protocol but only followed participants for 48 hours.

Results: The first study found that the two LAMAs provided clinically significant and sustained bronchoprotection for up to seven days, with the maximal degree of protection (16-fold increase in methacholine tolerance) provided at one hour post-treatment. The second study revealed that glycopyrronium and combination glycopyrronium/indacaterol each significantly reduced airway sensitivity and reactivity. The combination therapy also significantly reduced the maximal airway narrowing response to methacholine. Indacaterol alone only produced a mild and short-lived reduction in airway sensitivity.

Conclusion: LAMAs should be abstained from for at least seven days prior to undergoing MCT to ensure test accuracy. The LAMA glycopyrronium and the combination

glycopyrronium/indacaterol both show promising results in terms of improvements in airway sensitivity, reactivity, and maximal response characteristics of airway hyperresponsiveness and following further experimentation may become standard therapies for the treatment of poorly controlled asthma.

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AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
ASM	Airway smooth muscle
ATS	American Thoracic Society
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase 2
CysLT	Cysteinyl leukotrienes
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GRK	G-protein-coupled receptor kinase
IB	Ipratropium bromide
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
LABA	Long-acting β_2 agonist
LAMA	Long-acting muscarinic antagonist
LTRA	Leukotriene receptor antagonist
M_1	Muscarinic type-1
M_2	Muscarinic type-2
M_3	Muscarinic type-3
MCT	Methacholine challenge testing
MDRC	Methacholine dose-response curve
PC_{20}	Provocative concentration of methacholine causing a 20% reduction in FEV_1
PKA	Protein kinase A
PKC	Protein kinase C
PDE-4	Phosphodiesterase 4
PGE_2	Prostaglandin E2

REB	Research Ethics Board
SABA	Short-acting β_2 agonist
SAMA	Short-acting muscarinic antagonist
SR	Sarcoplasmic reticulum
Th2	T-helper-2
TNFα	Tumor necrosis factor α
uLABA	Ultra long-acting β_2 agonist

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Asthma

1.1.1 Overview

Asthma is a common non-communicable respiratory disease that affects approximately 300 million people worldwide (Global Initiative for Asthma 2016). Although great advancements have been made in terms of pharmaceuticals for treating asthma, these medications only suppress and do not cure this chronic inflammatory condition (Holgate 2008).

Asthma is a complex disease to study, as it has a wide range of causes, symptoms, severities, and triggers. While environmental and genetic factors have been identified as contributing factors, much of the pathophysiology behind this condition still requires further elucidation (Akdis & Agache 2013). Asthma can be exhibited by a number of symptoms (e.g. difficulty breathing, coughing, wheezing), which develop in response to triggers such as smoke, exercise, animals, and pollens (Global Initiative for Asthma 2016). A further complication is the existence of subpopulations of asthmatics who exhibit different physiological changes in response to their symptom triggers; atopic (i.e. extrinsic) patients experience allergen-induced asthma and their airway inflammation is produced by eosinophils, T-helper-2 (Th2)-type cells and immunoglobulin E (IgE) antibodies (Amin et al. 2000); the mechanisms underlying non-atopic (i.e. intrinsic) asthma remain largely unknown, however inflammation in such patients is produced predominantly by neutrophils (Amin et al. 2000). For the remainder of this thesis, information will be presented on the general asthma population and will not be divided into these subpopulations.

Asthma differs from another common respiratory condition, chronic obstructive pulmonary disease (COPD), in that airflow obstruction in asthmatics is typically reversible either spontaneously or with therapeutic intervention (Global Initiative for Asthma 2016). However, this may not always be the case, particularly in more severe patients (Boulet 2009). A further complication when diagnosing patients and choosing an appropriate therapy is the possibility of overlap between multiple respiratory conditions, such as between asthma and COPD (Gibson & Simpson 2009). Misdiagnosis could lengthen the time required to establish control over symptoms and improve patient quality of life and outcome.

1.1.2 Pathophysiology

Asthma is a heterogeneous condition that is characterized by several related phenotypes including airway hyperresponsiveness (AHR), airway inflammation, airway remodeling, and airflow obstruction. AHR refers to the quick and excessive bronchoconstriction experienced in response to stimuli that would not normally cause airway smooth muscle (ASM) contraction. Some of the physiological causes of AHR are receptor and/or ion channel dysfunction [e.g. elevated muscarinic type-3 (M_3) and muscarinic type-1 (M_1) receptor expression, muscarinic type-2 (M_2) receptor dysfunction], abnormal neurotransmitter activity (e.g. increased neuronal acetylcholine levels), and altered action potential propagation (Sterk & Bel 1989; Novelli et al. 2013). Inflammation, remodeling and obstruction of the airways can also influence AHR (Sterk & Bel 1989).

Studies have revealed a wide range of abnormalities in the activity and prevalence of inflammatory cells and mediators in diseased airways. Mast cells, macrophages, eosinophils, T-lymphocytes and neutrophils are among the identified inflammatory cells that influence the release of mediators such as leukotrienes and cytokines, all of which produce airway inflammation, asthma symptoms (e.g. coughing), airway remodeling, and AHR (Akdis & Agache 2013). For example, inflammatory mediators contribute to airway remodeling through epithelium shedding, which results in the loss of the barrier important for preventing the penetration of bronchoconstrictors through the airway wall (Naylor 1962; Barnes et al. 1985). Degradation of the epithelium also likely impedes the regulation of inflammatory mediator levels, as protective enzymes such as neutral endopeptidases are not being produced (Barnes 1996). Inflammatory mediators may also abnormally modulate neurotransmitters such as acetylcholine, leading to increased bronchoconstriction and mucus production (Barnes 1996).

Much remains to be determined with regards to the extent of airway remodeling in asthma but it is nonetheless an important factor to consider. It has been shown that airway wall remodeling progressively increases with age and/or duration of asthma compared to healthy controls through increases in ASM cell volume and number, and decreases in airway lumen (Ebina et al. 1993; Bai et al. 2000); these factors together can decrease airway distensibility (i.e. impede bronchodilation) and lead to irreversible airflow obstruction. Enhanced airway constriction in asthmatics also likely results from increased levels of myosin light-chain kinase

(Ammit et al. 2000) and the degradation of the extracellular matrix in ASM (which prevents the proper regulation of muscle shortening) (Bramley et al. 1995).

Despite its higher prevalence and greater severity in COPD patients, mucus hypersecretion also poses a problem in asthma. When produced in normal amounts, mucus is important for protecting the airway epithelium from harmful inhaled particles (Gosens et al. 2006). In diseased airways, acetylcholine release is increased and results in overstimulation of M_3 receptors and, to a lesser extent, M_1 receptors (Gosens et al. 2006). This triggers excessive mucus production by submucosal glands and goblet cells, which is further augmented by synergistic interactions between muscarinic receptors and epidermal growth factor (Gosens et al. 2006). Inflammatory mediators can contribute to mucus production by enhancing the differentiation of cells into goblet cells, thereby increasing the number of mucus-producing cells in the airways (Holgate 2008). In addition, these mediators can cause microvascular leakage, which leads to an increase in airway secretions, impaired mucociliary clearance, and mucosal edema (Holgate 2008). Not only is mucus produced in higher quantities, but its composition is also altered; mucin and other components of mucous are secreted at abnormal concentrations, yielding more viscous mucus that is not easily cleared from the airways (Alagha et al. 2014).

1.1.3 Asthma Pharmacotherapy

The treatment of asthma can be a complicated endeavor due to the wide range of respiratory drug classes available and asthma phenotypes expressed. Treatment guidelines for physicians follow a step-up format for exploring different medications until that most effective for a given patient is found (Global Initiative for Asthma 2016). The first step is short-acting β_2 agonist (SABA; e.g. salbutamol) rescue therapy as needed, which takes effect quickly to treat sporadic episodes of airflow obstruction (BouyssouCasarosaet al. 2010). β_2 agonists activate β_2 receptors in the airways, which trigger the production of cyclic adenosine monophosphate (cAMP) from intracellular adenylyl cyclase; cAMP is key for inducing ASM relaxation and bronchodilation (Gibb & Yang 2013).

If poor symptom control persists, the next treatment step involves inhaled corticosteroids (ICS; e.g. budesonide, fluticasone propionate) as controller medication. ICS are well established for their improvement of asthma symptom severity and frequency, thereby improving patient quality of life with minimal adverse effects and a high safety margin (Pedersen & O'Byrne 1997). ICS promote the production of anti-inflammatory proteins (e.g. neutral endopeptidases)

while suppressing the production of pro-inflammatory proteins (e.g. cytokines) (Pedersen & O'Byrne 1997).

If ICS therapy does not work or is not an option in a particular case, a leukotriene receptor antagonist (LTRA; e.g. montelukast) may be prescribed. LTRAs possess some anti-inflammatory properties that are complementary to that of ICS, as glucocorticoids do not attenuate inflammation produced by cysteinyl leukotrienes (CysLTs; e.g. LTC_4, LTD_4) (Lipworth 1999). LTRAs are primarily beneficial for their inhibition of LTC_4-induced ASM contraction (Lipworth 1999). LTRAs are therefore controller medications with both bronchodilator and anti-inflammatory properties.

Methylxanthines (e.g. theophylline) are another option in place of ICS. This drug class provides bronchodilation through the inhibition of phosphodiesterase-4 (PDE-4). Inhibition of this enzyme prevents the breakdown of cAMP and subsequently contributes to ASM relaxation. PDE-4 inhibition also reduces mucin production and inflammation by reducing the activation of monocytes, T cells and neutrophils (Shukla et al. 2009).

When treatment beyond ICS (or LTRA or methylxanthine) monotherapy is required, long-acting β_2 agonists (LABAs; e.g. formoterol, salmeterol) are the most common add-on therapy. LABAs have similar mechanisms of action to SABAs but they produce significantly longer bronchodilation (BouyssouCasarosaet al. 2010; Global Initiative for Asthma 2016); LABAs are more potent and have a longer side chain than the short-acting formulations, the latter of which allows the anchoring of drug particles near β_2 receptors in the airways (Löfdahl & Chung 1991). Beyond their bronchodilator activity, LABAs have been suggested to have some anti-inflammatory mechanisms, given that β_2 receptors are also on inflammatory cells such as mast cells where they inhibit the release of histamine, LTC_4/LTD_4, and PGD_2 (Butchers et al. 1991). A number of safety concerns have been raised regarding the long-term use of LABA monotherapy, as patients have been found to develop drug tolerance and experience more severe asthma exacerbations, which can prove fatal (Grove & Lipworth 1995; Wijesinghe et al. 2008). Attention shifted to the development of once-daily long-acting anti-asthma medications such as ultra long-acting β_2 agonists (uLABAs), with goals of minimizing side effects and increasing the likelihood of patient compliance (i.e. less frequent drug administration) (Matera & Cazzola 2007).

Despite their common use for treating COPD, inhaled long-acting muscarinic antagonists (LAMAs; e.g. tiotropium, glycopyrronium) have only recently become incorporated into asthma treatment regimens as add-on therapy in cases uncontrolled by ICS or ICS/LABA. At present, the only LAMA approved for this use is tiotropium (Peters et al. 2010; Kerstjens et al. 2011; Global Initiative for Asthma 2016). LAMAs inhibit cholinergic-induced ASM contraction and mucus production by submucosal glands and goblet cells through the blockage of M_1 and M_3 receptor activity (Kistemaker et al. 2012; Alagha et al. 2014). LAMAs also exhibit some anti-inflammatory properties such as the inhibition of acetylcholine-induced chemotactic activity by eosinophils, neutrophils, monocytes and macrophages (Kistemaker et al. 2012). Finally, this drug class has some anti-remodeling effects, as it reduces ASM cell proliferation, which prevents the thickening of ASM (Kistemaker et al. 2012).

The formulations at the highest step in the treatment guidelines for poorly controlled asthma are monoclonal antibodies (e.g. omalizumab, mepolizumab). Various molecules have undergone or are undergoing clinical trials, but only three have been approved. One is omalizumab, an anti-IgE molecule that inhibits the function of IgE effector cells and prevents the activation of the IgE allergic response (i.e. inhibits mast cell and basophil responses) (D'Amato et al. 2014). The other approved monoclonal antibodies are mepolizumab and reslizumab, anti-IL5 molecules that inhibit eosinophil recruitment (Fala 2016).

Given the heterogeneity of asthma, it is not surprising that several drug classes are established for the treatment of this condition. However, the wide availability of so many formulations can make it more time-consuming when trying to determine the most effective treatment for a given patient.

1.2 Bronchoprovocation Testing

A number of testing methods have been developed in an effort to improve accuracy in diagnosing or ruling out asthma. These tests are also valuable in research for investigating the physiological effects of asthma and of respiratory medications. Direct and indirect bronchoprovocation tests each provide benefits and disadvantages. Direct testing refers to the use of pharmacological agents (e.g. methacholine, histamine) to "directly" act on receptors in the airways to measure airway responses. These tests are more sensitive and so they are most useful for ruling out clinically current asthma when the result is negative. On the other hand, indirect testing uses stimuli (e.g. exercise, mannitol, hypertonic saline, adenosine monophosphate) to

trigger airway narrowing through an "indirect" route, such as via the stimulation of mediator release from inflammatory cells. Indirect challenges are more specific and are therefore more beneficial for confirming a diagnosis of asthma; however, these tests are also relatively insensitive and could therefore produce false negative results in mild or well-controlled asthmatics. Direct challenges are relatively safe and quick to perform for investigating new therapeutic options while indirect challenges are more appropriate when examining airway inflammation (Joos et al. 2003). Both are nonetheless useful for investigating therapeutic options. Our study employs the methacholine challenge, which is the most common method used for direct bronchoprovocation testing.

1.2.1 Overview of Methacholine Challenge Testing

Methacholine challenge testing (MCT) is used to measure direct AHR and to rule out an asthma diagnosis. Methacholine is a non-selective muscarinic receptor agonist that mimics the effects of acetylcholine on ASM by directly inducing bronchoconstriction when it binds the M_1 and M_3 receptors (Cockcroft 2007). A number of protocols for performing MCT have been developed over the years; two protocols are commonly used today, the two-minute tidal breathing protocol (Cockcroft et al. 1977) and the five-breath dosimeter protocol (modified from Chai et al. 1975). A previous study has shown that the latter procedure affects the results through deep inhalation-induced bronchoprotection (Cockcroft et al. 2005); the former procedure is used in our laboratory.

The two-minute tidal breathing protocol involves five-minute cycles composed of two minutes of inhalation of a concentration of methacholine followed by spirometry at 30 and 90 seconds post-inhalation. The next inhalation is commenced five minutes after the start of the previous inhalation. The cycles are performed with doubling concentrations of methacholine with the goal of determining the methacholine PC_{20} [the provocative concentration of methacholine that causes a 20% or greater fall in the forced expiratory volume in one second (FEV_1)].

MCT alone is not used to diagnose asthma, as it has greater sensitivity than specificity and it represents only one factor of asthma, AHR (Crapo et al. 2000). The sensitivity and negative predictive value of MCT (i.e. ability to exclude current asthma) are near 100% in patients whose methacholine PC_{20} is greater than 8-16mg/mL (Cockcroft 2010). However, the specificity and positive predictive value of MCT are close to 100% only when a patient's

methacholine PC_{20} is ≤1mg/mL (Cockcroft 2010). Based on this knowledge, it has been established that a methacholine PC_{20} greater than 16mg/mL is sufficient to exclude a diagnosis of current asthma with reasonable certainty (Cockcroft 2010). However, MCT alone is insufficient to confirm a case of current asthma unless the methacholine PC_{20} is ≤1mg/mL, in which case the test specificity and positive predictive value are almost 100% (Cockcroft 2010).

In order to preserve the accuracy of MCT results and to ensure patient safety, the American Thoracic Society (ATS) published a set of guidelines to standardize the testing procedure and to account for potential confounding factors (Crapo et al. 2000). Different medications have been found to influence the test results for varying durations and so, abstinence periods from drug classes are recommended. However, not all new medications have been examined for their duration of bronchoprotection against methacholine. As a result, some of the recommended withdrawal periods may be inaccurate or not indicated at all. In order to ensure adequate washout from different medications, studies must examine their duration of effect in human models.

1.2.2 Characteristics of the Methacholine Dose-Response Curve

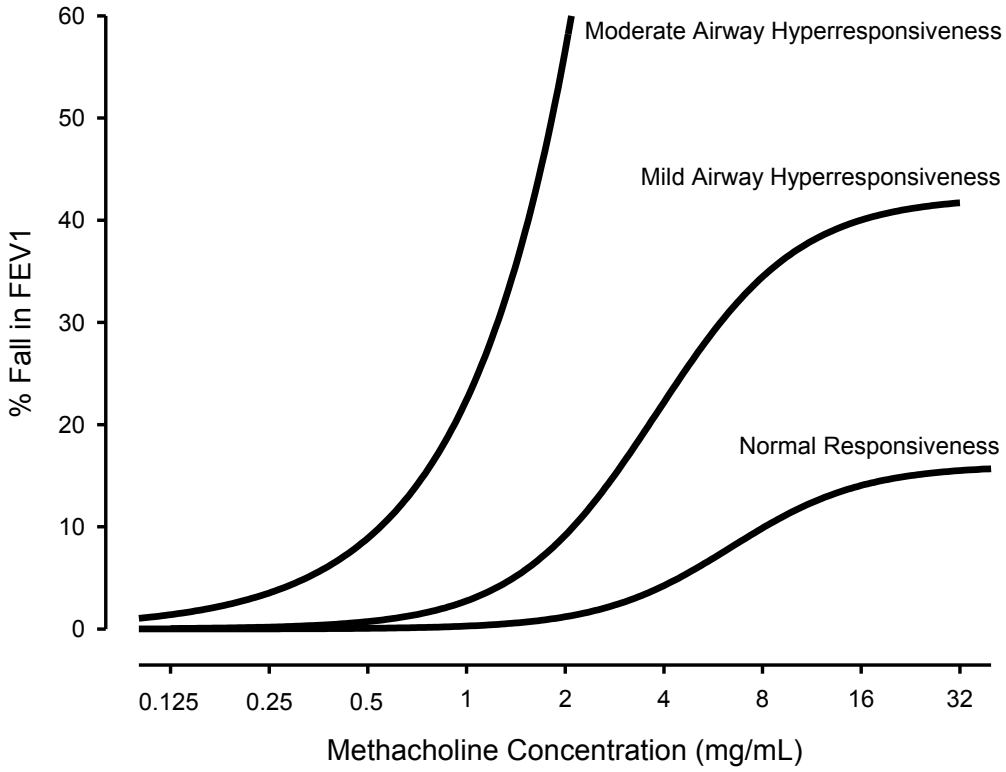

Figure 1.1. *Simulated methacholine dose-response curves illustrating different levels of airway responsiveness.*

Although studies typically report MCT results in terms of the PC_{20}, this parameter only describes airway sensitivity to methacholine. A less routinely used analytical tool is the methacholine dose-response curve (MDRC) of the percent fall in FEV_1 per methacholine concentration (mg/mL). The MDRC illustrates AHR in terms of airway sensitivity (i.e. horizontal position of curve), airway reactivity (i.e. slope of the curve), and maximal airway responsiveness (i.e. uppermost vertical placement of the curve). MDRCs can be particularly useful during drug development, as they quantify drug-induced changes in airway sensitivity, reactivity, and maximal responsiveness. In addition, the investigation of long-term use of a respiratory medication through MDRCs allows researchers to observe potentially harmful effects that may develop from chronic treatment (e.g. a steeper slope indicates worsening asthma exacerbations) (BelZwindermanet al. 1991; Bhagat et al. 1996; Wong et al. 1997).

1.2.2.1 Asthmatic Methacholine Dose-Response Curves

Compared to healthy controls, the MDRC of asthmatics is typically shifted to the left (i.e. increased airway sensitivity), has a steeper slope (i.e. increased airway reactivity), and a raised plateau or the complete loss of a measurable plateau (i.e. increased maximal constricting response) (Sterk & Bel 1989). The mechanisms underlying each of the three characteristic features of the MDRC differ with some overlap, hence how a drug can specifically influence only one portion of the curve.

The factors underlying the leftward, or horizontal, shift in the MDRC have been termed "prejunctional" mechanisms and consist of anything that enhances the effect of airway narrowing stimuli (Sterk & Bel 1989). These include: epithelial damage or malfunction (i.e. greater accessibility of stimuli to their receptor sites), abnormal autonomic neural control (i.e. increased cholinergic reflex activity), increased inflammatory cell number and activity (e.g. enhanced spontaneous mediator release from leukocytes, mast cells, and eosinophils), dysfunctional metabolism and/or absorption of inflammatory mediators and neurotransmitters (i.e. persistent inflammation and/or bronchoconstriction), and interactions between inflammatory cells, mediators, neural control and ASM (i.e. additive effects) (Sterk & Bel 1989).

"Postjunctional" mechanisms influence the maximal response/plateau of the MDRC and refer to anything that causes an increased response by the effector organ (Sterk & Bel 1989). Such mechanisms include: increased ASM contractility [from a disruption in receptor regulation, propagation of excitation, calcium handling, or increased ASM mass (hypertrophy or hyperplasia)], disruptions in viscous and elastic loads (which depends on the balance and interdependence of structural elements within the airway wall and lung parenchyma), swelling of the airway wall, and excessive intraluminal exudate and secretions (Sterk & Bel 1989).

The third characteristic of the MDRC is the slope. The steepening of the MDRC slope reflects a quicker increase in airflow obstruction following exposure to a constricting stimulus (i.e. a more severe exacerbation) (BelZwindermanet al. 1991). The specific physiological changes affecting airway reactivity remain unclear.

With this knowledge of the factors influencing the position and maximal response of the MDRC, it is possible to gain a better understanding of the effect of anti-asthma drugs on the airways of human models. The MDRC could also potentially serve as a tool for determining the best treatment for a patient.

1.2.2.2 Non-Asthmatic Methacholine Dose-Response Curves

The MDRC of non-asthmatics is expected to lie to the right outside the range of a positive methacholine challenge (i.e. PC_{20} >16mg/mL) (Crapo et al. 2000). It is anticipated that the curve slope is more flat and that airway narrowing is attenuated at a low percent fall in FEV_1 by forming a response plateau to methacholine (Woolcock et al. 1984). The overall MDRC is therefore expected to have a sigmoid shape. However, given that as high as 14.3% of the asymptomatic non-asthmatic population may have AHR (i.e. PC_{20} ≤16mg/mL), the MDRC of this population may not be as uniform as previously described (Jansen et al. 1997).

1.3 Bronchodilator Mechanisms of Action

1.3.1 Long-Acting Muscarinic Antagonists

As previously mentioned, LAMAs inhibit M_1 and M_3 receptors on ASM, which prevents cholinergic-induced effects. M_1 receptor blockage also inhibits parasympathetic neurotransmission in the ganglia and reduces mucus secretion by preventing electrolyte and water secretion by epithelial cells (Alagha et al. 2014). M_3 receptor antagonism in turn prevents mucus secretion from submucosal glands and inhibits ASM contraction. Unlike their short-acting counterparts (SAMAs; e.g. ipratropium) that are regularly prescribed for COPD, LAMAs possess receptor selectivity; LAMAs selectively inhibit M_1 and M_3 receptors while rapidly dissociating from M_2 receptors. This selectivity is important, as M_2 receptor expression contributes to bronchodilation through its negative feedback mechanism in preganglionic nerves and parasympathetic nerve terminals where it inhibits the excessive release of acetylcholine (Novelli et al. 2013; Alagha et al. 2014). LAMAs also have higher binding affinity and slower dissociation from M_1 and M_3 receptors than do SAMAs, which explains the significantly longer duration of effect.

Tiotropium and glycopyrronium are two potent antimuscarinic quaternary ammonia LAMAs that are commonly used as once-daily medications for COPD. The quaternary nitrogen in the LAMA structure largely minimizes side effects by preventing the systemic absorption of drug particles and the crossing of the blood-brain barrier (Hansel et al. 2005). Unlike tiotropium, glycopyrronium does not show kinetic selectivity for binding M_3 rather than M_2 receptors (Lipworth 2014). However, it still shows higher affinity for the M_1 and M_3 receptor-types than M_2 receptors. Higher doses of glycopyrronium (50 and 100μg) have shown equivalency to

tiotropium (18μg) in terms of degree and duration of bronchodilation in COPD patients with the exception that glycopyrronium has a more rapid onset (five minutes post-dose as opposed to 0.5-1 hour post-dose) (Verkindre et al. 2010).

Studies investigating the effects of LAMAs in human models have primarily examined this drug class for its bronchodilator properties and its ability to reduce the frequency and severity of asthma exacerbations (Kerwin et al. 2012). However, physiological investigations in humans are relatively rare and experimentation with human tissue is also lacking but crucial. A study examining cells obtained through endobronchial biopsies in patients with lung fibrosis found that tiotropium prevented airway remodeling by inhibiting acetylcholine-induced proliferation of fibroblasts (Pieper et al. 2007). If left unmitigated, fibroblasts contribute to ASM cell hyperplasia and alter the normal function of extracellular matrix proteins (Holgate 2008).

Physiological studies are mainly limited to animal models and not all LAMAs have been studied to a great extent in this fashion. When the effects of tiotropium were investigated in murine models of asthma challenged with ovalbumin, the acute model did not appear to derive any benefit; however, the chronic model showed several improvements in airway remodeling following treatment with tiotropium compared to untreated, ovalbumin-challenged mice including reduced peribronchial fibrosis, decreased ASM thickness, reduced M_3 receptor activity and increased M_2 activity (Kang et al. 2012). Previous studies in guinea pig models of allergic asthma have found that tiotropium partially or completely inhibits increased ASM mass (i.e. hyperplasia), contractility, and contractile protein expression, all of which are involved in increased acetylcholine-induced ASM remodeling (Gosens et al. 2005; Bos et al. 2007). In addition, these studies showed that this LAMA reduces mucus gland hypertrophy and airway eosinophilia (Gosens et al. 2005; Bos et al. 2007). A study in mouse models of asthma found similar results; tiotropium inhibited the production of eosinophils and Th2-type cytokines [e.g. interleukin (IL)-4, IL-5, and IL-13], prevented acute and chronic inflammation, and inhibited the thickening of ASM (Ohta et al. 2010). LAMAs have yet to be studied in human participants through traditional research techniques such as MCT and allergen challenge testing.

Glycopyrronium has also been shown to have several anti-inflammatory properties in studies with animal models. For example, pre-treatment with glycopyrronium has prevented the accumulation of inflammatory mediators [e.g. IL-1β, tumor necrosis factor-α (TNFα)] in the bronchoalveolar lavage fluid of mice that had been exposed to cigarette smoke (Shen et al.

2014). Shen et al also found that glycopyrronium prevents some airway remodeling by inhibiting the cigarette smoke-induced production of matrix metalloproteinase-9, a major enzyme involved in the degradation of the extracellular matrix (Shen et al. 2014).

These findings together suggest that LAMAs have the potential to benefit asthmatics in several ways, from reducing airway inflammation and remodeling to preventing bronchoconstriction.

1.3.2 Ultra-Long Acting β_2 Agonists

Physiological data on once-daily ultra long-acting β_2 agonists (uLABAs; e.g. indacaterol, olodaterol) in humans remain limited, as these add-on medications are relatively new for the treatment of COPD and are currently being investigating for use in asthmatics. These longer-acting β_2 agonists were designed to provide the optimal bronchodilation characteristic of this drug class with a good safety profile in a once-daily formulation to improve patient adherence. Bronchodilation via β_2 receptors seems to be produced through multiple mechanisms: increased cAMP and PKA levels triggering ASM relaxation through hyperpolarization of ASM cells, inhibition of myosin light-chain kinase, inactivation of contractile enzymes through mitogen-activated protein kinase phosphatase-1 mechanisms, and phosphorylation of inositol 1,4,5-triphosphate (IP_3) resulting in a reduction in intracellular calcium release from the endoplasmic reticulum of ASM cells (Giembycz & Newton 2006). Proposed mechanisms behind the long duration of action of uLABAs include: the presence of a long lipophilic side chain that anchors the drug near or within the β_2 receptor, strong binding of the receptor, low plasma clearance, or the lipid solubility of the drug allows its deposition and/or storage in the membrane of the target tissue (Beasley & Pearce 1993; Trifilieff et al. 2014).

ULABAs possess strong selectivity and affinity for only the β_2 receptor subtype, which results in fewer systemic side effects such as tachycardia (BouyssouCasarosaet al. 2010). These formulations also exhibit high intrinsic efficacy, which explains the rapid onset of action and behaviour as nearly full β_2-agonists (Trifilieff et al. 2014). These are highly desirable characteristics for full β_2 agonists, as they result in less cross-tolerance to SABA rescue medications - partial agonists can behave like antagonists if another agonist of higher efficacy for the same receptor is used (Beeh et al. 2007; Trifilieff et al. 2014).

Human studies on uLABA effects have mainly focused on time of onset and degree of bronchodilation in COPD patients. Physiological investigations in animal models are also

limited; experiments with mouse and guinea pig models of cigarette smoke-induced airway inflammation have shown that treatment with olodaterol significantly reduces TNFα levels and inhibits enzymes important for neutrophil function and for remodeling of the extracellular matrix of ASM (Wex et al. 2015). Following confirmation of the presence of β_2 receptors on human lung fibroblasts, a study demonstrated that olodaterol inhibited fibroblast proliferation and collagen synthesis, both of which are common airway remodeling factors in asthma (Lamyel et al. 2011).

Although some animal studies have suggested that uLABAs possess capabilities beyond bronchodilation, more investigations on this new drug type must be completed to clarify its functions in humans.

1.3.3 Combination LAMA/uLABA

Like the previously described monotherapies, combination LAMA/uLABA formulations have shown promising but limited results in asthmatic animal models. Although LAMAs and uLABAs act on different receptors, they both utilize the same pool of G proteins, leading to receptor crosstalk (Costa et al. 2014). Studies in animal models have shown this relationship clearly; mice deficient in an enzyme important for the cAMP pathway normally triggered by β_2 agonists were found to no longer respond to cholinergic stimulation (Hansen et al. 2000); another study found that M_3 receptor stimulation in hamster ovary eggs transfected with M_3 and β_2 receptors resulted in β_2 receptor phosphorylation and subsequent desensitization (Budd et al. 1999).

The finding that M_2 receptors predominate in fibroblasts where they likely inhibit cAMP production (even more so in asthmatic fibroblasts) also points to crosstalk, as treatment with tiotropium restored cAMP, the main secondary messenger triggered by β_2 receptors, to normal levels (Costa et al. 2014). This reflects that temporary inhibition of M_2 receptors by a LAMA augments β_2 agonist activity. Given that M_2 receptors trigger inhibitory G proteins while β_2 receptors influence stimulatory G proteins, these receptors clearly have a counteracting component (Proskocil & Fryer 2005). As a result, the combination of muscarinic antagonist and β_2 agonist could potentiate bronchodilation, as inhibition of M_2 receptors removes this receptor's inhibitory effect on β_2 receptors.

A study that investigated the combined effect of SABA and SAMA (salbutamol/ipratropium) in asthmatics revealed that this combination produced positive albeit

modest improvements in pulmonary function, a reduction in risk of future exacerbations, and fewer hospital admissions compared to either drug alone (Lanes et al. 1998). This suggests strong clinical relevance for the use of muscarinic antagonists and β_2 agonists in combination. In animal models, glycopyrronium and indacaterol together produced synergistic bronchoprotection against cholinergic-induced airway narrowing in guinea pig tracheal preparations (Kume et al. 2012). In guinea pig models of acute allergic asthma, tiotropium was found to synergistically enhance the protective effects of olodaterol against allergen-induced AHR by significantly reducing the early and late asthmatic response, and allergen-induced airway inflammation (Smit et al. 2014). This suggests that endogenous acetylcholine release considerably attenuates β_2 agonist responsiveness and further suggests that there is crosstalk between the two receptor types. Furthermore, in both precision-cut lung splices and beagle dog models that had acetylcholine-induced bronchoconstriction, combination LAMA/uLABA therapy provided significantly more bronchoprotection against carbachol and acetylcholine, respectively, than either drug as monotherapy (BouyssouSchnappet al. 2010; Schlepütz et al. 2013). In medium and small human isolated bronchi, glycopyrronium and indacaterol in combination synergistically inhibited cholinergic contractility, additively inhibited histamine contractility, reduced acetylcholine release from the airway epithelium, and increased cAMP in the bronchi and epithelial cells (Cazzola et al. 2016).

Synergism between muscarinic antagonists and β_2 agonists has been shown in clinical trials investigating combination LAMA/LABAs in COPD patients. Three studies documented synergistic improvements in maximal bronchodilation, pulmonary function during day- and night-time, and frequency of rescue therapy use following tiotropium/formoterol or tiotropium/salmeterol combination therapy in comparison with the respective monotherapies (van Noord et al. 2005, 2010; Di Marco et al. 2006). These findings together suggest that LAMA/uLABA combination therapy should produce several synergistic benefits, particularly as uLABA mechanisms may be disinhibited by muscarinic receptor blockage.

1.4 Influence of Different Respiratory Drug Classes on Methacholine Challenge and Dose-Response Curve Results

1.4.1 Inhaled Corticosteroids

The ATS guidelines for MCT recommend not withholding ICS therapy prior to testing, but studies have shown a significant shift in PC_{20} over time when these drugs are taken regularly.

Over a 12-week period of treatment with budesonide (800µg twice-daily), 14 atopic asthmatics showed a mean increase in PC_{20} of 1.4 doubling doses (Booms et al. 1997). A similar dose shift between one and two doubling doses was found in 51 asthmatics after 12 weeks of therapy with both 400µg twice-daily and 800µg twice-daily budesonide (Riccioni et al. 2003). Another investigation compared the dose shift in PC_{20} following four weeks of treatment with fluticasone at 250µg or 1000µg per day in 64 asthmatics and found that the mean dose shift was between one and two doubling concentrations (no dose-dependency) (Sumino et al. 2014). A longer term study followed asthmatic participants (n=16) for 12 months during which time they received budesonide therapy (200µg twice daily); after 3 months, participants showed a mean two-fold doubling dose shift in methacholine PC_{20}, which increased to a four-fold shift by 12 months (Juniper et al. 1990).

In order to better understand the effects of ICS on the airways, these drugs have been repeatedly studied for their influence on the MDRC. As monotherapy, they have been documented in several studies to produce a rightward shift and often a lower plateau (BelTimmerset al. 1991; Overbeek et al. 1996; Booms et al. 1997; Oga et al. 2001). Fluticasone propionate was shown to be very effective for decreasing the MDRC plateau in asthmatics after 6 weeks of treatment, an effect that was shown to persist after 12 weeks (Overbeek et al. 1996). The researchers also found a significant increase in methacholine PC_{20}, resulting in a rightward shift in the curve and a slight improvement in reactivity. Based on previous findings with regards to the function of ICS, the researchers suggested that this improvement in plateau resulted from a reduction in airway wall thickness in response to anti-inflammatory functions preventing mucosal swelling and/or plasma exudation. Similar findings were reported with beclomethasone dipropionate and it was disclosed that the impact of ICS therapy on airway sensitivity was more pronounced than on maximal responsiveness (Oga et al. 2001).

Previous studies specifically examined the effect of budesonide on reversing unlimited airway narrowing through the induction of a plateau on the MDRC. Out of 14 participants who did not previously show a plateau on their MDRC, nine (64%) produced a plateau after 12 weeks of treatment with ICS (Booms et al. 1997); a slight but insignificant decrease in MDRC slope was also reported. Another study took the opposite approach and enrolled 16 mild asthmatics with a measurable plateau prior to treatment; it was found that budesonide therapy still significantly reduced the maximal response to methacholine (BelTimmerset al. 1991). Both

publications suggested that budesonide reduces mucosal thickness and intraluminal exudate, which allows for the restoration of interdependence between the airway wall and parenchyma.

MDRCs have been proven useful for gauging which patients can tolerate a reduction in their ICS dosage without losing control of their asthma symptoms. One study found that 96% (23/24) of participants who initially showed a plateau on their MDRC did not deteriorate in asthma control following a 75% reduction in their ICS dosage (Prieto et al. 1999). In comparison, only 55% (12/22) of participants who did not have a baseline measurable plateau did not deteriorate. Overall, this suggests that the presence of a measurable plateau on the MDRC can be used as an objective indicator for safely reducing ICS dose. The study also indicated that the introduction of a plateau on the MDRC with continued ICS treatment is not necessary for achieving satisfactory control of asthma symptoms. Asthma symptomology must therefore stem from more than simply the loss of protective mechanisms against excessive airway narrowing.

1.4.2 Leukotriene Receptor Antagonists.

Although the ATS recommends abstaining from LTRAs for at least 24 hours before undergoing MCT (Crapo et al. 2000), this may in fact be longer than necessary. In a placebo-controlled study, 10mg montelukast was found not to significantly change responsiveness to methacholine at 1 or 25 hours post-treatment (Davis & Cockcroft 2005). When taken daily for 12 weeks, this same treatment and dose was found to produce only a dose shift of one doubling concentration of methacholine (Riccioni et al. 2003). When montelukast was examined for its effect on the MDRC of asthmatics, the results were also of low significance; montelukast was first investigated as monotherapy for its potential benefits in reducing the maximal airway response to methacholine, which revealed no significant improvement in AHR (Ulrik & Diamant 2009). The researchers next examined the effect of combination ICS plus montelukast therapy on the MDRC and found that the LTRA only produced a modest improvement in the maximal response plateau and sensitivity compared to ICS alone (Ulrik & Diamant 2010). It should be noted that LTRAs are similar to ICS in that these medications must be taken regularly for a number of weeks before reaching optimal anti-inflammatory effectiveness (Lipworth 1999). Neither drug type is designed for sporadic use in treating acute symptoms.

1.4.3 Methylxanthines

Given its anti-inflammatory and bronchodilator mechanisms, researchers examined the methylxanthine theophylline for its effect on methacholine-induced AHR. Following two months of treatment with theophylline (dose range of 250-375mg twice-daily), 28 asthmatics showed significant reductions in both airway sensitivity (dose shift of approximately one doubling concentration of methacholine compared to parallel placebo arm) and reactivity to methacholine (Page et al. 1998). The researchers confirmed that these albeit small effects were not simply the result of the drug's bronchodilator effect, as no significant change in baseline FEV_1 was observed. While this study demonstrated the modest effects of theophylline in comparison with ICS therapy, it also revealed that this drug, unlike β_2 agonists, did not worsen airway reactivity with long-term use.

1.4.4 Muscarinic Antagonists

The ATS guidelines suggest that LAMAs like glycopyrronium only provide significant bronchoprotection for up to 48 hours, perhaps one week in the case of tiotropium (Crapo et al. 2000). However, there are no references to corroborate these suggestions. The guidelines have been found to be inaccurate in the past with regards to muscarinic antagonists; although it is recommended that ipratropium be abstained from for a minimum of 24 hours, its protective effects have been found to subside within 12 hours at the standard dose of 40μg (Illamperuma et al. 2009).

Previous studies have shown that tiotropium and glycopyrronium each provide significant bronchoprotection against methacholine-induced bronchoconstriction, but no study has examined their complete duration of effect. Tiotropium bromide has been found to provide dose-dependent bronchoprotection for at least 48 hours in 12 patients (O'Connor et al. 1996). The doses of 10, 40 and 80μg tiotropium produced methacholine dose shifts of 5, 7.1 and 7.9 doubling concentrations at 2 hours, respectively, and of 2.2, 2.2, and 3.9 doubling concentrations at 48 hours, respectively. Four participants from this study also completed MCT at 72 hours post-tiotropium and all showed significant persistent bronchoprotection. Glycopyrronium has only been followed for 30 hours after treatment administration; in ten mild-to-moderate asthmatics, three doses of glycopyrronium (0.5, 1.0 and 2.0 mg) each produced significant, sustained, dose-independent bronchoprotection against methacholine for at least 30 hours (Hansel et al. 2005). At 2 hours,

each dose produced a shift in methacholine PC_{20} of approximately five doubling concentrations and by 30 hours, each dose produced a shift of at least two doubling concentrations.

No muscarinic antagonist, long- or short-acting, has been characterized for its impact on the MDRC thus far.

1.4.5 β_2 Agonists

Selective β_2 agonists, both short- and long-acting, have been extensively studied for the degree of bronchoprotection provided by different dosages against methacholine-induced bronchoconstriction. With the SABA salbutamol, 100µg has been found to induce methacholine dose shifts of 2.1 doubling doses at 30 minutes and 1.3 at 2 hours post-treatment (Higham et al. 1997); 200µg salbutamol in one study produced a dose shift of approximately two doubling doses at 1 hour, an effect that had dissipated within 12 hours (Derom et al. 1992), while a second study recorded a 3.5 doubling dose shift at 30 minutes post-treatment, an effect that had dissipated within 4 hours (Ramsdale et al. 1991); the latter finding was confirmed in another study when after 200µg salbutamol once daily for 10 days, participants showed a dose shift of 3.5 when MCT was performed 10 minutes following administration of one last 200µg dose of salbutamol (Jokic et al. 2001); 400µg salbutamol provided dose shifts of 3.7 doubling concentrations at 30 minutes and 2.3 at 2 hours post-dose (Higham et al. 1997). Salbutamol clearly produces dose-dependent bronchoprotection for less than 12 hours.

The LABA salmeterol has been investigated for its single dose and long-term effects in several studies; 25µg salmeterol produced doubling dose shifts of 1.9 at 30 minutes and 2.5 at 2 hours post-treatment (Derom et al. 1992). A dose of 50µg salmeterol has produced some variability in results; one study found a relatively consistent dose shift of approximately 2 doubling doses from 1 to 12 hours (Derom et al. 1992), two studies found a 3.3 doubling dose shift at 1 hour (Cheung et al. 1992; Bhagat et al. 1995), and a fourth reported a dose shift of 1 doubling concentration at 12 hours post-treatment (Booth et al. 1993). With a dose of 100µg salmeterol, the dose shift was approximately three doubling concentrations for the first 2 hours and significant bronchoprotection was still apparent at 12 hours with a doubling dose shift of two (Derom et al. 1992; Higham et al. 1997). Long-term studies found that twice-daily 25µg salmeterol produced a dose shift of one doubling concentration after 4 and 8 weeks of regular dosing (Cheung et al. 1992; Booth et al. 1993) while twice-daily 42µg salmeterol produced the same dose shift after 4, 12, and 24 weeks of treatment (Rosenthal et al. 1999). When usage was

stopped after eight weeks, Cheung et al found that no significant bronchoprotection remained two days after the final dose was taken (Cheung et al. 1992). Similarly, Rosenthal and colleagues found no rebound bronchoconstriction at three days after having ceased daily dosing (first follow-up visit post-treatment) (Rosenthal et al. 1999). Altogether, these findings suggest that salmeterol-induced bronchoprotection is dose-dependent and that the drug does not last more than 48 hours.

The LABA formoterol has also been characterized for its bronchoprotective effects in asthmatics; one hour after dosing, 24μg formoterol produced a dose shift of more than 3 doubling concentrations while 12μg and 6μg formoterol each produced a doubling dose shift of approximately 2.5. After two weeks of regular treatment, 24μg twice-daily, 12μg once-daily and 6μg twice-daily of formoterol each produced a dose shift of 0.5-1 doubling concentration of methacholine (Lipworth et al. 1998). Another study reported a doubling dose shift greater than four in asthmatics at 30 minutes post-dosing with 24μg formoterol, which persisted for at least 12 hours at which time the dose shift was approximately three doubling concentrations (Ramsdale et al. 1991). A dose of 12μg formoterol produced slightly less significant results, with dose shifts of three to four doubling concentrations at 30 minutes and of two to three doubling concentrations at 12 hours post-treatment (Ramsdale et al. 1991). Formoterol at these doses therefore generates similar bronchoprotection to salmeterol, but no study has examined its full duration of effect.

The full duration of bronchoprotection against methacholine provided by uLABAs (e.g. indacaterol, olodaterol) is not yet known. Olodaterol has only been followed for 32 hours after a single dose, at which time it was shown to provide significant persistent bronchoprotection (O'Byrne et al. 2009). At dosages of 2, 5, 10, and 20μg, olodaterol produced methacholine dose shifts ranging from 2.1 to 4.2 doubling concentrations at 0.5 hour post-treatment in a dose-dependent manner. At 32 hours, the range in dose shift was 1.2 to 2.7 doubling concentrations. Based on the current guidelines, it is assumed that uLABAs will provide bronchoprotection for up to 48 hours, presumably based on LABA data.

When examined for their effect on the MDRC of asthmatics, both SABAs and LABAs seem only to reduce airway sensitivity to bronchoconstricting stimuli (i.e. a rightward shift in the MDRC). No MDRC has been generated for uLABAs such as olodaterol as of yet. When the effect of the SABA salbutamol (200μg) was compared with that of the LABA salmeterol (50μg),

each taken twice daily for four days, the former produced a larger rightward shift (Wong et al. 1997). This difference is not surprising given the previously mentioned findings regarding the dose shift produced by each treatment as well as the fact that salbutamol has a significantly faster onset and MCT was performed 15 minutes after final dosing for both treatment arms. Neither drug influenced the maximal response to methacholine, but each produced an increase in slope. Two similar studies confirmed that regular use of salbutamol steepens the MDRC slope (BelZwindermanet al. 1991; Bhagat et al. 1996), one of which corroborated that salbutamol shifts the MDRC to the right but has no effect on the maximal response (BelZwindermanet al. 1991). Bel, Zwinderman and colleagues found that the rightward shift was not correlated with the degree of bronchodilation achieved and that the most sensitive subjects at baseline experienced the greatest rightward shift in their MDRC.

The steepening of the MDRC slope is concerning, as it illustrates that more rapid airway obstruction occurs following exposure to a bronchoconstricting stimulus. To explain this observation, it was suggested that regular use of β_2 agonists increases AHR due to excessive release of mediators from persistently stimulated β_2 receptors on mast cells (Bhagat et al. 1996). It was also suggested that ASM β_2 receptors become dysfunctional and no longer efficiently promote ASM relaxation, thereby contributing to the loss of protection afforded by β_2 agonists against methacholine (Bhagat et al. 1996).

1.5 Introduction Summary

Asthma is a complex heterogeneous disease for which there is no cure. Despite the availability of a number of treatment options, the variability in phenotypes among asthma patients can make it a time-consuming process to find the right therapy. Research still lags in characterizing the various effects of respiratory medications on diseased airways, particularly in human models, which further impedes our ability to treat asthma based on phenotype. Direct provocation testing such as MCT can be utilized as a tool in respiratory clinics for assessing AHR and in research for determining the physiological effects of anti-asthma drugs on diseased airways. However, to maintain test standardization and ensure the accuracy of MCT results, new treatments must be characterized for the degree and duration of bronchoprotection that they afford in order to establish an appropriate abstinence period prior to testing. The examination of each asthma drug class through MDRCs is also important, as these visual representations of AHR provide preliminary information on the physiological changes elicited by each drug in

humans. Recent pharmaceutical developments that lack MCT data include LAMAs, uLABAs, and combinations of LAMA/uLABA, each of which shows promise in addressing certain pathophysiologies not adequately mitigated by previous treatment regimens.

1.6 Rationale

The recent addition of the LAMA tiotropium to the standard treatment hierarchy for asthma preceded certain investigations in human models that are important for ensuring accuracy in diagnostic and research techniques in asthmatics. For continued standardization of the commonly performed MCT procedure, all respiratory medications should be assessed for their duration of bronchoprotection against methacholine in order to deduce suitable washout periods. This information is not yet known for LAMAs and so the first project of this thesis examined the duration and degree of bronchoprotection provided by two LAMAs, tiotropium and glycopyrronium.

Newer respiratory medications, such as LAMAs and uLABAs, have yet to be characterized for their effects on the MDRC of asthmatics. This information is beneficial for quantifying the effects, if any, of the treatments on airway reactivity and maximal responsiveness while supplementing data on airway sensitivity, which may have been previously investigated through methacholine PC_{20} measurements. Results from a study of this nature could inform future physiological studies and may eventually help to shape clinical decision making. Despite the fact that LAMAs and uLABAs are not intended for monotherapeutic use, it is worthwhile to characterize both their individual and combined effects to determine what changes each produces on the asthmatic MDRC and whether synergism is in fact observed as has been found in animal models. The second study of this thesis investigated the effects of the LAMA glycopyrronium and the uLABA indacaterol, alone and in combination, on the MDRC of mild asthmatics.

2.0 DURATION OF BRONCHOPROTECTION OF THE LONG-ACTING MUSCARINIC ANTAGONISTS TIOTROPIUM AND GLYCOPYRRONIUM IN MILD ASTHMATICS

2.1 Objective

The purpose of this study was to determine the duration and degree of bronchoprotection provided by two LAMAs, tiotropium and glycopyrronium, against methacholine-induced bronchoconstriction in mild asthmatics. This information could then be incorporated into MCT

standardization guidelines, which currently provide a recommended abstinence period from LAMAs that has not been scientifically validated.

2.2 Hypothesis

The two LAMAs will provide equivalent and significant bronchoprotection against methacholine-induced bronchoconstriction for more than 48 hours.

2.3 Relationship to Thesis

This chapter describes the first study investigating the complete duration of bronchoprotection provided by the LAMAs tiotropium and glycopyrronium against inhaled methacholine. This work was published in Respiratory Medicine (Appendix A) and has been cited in the 2017 MCT guidelines (Coates et al. 2017).

2.4 Abstract

Despite the recent recommendation of the LAMA tiotropium for use in poorly controlled asthma, the duration of bronchoprotection provided by this drug class and whether the various formulations of LAMAs differ remain unknown. The 1999 standardization guidelines for MCT suggest that LAMAs should be avoided for 48 hours (perhaps 1 week in the case of tiotropium) prior to testing to ensure proper test interpretation. However, these suggestions are based on findings with LABAs. Our objectives were to determine and compare the duration and degree of protection afforded by a single dose of two different LAMAs, tiotropium and glycopyrronium, against methacholine-induced bronchoconstriction.

Thirteen mild-to-moderate asthmatics [8 male; baseline FEV_1 >65% of predicted; baseline PC_{20} ≤8mg/mL] completed this randomized, double blind, double dummy, crossover study. Methacholine challenges were performed before treatment administration (5µg tiotropium or 50µg glycopyrronium) and at 1, 24, 48, 72, 96 and 168 hours (7 days) post-treatment. Treatment administrations were separated by at least 11 days.

Both drugs provided significant bronchoprotection, each producing an increase in mean PC_{20} that was greater than 16-fold, or four doubling concentration, by one hour post-dose. Tiotropium still provided statistically significant protection on day seven (p=0.0282) while glycopyrronium provided bronchoprotection until day seven (p=0.0590). Tiotropium provided statistically superior bronchoprotection at 24 and 72 hours compared to glycopyrronium.

To minimize the occurrence of false negatives, MCT guidelines should be updated to recommend a minimum one-week abstinence period from all LAMAs. MCT also differentiated

between tiotropium and glycopyrronium with respect to the degree and duration of bronchoprotection provided by each.

2.5 Introduction

Asthma is a respiratory condition that causes either persistent or sporadic airway hyperresponsiveness (AHR) in response to a variety of triggers (Lougheed et al. 2012). Although the exact cause of asthma remains unknown, it is believed that genetic and environmental factors contribute to its development (Kim & Mazza 2011). In order to treat patients with varying symptoms and severity of asthma, a range of pharmaceutical agents have been developed. Due to increased demand for more effective treatment regimens, research has now begun to examine the use of LAMAs (Peters et al. 2010; Kerstjens et al. 2011). LAMAs function by selectively inhibiting muscarinic M_1 and M_3 receptors and slowly dissociating from them, thereby generating a long duration of action (Lipworth 2014). Unlike the SAMA ipratropium bromide (IB), LAMAs are more selective and do not greatly inhibit M_2 receptors, which are key for preventing the excessive presynaptic release of the bronchoconstrictor acetylcholine. A number of LAMAs such as tiotropium and glycopyrronium have been developed and are approved for use in COPD. Currently, the only LAMA included in the Global Initiative for Asthma report 'Global Strategy for Asthma Management and Prevention (2016 update)' is tiotropium (Global Initiative for Asthma 2016).

Given the incorporation of LAMAs into asthma treatment regimens, it is important to know the impact of such medications on diagnostic tests. One of the most common diagnostic tests used in asthma research and clinical practice is the methacholine challenge. This test is typically used for refuting a physician diagnosis of asthma (Crapo et al. 2000). A number of factors, including concomitant medications, have been found to influence the accuracy of this diagnostic test, thereby contributing to false negative results.

To establish a standard testing protocol for accuracy and safety purposes, the ATS published a set of guidelines for MCT which includes suggested abstinence periods from several classes of pharmaceuticals (Crapo et al. 2000). Due to the various durations of pharmacological effects, the ATS guidelines suggest withholding long-acting bronchodilators for 48 hours and possibly one week for tiotropium (Crapo et al. 2000). However, the references provided to support these washout periods pertained to the duration of action of single doses of LABAs (Derom et al. 1992; Cockcroft & Swystun 1997). It may be inappropriate to generalize these

results to all long-acting bronchodilators, as LABAs and LAMAs have completely different mechanisms of action. In addition, few studies have examined the duration of effect of single doses of LAMAs in asthmatics and no study has captured the effect in its entirety.

2.6 Methods

2.6.1 Participants

Participants were first recruited through our contact list of previous participants in our laboratory. To achieve our target sample size, the remainder of participants was recruited from the campus community through a Research Ethics Board (REB)-approved ad posted on the online university news portal (PAWS). This study was approved by the University of Saskatchewan Biomedical REB (Bio-REB 15-254; Appendix B) and was registered with clinicaltrials.gov (NCT02622243). Prior to enrolling in the study, each participant was given the opportunity to ask questions before providing written informed consent.

Eligible participants were at least 19 years of age, had a methacholine $PC_{20} \leq 8$mg/mL, had a baseline % predicted $FEV_1 \geq 65\%$, and were either nonsmokers or ex-smokers with less than ten pack-years history. Individuals were ineligible if they had taken any anticholinergic agent within 30 days or if they had suffered an upper respiratory tract infection within 4 weeks of study entry. ICS monotherapy was permitted as long as it was taken regularly at a stable dose for at least 30 days prior to study enrollment. Salbutamol rescue therapy was allowed but was abstained from for six hours before all lab visits. Due to known contraindications for MCT and for the study treatments, the following were excluded: pregnant or nursing women, and those suffering from cardiovascular or prostate or kidney or urinary retention problems, or glaucoma.

2.6.2 Methacholine Challenge Test

The two-minute tidal breathing protocol for MCT was performed as outlined in the ATS guidelines for MCT (Crapo et al. 2000):

1. The following methacholine concentrations in mg/mL were prepared in advance by diluting either 1600mg or 1280mg Provocholine® (Methapharm, Inc., Brantford, ON, CA) powder with 0.9% saline: 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128.
2. Baseline spirometry was performed to obtain three reproducible FEV_1 measurements.
3. 2mL of saline diluent (0.9%) was inserted into a Bennett-Twin jet nebulizer (Puritan Bennett Corporation, Carlsbad, CA) that had been calibrated to give an output volume of

0.13mL/min prior to beginning the study. A participant used the same nebulizer for all of their testing.

4. The participant calmly breathed in nebulized aerosol for two minutes via a facemask while their nose was clipped.
5. At 30 seconds and 90 seconds post-inhalation, the participant's FEV_1 was measured. A third FEV_1 measurement was obtained at 150 seconds post-inhalation if the FEV_1 value at 90 seconds was >5% less than the value measured at 30 seconds.
6. At 5 minutes after the start of the previous inhalation cycle, the next cycle was started with 2mL of the lowest concentration of methacholine and steps 4-5 were repeated. A participant received the same starting concentration of methacholine for each methacholine challenge.
7. Steps 4-6 were repeated with doubling concentrations of methacholine until the PC_{20} was attained (i.e. after ≥20% fall in FEV_1), until the highest methacholine concentration (256mg/mL*) had been administered, or if the participant wished to stop. *To administer the 256mg/mL concentration of methacholine, 2mL of 128mg/mL methacholine was inhaled for four minutes.
8. Following a baseline methacholine challenge, participants were given their randomized study treatment. Following post-treatment MCT, participants were offered 200µg salbutamol to quickly reverse the methacholine-induced bronchoconstriction.

2.6.3 Study Design

This was a randomized, double blind, double dummy, crossover study. For each treatment arm, participants underwent baseline MCT followed by treatment administration and MCT at 1, 24, 48, 72, and 96 hours post-treatment. Each methacholine challenge was performed at the same time of day (± two hours). It was subsequently identified that the treatments could last longer than 96 hours. It was also found that the first participant developed a response plateau (i.e. three data points within 5%) around a 20% fall in FEV_1. As a result, a study amendment was drafted and approved (Appendix C) to allow for MCT at 7 days (or 168 hours) post-treatment. The amendment also allowed for the administration of one additional concentration of methacholine if a participant showed signs of developing a plateau (i.e. two data points within 5% when a 20% fall in FEV_1 was reached). Initially, testing on day 7 was only performed in participants who showed persistent bronchoprotection at 96 hours. It was soon recognized

however, that statistical analysis would be weakened if a participant had data until day 7 on one drug, but only data until 96 hours post-treatment on the other study drug. Following this, all remaining participants underwent MCT on day seven for both treatments. The treatment administrations were separated by a minimum 11-day washout.

2.6.4 Study Drugs and Blinding

Inhalers were pre-loaded with active and placebo capsules (Breezhaler®) or canisters (Respimat®); participants and the administering investigator were blinded to the treatment and randomization scheme. For each treatment, participants took two inhalations from a Respimat® and two inhalations (from the same capsule) from a Breezhaler®; one device contained an active treatment and the other a placebo. Participants were advised to hold their breath for five seconds after each inhalation. The glycopyrronium treatment arm consisted of a dose of 50µg and the tiotropium treatment arm consisted of a dose of 5µg.

2.6.5 Statistical Analysis

A sample size of 13 participants provided a 99% study power for detecting a half concentration difference in methacholine PC_{20}. The methacholine PC_{20} data were calculated with **Formula 2.1** (Cockcroft et al. 1983):

$$\textit{Methacholine } PC_{20} = \textit{antilog}\left[\log C_1 + \frac{[(\log C_2 - \log C_1)(20 - R_1)]}{R_2 - R_1}\right]$$

C_1 = Next-to-last concentration of methacholine administered;

C_2 = Last concentration of methacholine administered (the concentration producing a 20% or greater fall in FEV_1);

R_1 = Percent fall in FEV_1 post-C_1;

R_2 = Percent fall in FEV_1 post-C_2;

If the maximum methacholine concentration was administered and the fall in FEV_1 was only between 10-20%, the PC_{20} was extrapolated using **Formula 2.2** (Jokic et al. 1998):

Methacholine PC_{20} = [20 /last percent fall in FEV_1] x final concentration administered;

If the FEV_1 fell by less than 10% at the maximum methacholine concentration (256 mg/mL), the methacholine PC_{20} was arbitrarily set at 1024mg/mL. The PC_{20} values were then log-transformed and used to assess bronchoprotection by calculating the dose shift in methacholine PC_{20} with **Formula 2.3** (O'Connor et al. 1992):

PC_{20} dose shift = (Δlog methacholine PC_{20})/0.3;

Using Statistix 9 (Analytical Software, Tallahassee, FL, USA), paired t-tests were performed to compare treatment differences in PC_{20} dose shift and in baseline FEV_1 (using the highest of the three baseline FEV_1 measurements). Repeated measures analysis of variance (ANOVA) was performed with Statistix 9 to compare dose shift and pre-challenge FEV_1 data between the two drugs. A dose shift from baseline of at least one doubling concentration was considered clinically significant (Dehaut et al. 1983). Graphs were generated with SigmaPlot 10 (Systat Software, Inc., Richmond, CA, USA). Significance was two-sided and set at 0.05. Results are reported as mean data ± standard error of mean (SEM), unless otherwise stated.

2.7 Results

2.7.1 Participants

Table 2.1. *Participant demographics*

Participant	Gender	Age (yrs.)	Height (cm)	Pre-GLY FEV_1 (% Predicted)	Pre-TIO FEV_1 (% Predicted)	PC_{20} Pre-GLY (mg/mL)	PC_{20} Pre-TIO (mg/mL)
1	M	69	168	72	77	1.0	1.4
2	M	39	178	88	85	1.2	0.64
3	M	22	183	83	82	6.4	7.2
4	F	22	173	96	96	0.98	1.2
5	M	31	196	99	96	1.1	3.4
6	F	27	168	102	96	1.5	2.4
7	M	24	178	100	99	1.1	1.1
8	M	29	185	105	105	10.0	7.0
9	M	44	175	95	95	7.1	7.6
10	F	28	160	89	91	1.4	3.4
11	M	20	185	76	94	0.78	0.73
12	F	21	170	79	80	0.66	1.5
13	F	24	147	68	76	0.36	0.16
Mean:	62% M	31 [13]	174 [12]	89 [12]	90 [10]	1.5*	1.8*

**, geometric mean; [ ], standard deviation; GLY, glycopyrronium; TIO, tiotropium; FEV_1, forced expiratory volume in one second; PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV_1*

A total of 18 participants enrolled in the study and 13 followed it to completion (see Table 2.1). Five participants were excluded; four did not have a positive methacholine challenge and one did not have an adequate baseline FEV_1. No unexpected serious adverse effects occurred. Reported side effects were mild and included topical dryness, shortness of breath, chest tightness, muscle stiffness, and headaches. All were known side effects of either drug or of MCT and subsided without the need for intervention. No participants were taking ICS as a monotherapy and all were nonsmokers.

2.7.2 Bronchoprotection

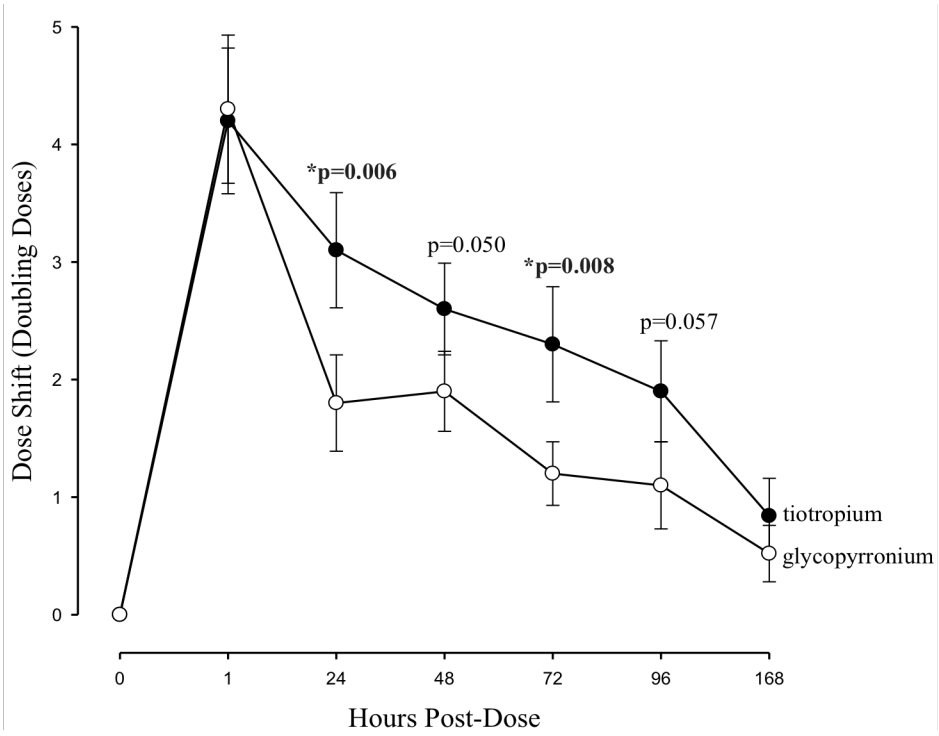

Figure 2.1. *Mean dose shift and standard error at various time points following treatment with tiotropium (TIO) and glycopyrronium (GLY). The sample size was 13 except for TIO at 96 hours (n=12), TIO at 168 hours (n=10) and GLY at 168 hours (n=9). P values represent the difference between the treatment arms at the given time points.*

Table 2.2. *Methacholine PC_{20} data*

	Methacholine PC_{20} (mg/mL) at:						
	Baseline	1 hour	24 hours	48 hours	72 hours	96 hours	168 hours
Tiotropium	1.8	33	15	11	9.1	7.0	3.8
Glycopyrronium	1.5	30	5.4	5.6	3.5	3.2	2.4

Baseline and post-treatment PC_{20} data presented as geometric means

PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV_1

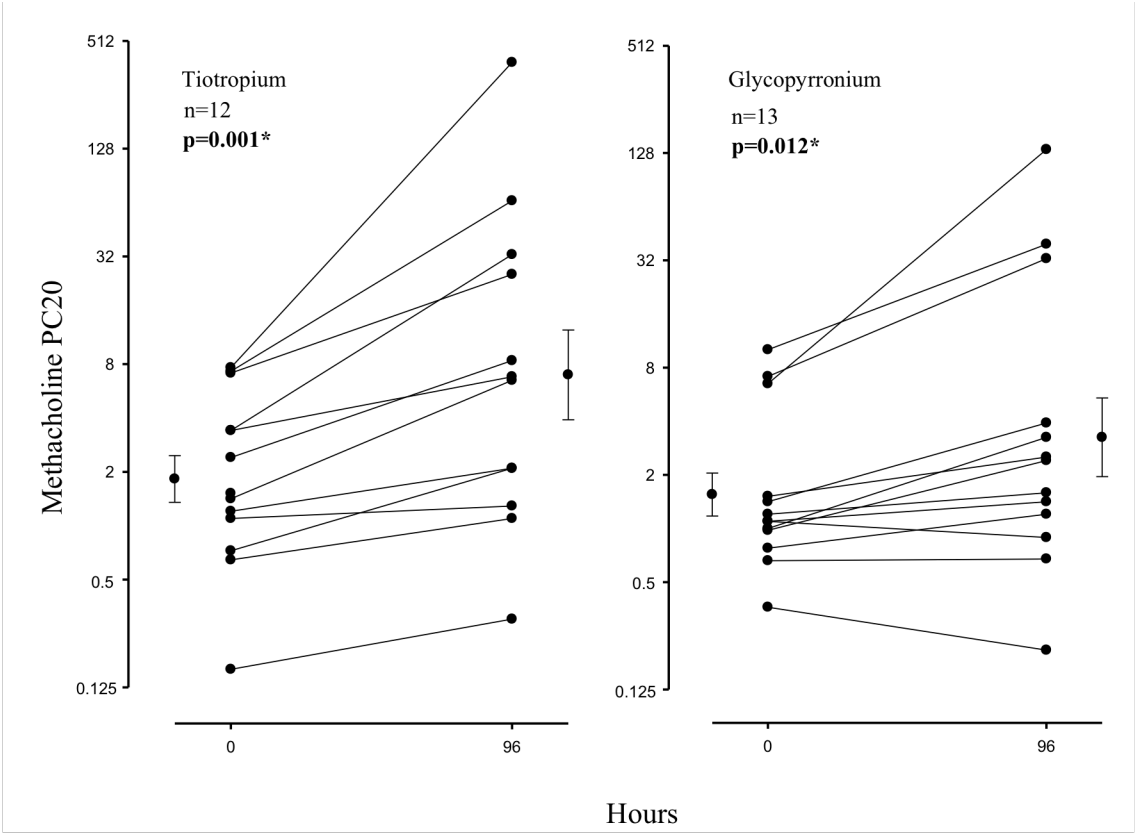

Figure 2.2. *Individual methacholine PC_{20} values pre-treatment and at 96 hours post-dose for each drug. Single data points represent geometric mean and SEM values. P values represent the difference between the PC_{20} values at 96 hours versus those values at baseline within the same treatment arm.*

Dose shift data and methacholine PC_{20} values for both treatments are shown in Figure 2.1

and Table 2.2, respectively. Both treatments significantly increased the amount of methacholine required to achieve a 20% fall in FEV_1 by more than 16-fold at one hour. Tiotropium and glycopyrronium each continued to provide clinically significant bronchoprotection at 96 hours (p-0.001 and p=0.012, respectively), as shown in Figure 2.2. At 168 hours, tiotropium provided statistically significant bronchoprotection (p=0.028; dose shift=0.84; n=10) while glycopyrronium did not (p=0.059; dose shift=0.52; n=9).

Tiotropium appears to be superior to glycopyrronium until the 168-hour time-point. Tiotropium provided significantly more bronchoprotection than glycopyrronium at 24 hours (p=0.006) and 72 hours (p=0.008), and approached statistical significance at 48 hours (p=0.050) and 96 hours (p=0.057). Paired t-tests of the dose shift produced by each drug at 1 and 168 hours post-treatment showed no statistical difference between the two treatments (p=0.865 and p=0.212 respectively).

2.7.3 Bronchodilation

Table 2.3. *Mean peak FEV_1 data*

	Mean peak FEV_1 values at:						
	Baseline	1 hour	24 hours	48 hours	72 hours	96 hours	168 hours
Tiotropium	3.70	3.70 (0.976)	3.82 (0.058)	3.71 (0.931)	3.66 (0.506)	3.75 (0.817) n=12	3.36 (0.158) n=10
Glycopyrronium	3.65	3.75 (0.053)	3.74 (0.005*)	3.70 (0.174)	3.65 (0.880)	3.66 (0.671)	3.40 (0.697) n=9

*( ), P values; *, statistical significance; FEV_1, forced expiratory volume in one second*
Sample sizes are specified if different from n=13

Mean peak FEV_1 data only differed significantly from baseline at 24 hours post-glycopyrronium, as shown in Table 2.3. In terms of mean shift in peak FEV_1 [95% confidence interval], glycopyrronium provided an average increase in lung volume of 90 mL, from 3.65L [3.00, 4.29] at baseline to 3.74L [3.12, 4.37] at 24 hours. Borderline statistically significant shifts

in mean peak FEV_1 from baseline were observed with glycopyrronium at 1 hour (p=0.053) and with tiotropium at 24 hours (p=0.058). No significant difference in mean FEV_1 was observed between the two treatments at any time-point.

2.7.4 Post hoc analysis of methacholine dose-response curves

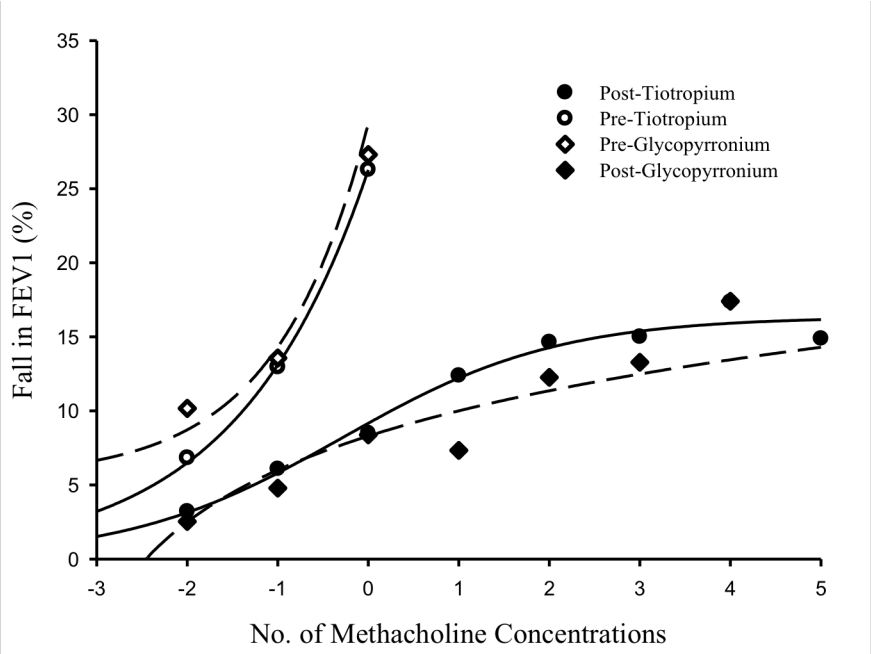

Figure 2.3. *Comparison of the mean MDRCs pre-treatment versus one hour post-treatment with tiotropium and glycopyrronium, respectively. Zero was set as the methacholine concentration that caused the minimum 20% fall in FEV_1 at baseline. Any lower concentrations administered were plotted as negative x-values and any higher concentrations were plotted as positive x-values. Best-fit curves were then generated. Data points with a sample size less than six were excluded.*

Figure 2.3 shows that the mean baseline MDRC prior to each treatment arm was exponential-shaped and did not appear to plateau. At one hour post-treatment, both drugs shifted the curve to the right, reduced the slope, and produced a response plateau between a 10-15% fall in FEV_1.

2.8 Discussion

The gap in knowledge regarding the duration of bronchoprotection provided by LAMAs against methacholine in asthmatics necessitated that a study of this nature be performed to

inform MCT standardization guidelines; this is particularly important now that a LAMA (tiotropium) is recommended for use in this patient population (Global Initiative for Asthma 2016). Previously, O'Connor et al found that different dosages of tiotropium provided bronchodilation for up to 24 hours and dose-dependent bronchoprotection for at least 48 hours in 12 mild-to-moderate asthmatics (O'Connor et al. 1996). With a reduced sample size of four, they also found persistent bronchoprotection at 72 hours post-treatment. Hansel et al performed a similar study comparing bronchoprotection provided by IB versus three different dosages of glycopyrronium in ten mild-to-moderate asthmatics (Hansel et al. 2005). They found that while IB provides more bronchoprotection at 2 hours post-treatment, bronchoprotection by glycopyrronium is greater at 12, 24 and 30 hours. Unfortunately, neither of these studies followed their participants until the treatments had completely worn off and so, the complete duration of bronchoprotection provided by each drug remained unknown.

Our findings show that bronchoprotection provided by glycopyrronium lasts up to seven days while tiotropium still provides statistically significant bronchoprotection at seven days. However, the dose shift in methacholine PC_{20} at seven days after each treatment was not clinically significant, as it was less than one doubling concentration in both cases. Initially, participants only completed day 7 of testing if they still showed significant bronchoprotection at 96 hours. This resulted in reduced sample sizes on day seven for both treatments and it is possible that neither drug may have been found to be significant one week after drug administration had all participants completed day seven testing. Nonetheless, these findings suggest that current guidelines for MCT need to be updated to account for the significantly longer duration of bronchoprotection provided by LAMAs than the currently suggested 48 hours and "perhaps 1 wk. for tiotropium" (Crapo et al. 2000).

The secondary objective of this study was to compare the two LAMAs in order to determine if there is any difference in bronchoprotection between the two treatments. Since the two study drugs are typically used in COPD patients, more data has been collected on their treatment effects in that patient population. Glycopyrronium has been found to provide a dose-dependent bronchodilator effect, with higher doses (i.e. 50-115.2μg) producing comparable effects to the recommended tiotropium dosage of 18μg in COPD patients (Verkindre et al. 2010; Kerwin et al. 2012; Rennard et al. 2014). The main difference observed between the two treatments was that glycopyrronium provides more rapid and significant bronchodilation from

five minutes to four hours post-treatment (Verkindre et al. 2010; Kerwin et al. 2012). This presented the hypothesis that these two drugs would exert similar durations of bronchoprotection and bronchodilation in asthmatics undergoing MCT.

Our results reflect that while their overall duration is similar in terms of clinical significance, tiotropium provides superior bronchoprotection at almost all time points, and its superiority reached statistical significance at 24 and 72 hours post-treatment. Reasons for this difference may be that the dosage of tiotropium administered was that recommended for asthmatics while glycopyrronium was administered per guidelines for COPD patients. Although glycopyrronium is somewhat more selective for M_3 receptors than for M_1 and M_2 types, only tiotropium has kinetic selectivity for M_3 over M_2 receptors, which could lead to a significant difference in perceived bronchoprotection between the two treatments (Moulton & Fryer 2011). If glycopyrronium binds M_2 receptors for a longer period of time, this could produce prolonged inhibition of this receptor-type's normal function as a moderator of acetylcholine in the airways. The result could be a reduction in overall bronchoprotection, as elevated acetylcholine levels could promote some bronchoconstriction.

It has also been suggested that differences in receptor binding kinetics between tiotropium and glycopyrronium only explain some of the variation seen in their respective durations of action (Fogarty et al. 2011). Drug rebinding may prolong the duration of action of pharmaceuticals, as freshly dissociated drug molecules could rebind to receptors further down the target tissue rather than being immediately metabolized (Sykes et al. 2012). Given that some drug molecules would be lost to metabolism with each dissociation and rebinding event, glycopyrronium could lose effect more quickly, as it dissociates more rapidly than tiotropium. Traditional pharmacokinetic data with these LAMAs are not applicable given the present study context of single dose, inhaled treatment in the airways of a live human model. As such, we can only speculate regarding the influence of receptor binding kinetics in distinguishing tiotropium from glycopyrronium. Another potential factor contributing to the different effects of each drug could be their respective delivery methods. It has been argued that soft mist inhalers deliver pharmaceuticals more efficiently than do dry powder inhalers, leading to more drug deposition in the airways even in the event of poor inhalation technique (Brand et al. 2008).

Neither drug produced a strong bronchodilator effect, which is contradictory to results obtained in previous studies in mild asthmatics (O'Connor et al. 1996; Hansel et al. 2005). Only

glycopyrronium provided statistically significant bronchodilation at one time-point, 24 hours post-treatment. However, this effect was not clinically significant and only represented an increase in volume of 97mL while the ATS guideline for significant post-bronchodilator FEV_1 is a 200mL increase (and 12%) (American Thoracic Society 1995). This lack of significant bronchodilation in the current study may be explained by the much greater dosages of the treatments administered in previous studies. It should also be noted that the participants were mildly asthmatic and so, they likely had relatively low airway constriction at rest. Therefore, the airways may not have had much capacity to dilate further. Nonetheless, while this study's findings do not suggest that the two LAMAs are effective bronchodilators in mild asthmatics, they both provide significant bronchoprotection, particularly at one hour post-treatment where both drugs produced a dose shift of four doubling concentrations of methacholine (or a 16-fold increase in mean PC_{20}). This is comparable to the duration of bronchoprotection provided by other drug types; at 30 minutes post-dose, the LABA formoterol (24µg) provides 20-fold bronchoprotection and the uLABA olodaterol (20µg) provides 19-fold protection (Ramsdale et al. 1991; O'Byrne et al. 2009).

In addition to the anticipated shift of the MDRC to the right compared to the pre-treatment curve, both LAMAs unexpectedly produced a reduced maximal response to methacholine. The reduction in maximal response reflects a decrease in what appeared at baseline to be unlimited AHR (Sterk & Bel 1989). The rightward shift reflects a reduction in airway sensitivity to methacholine, which is unsurprising given the binding of LAMAs to M_1 and M_3 receptors, thereby preventing the binding of bronchoconstrictors (Sterk & Bel 1989; Beasley et al. 2009). Potential mechanisms behind the decrease in maximal response to methacholine include reduced ASM contractility and inhibition of mucus secretion responsible for airway wall swelling (Sterk & Bel 1989). Tiotropium has also been found to trigger anti-inflammatory mechanisms in COPD patients (Holownia et al. 2010). This function may contribute to both the rightward shift and plateau in the MDRC, as the airways are less sensitive to stimuli (i.e. lower release of inflammatory mediators) and swelling is reduced. In comparison, ICS also produce a lower maximal response and a rightward shift in the MDRC while β_2 agonists simply produce a rightward shift (Wong et al. 1997; Oga et al. 2001). Further investigation into the impact of LAMAs on the MDRC of asthmatics could expand our current knowledge on the physiological effects of these medications and could have implications for clinical decision-making.

Overall, the results from this study provide objective data indicating that the current guidelines underestimated the complete duration of clinically significant bronchoprotection by LAMAs, although the undocumented caveat for one week for tiotropium did prove accurate. The remaining question is whether all LAMAs act roughly equivalently to tiotropium or glycopyrronium and so, in order to better account for the length and degree of bronchoprotection of this drug class, it would be recommended that all LAMAs be abstained from for a minimum of one week prior to undergoing MCT to avoid false negative or difficult-to-interpret test results.

3.0 THE EFFECT OF GLYCOPYRRONIUM AND INDACATEROL, AS MONOTHERAPY AND IN COMBINATION, ON THE METHACHOLINE DOSE-RESPONSE CURVE

3.1 Objectives

The purpose of this study was to characterize the physiological effects of a LAMA (glycopyrronium) and a uLABA (indacaterol), as monotherapy and in combination, based on the changes each elicited on the MDRCs of mild asthmatics. A secondary objective was to compare these asthmatic MDRCs with a non-asthmatic control MDRC to better visualize how the study drugs may (or may not) improve airway function towards a "normal" level of responsiveness.

3.2 Hypotheses

The LAMA (glycopyrronium) will significantly reduce airway sensitivity (i.e. rightward shift), reactivity (i.e. smaller slope), and maximal responsiveness (i.e. low response plateau) to methacholine, as found *post hoc* in the previous study. The uLABA (indacaterol) will only produce a rightward shift, as has been found with both SABAs and LABAs. The combination therapy will produce synergistic improvements in airway sensitivity, reactivity, and maximal responsiveness, based on current knowledge of synergistic interactions in animal models and in clinical trials of LAMA/LABA combinations in COPD patients.

3.3 Relationship to Thesis

This chapter discusses the second study undertaken for this Master of Science thesis, which sought to confirm the previous findings with glycopyrronium while also investigating two other treatments: indacaterol, and combination glycopyrronium/indacaterol. This study focused on the use of MDRCs to distinguish the bronchoprotective mechanisms of the two drug classes administered as monotherapy and in combination.

3.4 Abstract

MDRCs can be useful for illustrating pharmacologic bronchoprotection against methacholine-induced AHR by quantitating changes in airway sensitivity (position), reactivity (slope), and maximal responsiveness. Our objective was to determine the influence of single-dose glycopyrronium (LAMA) and indacaterol (uLABA), as monotherapy and in combination, on the MDRC of mild asthmatics and to compare these findings with a non-asthmatic control curve.

This was a randomized, double blind, double dummy, three-way crossover study. For asthmatic participants (n=14), each treatment arm included a baseline methacholine challenge, drug administration, and repeat methacholine challenges at 1, 24, and 48 hours. Non-asthmatic control participants (n=22) underwent a single methacholine challenge and did not receive any study treatment.

Treatment effects on the MDRCs were first explored as mean data. Compared to baseline, indacaterol produced a slight reduction in sensitivity to methacholine at one hour; glycopyrronium significantly reduced airway sensitivity and reactivity as well as slightly decreased maximal responsiveness at all time-points. The control and one-hour glycopyrronium curves are nearly identical. Only combination therapy appeared to generate a true response plateau. Combination therapy also decreased airway sensitivity for at least 48 hours and reduced airway reactivity for 24 hours.

When graphed individually, the asthmatic participants' one-hour curves varied not only with respect to which drug provided the more favourable response but also with respect to how a specific drug altered the characteristics of the MDRC.

Among the non-asthmatic participants, 32% (7/22) were found to be hyperresponsive to methacholine or saline and were not included as controls.

MDRCs differentiate the bronchoprotective mechanisms triggered by different classes of asthma medications. In general, the only effect following indacaterol treatment was decreased sensitivity. On the other hand, glycopyrronium and combination therapy provided significant persistent bronchoprotection against methacholine, but only the latter protected against excessive airway narrowing (i.e. maximal responsiveness). Assessment of bronchoprotection using MDRCs may be useful during treatment development when performing superiority, equivalence, and/or non-inferiority trials.

3.5 Introduction

As a heterogeneous condition with multiple phenotypes, asthma is a complicated disease to research. Although pharmaceutical agents are thoroughly studied for their safety and efficacy in animal models and clinical trials, investigations into their physiological effects in human *in vivo* models are often lacking. For example, new therapeutic interventions are often unknown for their influence on common diagnostic tests such as MCT for AHR.

MCT is a common research technique that can be used to assess pharmacologic bronchoprotection against cholinergic-induced AHR. Such effects can be evaluated based on the resulting improvement in methacholine sensitivity through an increase, or dose shift, in methacholine PC_{20}. Drug effects can also be studied through the generation of MDRCs, which illustrate not only airway sensitivity (i.e. position), but also airway reactivity (i.e. slope) and maximal responsiveness. Compared to that of a healthy airway, the typical asthmatic MDRC is shifted to the left (i.e. increased sensitivity), has a steeper slope (i.e. increased reactivity) and shows a higher maximal constricting response (i.e. excessive airway narrowing) (Sterk & Bel 1989; Wong et al. 1997).

Underlying physiology causing increased airway sensitivity includes epithelial damage and dysfunction, increased cholinergic neural activity, and airway inflammation (Sterk & Bel 1989). Greater maximal airway responsiveness can result from increased ASM contractility, swelling of the airways, airway remodeling, and/or excessive mucus secretion (Sterk & Bel 1989). With this knowledge, it is possible to elucidate the mechanisms of action of drugs based on the changes they produce on the MDRC.

Three new or emerging therapies for poorly controlled asthma include LAMAs, uLABAs, and combination LAMA/uLABA. The previous study found that a single dose of each of the LAMAs tiotropium and glycopyrronium produced not only a rightward shift but also, *post hoc*, a low response plateau on the MDRC (Blais et al. 2016). Contrastingly, use of SABAs and LABAs have only been shown to improve airway sensitivity through a rightward shift on the MDRC (BelZwindermanet al. 1991; Wong et al. 1997). Whether uLABAs such as indacaterol produce the same result is unknown. While combination LAMA/uLABA therapy has generated promising results in animal models of asthma (BouyssouSchnappet al. 2010; Schlepütz et al. 2013; Costa et al. 2014; Smit et al. 2014), its influence on MCT and, more specifically, on the MDRC in humans is still unclear.

3.6 Methods

3.6.1 Participants

Participants were either recruited through our contact list of previous participants in the Asthma Research Lab or were recruited from the University of Saskatchewan community via an REB-approved ad posted on the online university news portal (PAWS). This study was approved by the University of Saskatchewan Biomedical REB (Bio REB 16-205; Appendix D) and was registered with clinicaltrials.gov (NCT02953041). Prior to beginning the study, each participant was given the opportunity to ask questions before providing written informed consent.

3.6.1.1 Asthmatic Participants

Eligible asthmatic participants were 18 years of age or older, had a methacholine $PC_{20} \leq$ 8mg/mL, and had a baseline % predicted FEV_1 of 65% or greater. In addition, eligible participants could not have suffered from an upper respiratory tract infection or from allergy-induced asthma symptoms within four weeks prior to the study. Exsmokers or current smokers could participate as long as they had less than ten pack-year history. Current smokers abstained from smoking completely on each test day prior to their study visit. An individual was ineligible to participate if they had taken any cholinergic agent or LABA within ten days prior to beginning the study. Participants taking regular ICS (monotherapy) were allowed to participate provided they had been taking their medication in the same dose regularly for at least 30 days. Salbutamol was allowed as rescue therapy but was withheld for six hours prior to all study visits. Due to known contraindications for MCT and the study treatments, the following were excluded: pregnant or nursing mothers, those with cardiovascular, kidney, prostate or urinary retention problems and those with hypokalemia, diabetes, or glaucoma.

3.6.1.2 Non-Asthmatic Participants

Eligible non-asthmatic participants were 18 years of age or older, were nonsmokers (or former smokers with less than ten pack-years history) and had a negative methacholine PC_{20} (>16mg/mL). An individual could not participate if they were pregnant or nursing, if they had cardiovascular problems or if they had suffered an upper respiratory tract infection within four weeks of the study. Individuals were also excluded if they had a personal or first-degree family history of respiratory illness.

3.6.2 Methacholine Challenge Testing

The two-minute tidal breathing protocol for MCT was performed as outlined in the previous chapter (section 2.6.2) with the following changes to the protocol steps:

Step 1: The maximum concentration prepared was 64mg/mL.

Step 7: Doubling concentrations of methacholine were administered until the highest concentration (128mg/mL*) had been administered, when a plateau response was achieved (three data points within 5%, provided that a minimum 10% fall in FEV_1 had been attained), when a participant's FEV_1 dropped by 40% from the lowest FEV_1 post-saline, or if the participant wished to stop. *To administer the 128mg/mL concentration, 2mL of 64mg/mL concentration was inhaled for four minutes.

Step 8: For non-asthmatic participants, following their methacholine challenge, they were given the option of relieving any methacholine-induced bronchoconstriction with 200μg salbutamol.

3.6.3 Study Design

This was a randomized, double blind, double dummy, three-way crossover study. A non-asthmatic group was recruited to serve as a control for "normal" methacholine responsiveness and did not receive any study medications. On test day, all participants avoided exercise prior to each scheduled study visit.

3.6.3.1 Protocol for Asthmatic Participants

Asthmatic participants underwent three treatment arms, each consisting of four methacholine challenges (Table 3.1). Each treatment arm entailed three study visits at the same time of day (± two hours) on three consecutive days. The first day of each treatment arm took approximately three hours; first, a baseline methacholine challenge was performed to establish the participant's methacholine PC_{20} at rest. While under observation, participants then self-administered one of the blinded study treatments. Participants were instructed to hold their breath for five seconds after each inhalation of the study medication to ensure drug deposition. A note was made if the participant coughed following drug inhalation and the specific inhaler causing the cough was marked to take into account the possibility of the drug particles being coughed out. Participants then underwent MCT at 1, 24, and 48 hours post-treatment. Testing at 24 and 48 hours took approximately 1 hour. Following a minimum ten-day washout between treatment

administrations, the same procedure was repeated with the second study treatment. Following a second ten-day washout, the procedure was again repeated with the third study treatment.

Table 3.1. *Treatment visit schedule for asthmatic participants*

Visit 1 (≈3 hours)	▪ Breakdown of study procedure and requirements ▪ Signing of the consent form ▪ Baseline methacholine challenge ▪ Treatment 1 inhalation (provided participant meets eligibility criteria) ▪ Methacholine challenge at 1 hour post-treatment
Visit 2 (≈1-1.5 hours)	▪ Methacholine challenge at 24 hours post-treatment
Visit 3 (≈1-1.5 hours)	▪ Methacholine challenge at 48 hours post-treatment
Washout (minimum 10 days between treatment administrations)	

3.6.3.2 Protocol for Non-Asthmatic Participants

Each participant underwent a single methacholine challenge, which took approximately one hour.

3.6.4 Study Drugs and Blinding

Active treatments and matching placebos were pre-loaded into Breezhaler® inhalers; both participants and the administering investigator were blinded to the treatment and randomization. Each treatment involved administering the contents of two inhalers; for the LAMA monotherapy, one inhaler contained a glycopyrronium capsule (Seebri®) and the other contained a placebo capsule. For the uLABA monotherapy, one device contained an indacaterol (Onbrez®) capsule and the other contained a placebo capsule. For the combination therapy, one inhaler contained a glycopyrronium capsule and the other contained an indacaterol capsule. Each glycopyrronium capsule contained a 50μg dose and each indacaterol capsule contained a 75μg dose.

3.6.5 Statistical Analysis

With a sample size of 15 asthmatic participants, the power of this study was 99% for detecting a half concentration difference in methacholine responsiveness. MDRCs were graphed with SigmaPlot 10 as the percent fall in FEV_1 per methacholine concentration administered. Best-fit curves were then generated. Mean control MDRC slopes as well as mean asthmatic MDRC slopes after each treatment and at each time-point were calculated through linear regression analysis using Excel® 2011 (Microsoft Corp., Redmond, WA, USA); the data points included were those from the two concentrations of methacholine that at baseline had been administered prior to achieving a minimum 20% fall in FEV_1, plus all subsequent data points. If a response plateau formed, the data points up to the first of three plateau points were included for slope calculation. Slope values were then compared with paired t-tests using Excel® 2011. Slopes are presented with the units percent fall in FEV_1/methacholine concentration.

Methacholine PC_{20} data were calculated by algebraic formula (see Formula 2.1). If a participant's FEV_1 fell between 10-20% after the last concentration of methacholine (128 mg/mL), their PC_{20} was extrapolated (see Formula 2.2). If a participant's FEV_1 fell less than 10% from baseline after the final methacholine concentration, their PC_{20} was arbitrarily set at 256mg/mL. Methacholine PC_{20} data were log-transformed and used to assess bronchoprotection through the dose shift in methacholine PC_{20} from baseline (see Formula 2.3). Bronchoprotection was deemed to be clinically significant if the dose shift was at least one doubling concentration of methacholine greater than the baseline PC_{20} (Dehaut et al. 1983). Baseline FEV_1 measurements for each treatment and time-point were averaged within each participant. Using Statistix 9, a two-way ANOVA (subject/treatment) and all pairwise comparisons [least significant difference (LSD)] were performed to determine the differences in dose shift and baseline spirometry data. Significance was two-sided and set at 0.05. Results are presented with 95% confidence intervals, unless otherwise stated.

The non-asthmatic data were analyzed separately from the asthmatic results. Non-asthmatic participants with a negative methacholine challenge (i.e. PC_{20} >16mg/mL) were classified as non-hyperresponsive. Non-asthmatic participants with a positive test (i.e. PC_{20} ≤16mg/mL) or with a high degree of responsiveness to the saline control inhalation (i.e. >20% fall in FEV_1) were classified as hyperresponsive. When possible, methacholine PC_{20} data were calculated as described earlier. Individual slopes were calculated as described previously with

some modifications; if no plateau developed, all data points were included, and the methacholine concentrations were log-transformed. Slopes are presented with the units % fall in FEV_1/(log mg/mL).

3.7 Results

3.7.1 Participants

3.7.1.1 Asthmatic Participants

Table 3.2. *Asthmatic participant demographics*

Participant	Gender	Age (years)	Height (cm)	Mean Baseline FEV_1 (L)	Mean Baseline FEV_1 (% Predicted)	Mean Baseline Methacholine PC_{20} (mg/mL)
01	F	24	157	2.24	74	0.24
02	M	40	178	3.49	83	0.92
03	M	20	169	3.61	84	3.6
04	F	28	168	3.32	97	3.8
05	M	27	173	3.14	73	0.22
06	F	23	173	3.55	95	2.0
07	F	21	163	3.70	111	2.4
08	F	21	173	3.65	98	3.7
09	F	29	160	2.62	84	2.0
10	M	32	185	3.41	72	1.8
11	M	70	168	2.14	78	1.7
12	M	22	173	4.17	95	3.1
13	F	18	130	1.62	73	1.3
14	M	25	172	4.23	99	2.1
15	F	30	160	2.98	96	3.2
16	M	23	180	4.29	90	2.4
Mean:	50% Male	28 [12]	168 [12]	3.26 [0.77]	88 [12]	2.2*

[ ], standard deviation;, geometric mean; FEV_1, forced expiratory volume in one second; PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV_1*

Mean Baseline MCh PC_{20} – determined by averaging the pre-treatment baseline PC_{20}'s

A total of 16 asthmatics were recruited (Table 3.2) and 14 completed the study; one participant was withdrawn prior to the second randomized treatment due to significant improvement in their baseline PC_{20} in the non-hyperresponsive range (i.e. PC_{20} >16mg/mL). Another participant was withdrawn prior to the third randomized treatment due to worsening asthma symptoms. Reported side effects were mild and all subsided without need for intervention. Reported side effects included tremors, cold-like symptoms, headaches, fatigue, dizziness, flushing, and throat irritation. These adverse effects are known to sometimes occur with MCT or with the study treatments. Upon treatment inhalation, eight participants coughed immediately after inhaling the uLABA monotherapy, one coughed after the LAMA monotherapy and two coughed after the uLABA-containing inhaler of the combination therapy.

3.7.1.2 Non-Asthmatic Participants

Table 3.3. *Non-hyperresponsive non-asthmatic participant demographics*

Participant	Gender	Age (years)	Height (cm)	Mean Baseline FEV_1 (L)	Mean Baseline FEV_1 (% Predicted)
H01	F	25	163	3.64	112
H02	F	22	168	3.10	88
H03	F	18	180	3.31	82
H04	F	36	163	2.57	83
H05	M	26	180	4.83	103
H06	M	20	170	3.33	77
H07	M	20	170	3.77	88
H08	M	27	170	4.78	115
H09	M	29	164	3.43	90
H10	M	34	175	4.15	98
H11	F	47	173	3.30	101
H12	F	20	170	4.00	111
H13	F	22	168	3.44	99
H14	F	35	157	3.30	113
H15	M	64	178	2.90	84
Mean:	47% Male	30 [12]	170 [7]	3.59 [0.63]	96 [13]

[ ], standard deviation; FEV_1, forced expiratory volume in one second

Table 3.4. *Hyperresponsive non-asthmatic participant demographics (n=7)*

Subject	Age (yrs.)	Gender	Height (cm)	Weight (kg)	Mean Baseline FEV_1 (L)	Mean Baseline FEV_1 (% predicted)	PC_{20} (mg/mL)
1	20	M	178	73	3.58	76	n/a
2	20	F	169	59	3.03	84	11
3	29	M	173	70	3.21	76	15
4	22	M	185	95	5.86	118	3.0
5	22	M	180	80	4.40	92	4.2
6	23	F	172	72	3.58	98	3.1
7	20	F	173	59	3.47	93	6.4
Mean:	22 [3]	4 M (57%)	176 [6]	73 [12]	3.88 [0.98]	91 [15]	7.1 [4.9]

[ ], standard deviation; FEV_1, forced expiratory volume in one second; PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV_1

Twenty-two participants enrolled in the study; fifteen were non-hyperresponsive (Table 3.3) and were used to generate the control MDRC. Seven participants (32%) were hyperresponsive (Table 3.4), with six having a positive methacholine challenge and one experiencing a significant response to the saline control (i.e. 27% fall in FEV_1). If a participant's methacholine challenge result was positive (PC_{20}<16mg/mL), they were not included in the control group for comparison with the asthmatic results. Both groups of non-asthmatics reported some allergies; of the non-hyperresponsive group, one had an allergy to penicillin and one to cats (topical – hives); of the hyperresponsive group, one had an allergy to grass (topical – rash), one to peanuts (anaphylaxis), and one to spring pollens (disclosed post-MCT - possibly undiagnosed allergic rhinitis). Three former smokers were non-hyperresponsive, and no former smokers were hyperresponsive.

3.7.2 Methacholine Dose-Response Curves and Slopes

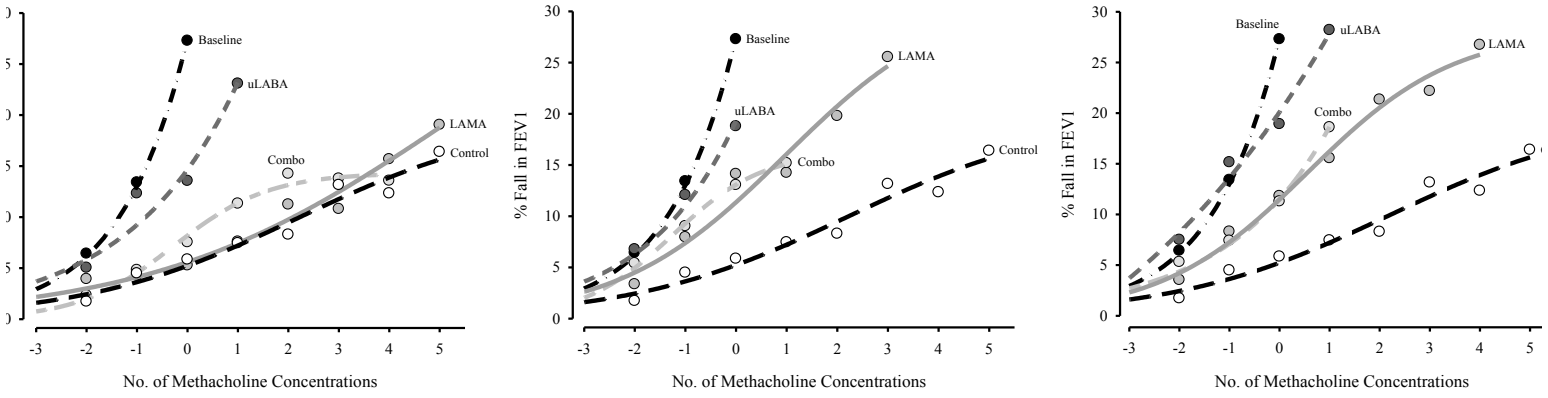

Figure 3.1. *Mean non-asthmatic (control), baseline and post-treatment MDRCs at 1 hour (**A**), 24 hours (**B**), and 48 hours (**C**). The methacholine concentration causing a minimum 20% fall in FEV_1 at baseline is designated as zero on the x-axis (i.e. corresponds to the final data point on baseline curves). Mean responses below (three) and above (five) comprise the post-treatment dose-response curves. Only data points with at least n=8 are included. The sample size for each curve is: n=15 for LAMA, n=16 for uLABA, and n=14 for combo.*

Mean asthmatic MDRCs for baseline, 1, 24, and 48 hours post-treatment for each drug and the mean non-asthmatic control MDRC are depicted in Figure 3.1 A-C. The mean non-asthmatic control MDRC and the mean combo MDRC at one hour post-dose meet the study definition of a plateau (i.e. last three data points within 5%); both MDRCs formed a plateau between a 10-15% fall in FEV_1.

In terms of mean slopes (m), the uLABA (m=5.5, 6.0, and 6.6 at 1, 24, and 48 hours, respectively) did not differ significantly from baseline (m=10.4) at any time-point. Only the one-hour LAMA slope (m=1.7) was statistically similar to the control slope (m=2.0; p=0.514). The LAMA at 1, 24 (m=4.2), and 48 hours (m=3.8) differed significantly from baseline (p=0.003, 0.027, and 0.016, respectively). The combo differed significantly from baseline at 1 (m=3.0; p=0.021) and 24 hours (m=3.3; p=0.039), but not at 48 hours (m=4.4; p=0.067).

3.7.2.1 Individual Asthmatic Methacholine Dose-Response Curves

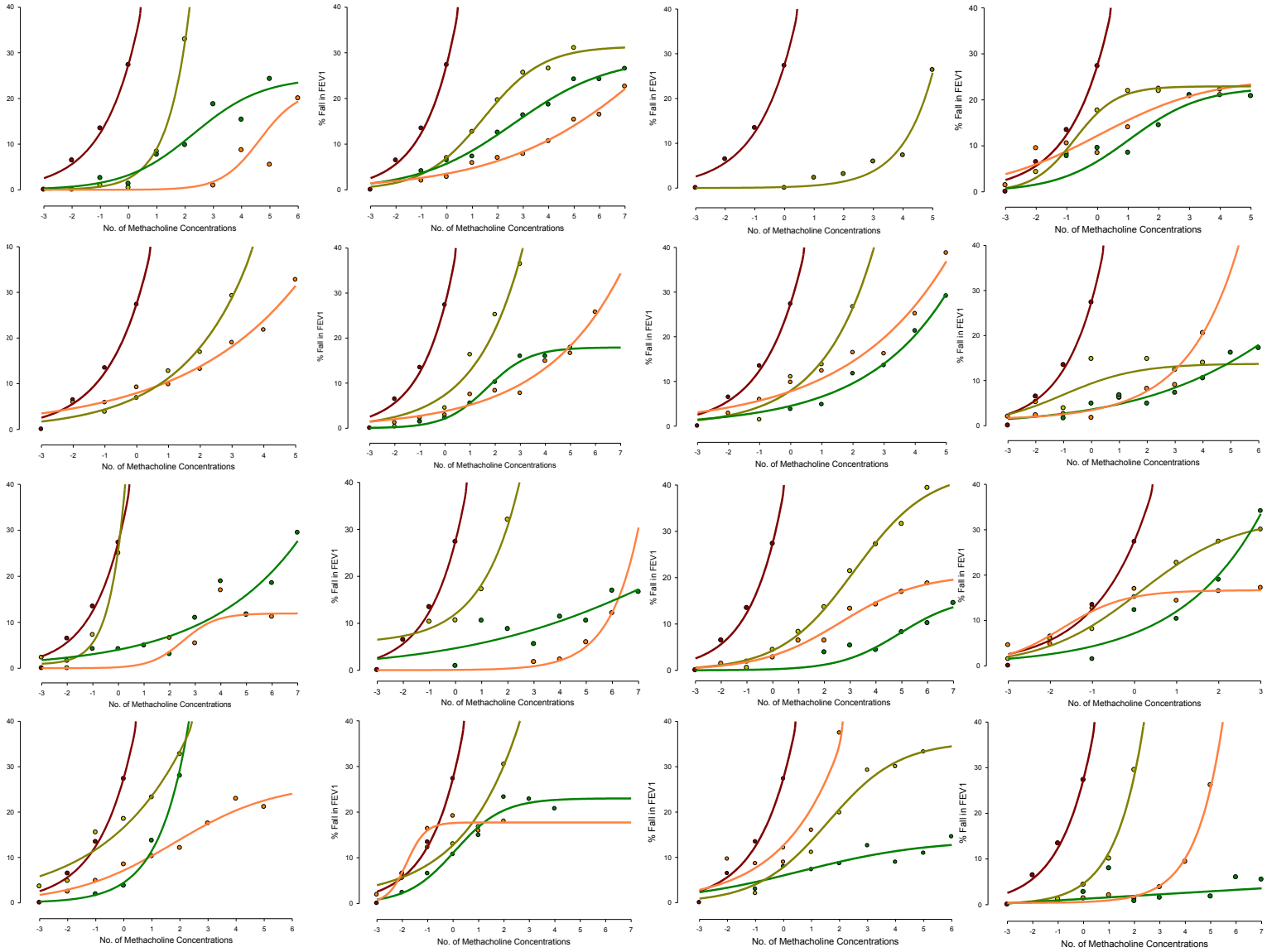

Figure 3.2. *Comparison of the mean asthmatic baseline MDRC with the one-hour post-treatment curves, graphed separately for each participant. The methacholine concentration causing a minimum 20% fall in FEV_1 at baseline is designated as zero on the x-axis (i.e. corresponds to the final data point on baseline curves). Higher and lower methacholine concentrations administered are plotted as positive and negative x-values, respectively. Red = mean baseline; yellow = uLABA; orange = LAMA; green = combo*

Individual asthmatic participants differed in terms of both the drug(s) producing the more favourable response and the changes in characteristics on the MDRC elicited by each treatment (Figure 3.2). Some participants did not reach a response plateau (i.e. three consecutive data points within 5%) with any treatment (i.e. A, G, J, M), while others developed a response plateau after one (i.e. B, F, H, I, K, L, O, P), two (i.e. N) or all three (i.e. D) treatments. The uLABA did not always appear to be the least beneficial medication (i.e. D, H, O). In the same fashion, the combo did not always appear to be the more beneficial treatment (i.e. A, B, I, J, M).

3.7.2.2 Individual Non-Asthmatic Methacholine Dose-Response Curves

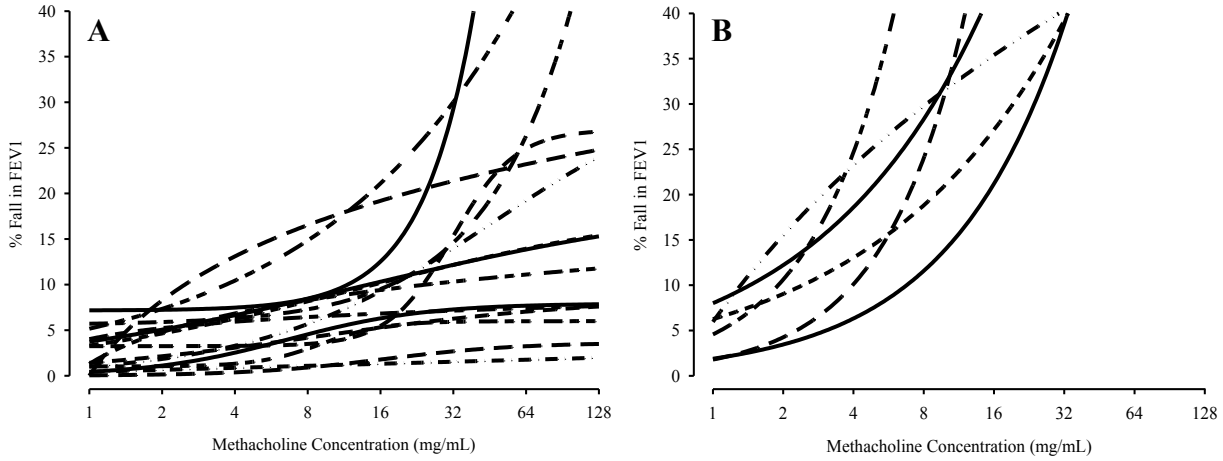

Figure 3.3. *Individual MDRCs of 15 non-hyperresponsive (**A**) and 6 hyperresponsive (**B**) non-asthmatic participants.*

The non-asthmatic response to cholinergic stimulation could be divided into four subgroups; non-hyperresponsive participants (Figure 3.3.A) could be subdivided into a plateau group (n=8; mean slope=4.4 ±1.1), a near-plateau group that was one data point shy of a plateau (n=3; mean slope=11.6 ±2.9), and a non-plateau group (n=3; mean slope=15.1 ±6.6); the hyperresponsive participants (Figure 3.3.B) could be grouped together (n=7; mean slope=24.6 ±2.5).

3.7.3 Methacholine Dose Shifts

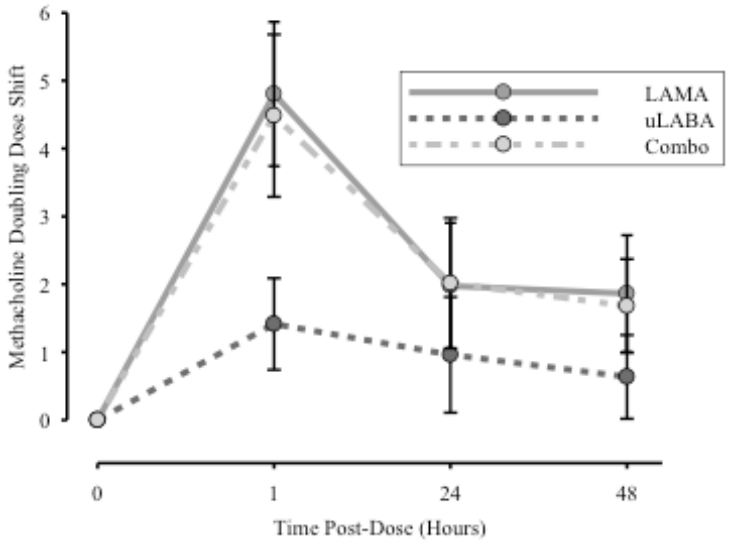

Figure 3.4. *Mean methacholine dose shifts and their respective 95% confidence intervals for each treatment arm at 1, 24, and 48 hours post-dosing. The sample size for each treatment is: n=15 for LAMA, n=16 for uLABA, and n=14 for combo*

Table 3.5. *Proportion of participants with a dose shift ≥ 1 doubling concentration post-treatment*

Time Post-Dose	LAMA, n=15	uLABA, n=16	Combo, n=14
1 hour	100% (15/15)	62.5% (10/16)	100% (14/14)
24 hours	73.3% (11/15)	37.5% (6/16)	64.3% (9/14)
48 hours	80% (12/15)	18.8% (3/16)	64.3% (9/14)

LAMA; long-acting muscarinic antagonist; uLABA, ultra-long acting β_2 agonist; Combo, combination LAMA/uLABA

Mean methacholine dose shifts from baseline at 1, 24, and 48 hours post-treatment are illustrated in Figure 3.4. Geometric mean baseline PC_{20}'s are 2.13 [1.32-2.95] for the LAMA, 2.47 [1.83-3.11] for the uLABA, and 1.78 [1.21-2.34] for the combo therapy. The uLABA and combo were statistically different (p=0.024) from each other.

The LAMA and combo treatments provided clinically significant protection against methacholine-induced bronchoconstriction at 1, 24 and 48 hours through dose shifts of approximately five, two, and two doubling concentrations, respectively. The uLABA only

provided clinically significant bronchoprotection at one hour with a dose shift of approximately 1.5 doubling concentrations. All dose shifts post-LAMA and post-combo are equivalent. Both treatments differ significantly from the uLABA at 1 ($p<0.00001$) and 48 hours ($p=0.012$). The LAMA and uLABA differ significantly at 24 hours ($p=0.049$). Table 3.5 describes the proportion of participants receiving clinically significant bronchoprotection from each treatment over 48 hours.

3.7.4 Bronchodilation

Table 3.6. *Mean baseline and post-dose shifts in FEV_1*

Treatment:	Baseline FEV_1 (L)	FEV_1 1 hour (L)	FEV_1 24 hours (L)	FEV_1 48 hours (L)
LAMA	3.25 [2.77-3.73]	3.31 [2.86-3.76]	3.33 [2.87-3.79]	3.26 [2.82-3.71]
uLABA	3.32 [2.91-3.72]	3.31 [2.91-3.72]	3.34 [2.94-3.73]	3.29 [2.89-3.69]
Combo	3.16 [2.70-3.62]	3.25 [2.77-3.72]	3.26 [2.78-3.74]	3.32 [2.83-3.81]

[ ], 95% confidence intervals; FEV_1, forced expiratory volume in one second; LAMA, long-acting muscarinic antagonist; uLABA, ultra-long acting β_2 agonist; Combo, combination LAMA/uLABA

Baseline and post-treatment FEV_1 data are described in Table 3.6. Mean baseline FEV_1 results for the LAMA, uLABA and combo did not differ significantly ($p=0.060$). No treatment produced significant bronchodilation; the combo produced the greatest improvement in FEV_1 (160mL increase from baseline at 48 hours post-treatment).

3.8 Discussion

The investigation of drug effects through changes on MDRCs provides interesting information on the bronchoprotective mechanisms while also differentiating individual responses. Beginning with the examination of the mean data, each treatment provided a distinct set of changes to the mean asthmatic MDRC. The LAMA glycopyrronium significantly reduced airway sensitivity and reactivity for at least 48 hours, and mildly reduced maximal responsiveness at 1 hour. The large degree of bronchoprotection and the lack of significant

bronchodilation observed post-LAMA confirm the findings of the first study and reinforce the fact that LAMA therapy could be clinically beneficial for some asthmatics (Blais et al. 2016). The minimal improvement in FEV_1 capacity was again unsurprising given the mild (if any) airway constriction at rest in the study population. Interestingly, the one-hour post-LAMA MDRC is nearly identical to the non-asthmatic control curve in terms of position and slope, but lacks a response plateau. Combination therapy does not appear to act synergistically or additively with respect to airway sensitivity and reactivity, as LAMA monotherapy produced the largest rightward shift (i.e. a doubling dose shift of approximately five) and reduction in slope. The shift in methacholine PC_{20} following LAMA treatment was not surprising as this drug is reported to be a competitive inhibitor of cholinergic activity. However, the shape of the one-hour LAMA curve indicates both non-competitive (decrease in maximal response) and competitive (rightward shift) antagonism may be occurring and that various mechanisms may be involved.

Several potential mechanisms may explain the MDRC changes observed post-LAMA. A study investigating the effects of glycopyrronium in guinea pig trachea preparations observed the blockage of calcium release and signaling, which resulted in the inhibition of ASM contractility (Fukunaga et al. 2016). This may have been a factor in the small reduction in maximal responsiveness observed at one hour. Although the study timeline may have been too short for anti-airway remodeling mechanisms to have a significant impact, it is possible that the LAMA prevented airway remodeling to some degree, which would again reduce the maximal response attained; in guinea pig trachea preparations, tiotropium was observed to inhibit airway wall thickening and the hypertrophy of mucous glands (Bos et al. 2007).

In terms of the significant improvement in airway sensitivity post-LAMA, competitive receptor binding and anti-inflammatory properties may be involved. The prevention of cholinergic binding to M_1 and M_3 receptors in particular could inhibit any abnormal autonomic cholinergic control (i.e. excessive bronchoconstriction). Anti-inflammatory effects may be less likely given the single dose, short follow-up study design. However, it is possible that glycopyrronium is inhibiting the release and activity of various inflammatory mediators. In mouse models of COPD, this LAMA prevented the accumulation of IL-1β and TNFα that normally occurs after cigarette smoke exposure (Shen et al. 2014). Tiotropium has also been shown to possess anti-inflammatory properties; in mouse models of asthma that had been acutely or chronically challenged with ovalbumin (used to induce AHR and airway remodeling), pre-

treatment with tiotropium inhibited the production of eosinophils and Th2 cytokines, which are common inflammatory mediators involved in the allergic response during an asthma exacerbation (Ohta et al. 2010).

With regards to reduced airway reactivity, only the LAMA at one hour produced a slope that was statistically similar to the control curve slope. This is a desirable feature of a respiratory medication, as reactivity represents the speed of onset of asthma symptoms during an exacerbation, also referred to as "breakthrough" bronchoconstriction (Löfdahl & Chung 1991; Wong et al. 1997; Lipworth et al. 1998). The LAMA-induced improvement in airway reactivity is likely the result of a combination of mechanisms involved in airway sensitivity and maximal responsiveness. Altogether, the significant and persistent bronchoprotective mechanisms triggered by LAMA therapy merit further investigation to inform clinical decision-making as LAMAs become more commonly used for asthma.

The effects produced by the uLABA indacaterol draw a sharp contrast to those observed post-LAMA; the uLABA only produces a mild reduction in airway sensitivity (i.e. a 1.5 doubling dose shift) at 1 hour and loses clinical significance by 24 hours. This low degree of bronchoprotection was unexpected given that the uLABA olodaterol has been shown to produce at least 32 hours of significant bronchoprotection against methacholine (O'Byrne et al. 2009). SABAs (e.g. salbutamol) and LABAs (e.g. formoterol, salmeterol) have also been shown to reduce airway sensitivity more significantly than was found with indacaterol; at 1 hour post-dose, 200μg salbutamol produced a dose shift of 2 (Derom et al. 1992), 50μg salmeterol produced a dose shift of 2-3.3 (Cheung et al. 1992; Derom et al. 1992; Bhagat et al. 1996), and 24μg formoterol produced a dose shift of 4 doubling concentrations of methacholine (Ramsdale et al. 1991). The uLABA indacaterol did not appear capable of outcompeting methacholine-induced bronchoconstriction for long. It is possible that coughing soon after treatment inhalation may have led to the exhalation of a significant amount of the drug particles. Coughing occurred in half of the study sample during the uLABA monotherapy arm and was rare in the other two treatment arms. This observation has been made previously, where a large clinical trial of n=416 reported that 17.8% of participants coughed within 15 seconds of having inhaled indacaterol (Feldman et al. 2010).

A physiological theory for the rapid loss of any uLABA-induced effects may be the result of receptor desensitization; when activated for a prolonged period of time, β_2 receptors

eventually trigger their own desensitization. β_2 receptors initiate an intracellular pathway that leads to protein kinase A (PKA) production. As PKA accumulates, it activates G-protein-coupled receptor kinases (GRKs) that phosphorylate and uncouple activated β_2 receptors by triggering the recruitment of β-arrestin, a molecule that deactivates G_s proteins (Shenoy & Lefkowitz 2003). Unimpeded M_3 receptor activity also desensitizes β_2 receptors, as M_3 receptor activation promotes the G_q protein-mediated production of protein kinase C (PKC); PKC has a similar influence on β_2 receptors as PKA, and additionally reverses the β_2 agonist-induced inhibition of myosin light-chain kinase, an important enzyme for triggering ASM contraction (Barisione et al. 2010).

Despite the lack of any apparent synergistic effects on airway sensitivity and airway reactivity, combination therapy did alter maximal responsiveness. Both LAMA monotherapy and LAMA/uLABA combination therapy produced virtually identical dose shifts in methacholine responsiveness at all time-points, but only the combination produced a response plateau similar to that observed with the control group. Perhaps no further significant reduction in airway sensitivity and reactivity is possible beyond that produced by the LAMA therapy.

A theory for the synergistic development of a response plateau relies on the increased activity of β_2 receptors as a result of M_2 and M_3 inhibition by the LAMA. M_2 receptors, when activated, inhibit cAMP formation and stimulate the opposite G protein (G_i) to that triggered by β_2 receptor activity. Therefore, through the concomitant use of a muscarinic antagonist with a β_2 agonist, the latter's stimulation of cAMP production could be unimpeded, and M_3 receptors are unable to trigger β_2 receptor desensitization as discussed previously. With full, unobstructed β_2 receptor activity, it is possible that the uLABA and LAMA initiate complimentary inhibition of calcium signalling, which results in a stronger block on ASM contractility. The uLABA is anticipated to block calcium through its stimulation of the production of PKA, which deactivates IP_3 and consequently inhibits calcium release from the sarcoplasmic reticulum (SR) (Barisione et al. 2010). PKA also promotes calcium/sodium exchange, which depletes cells of intracellular calcium, and stimulates both sodium/potassium ATPase and calcium-activated potassium channels (Gunst & Stropp 1988; Twort & van Breemen 1989; Kume et al. 1992). These mechanisms together result in the hyperpolarization of ASM cells and the inhibition of airway contractility. LAMAs in turn prevent IP_3 production through their blockage of M_3 receptor activity, which then prevents intracellular calcium levels from increasing sufficiently to trigger

contractile mechanisms (Meurs et al. 1988; Buels & Fryer 2012). LAMA blockage of M_2 receptors also reduces ASM contractility, as the inhibition of M_2-stimulated G_i protein activation prevents cyclic ADP-ribose from mediating calcium release via the ryanodine receptor channels in the SR (Jude et al. 2008). These known mechanisms of action for uLABAs and LAMAs should together block airway contractility and consequently excessive airway narrowing to a greater degree than either monotherapy.

It is interesting to note that while the LAMA and combo therapies produced equivalent mean dose shifts at all time-points, the LAMA shifted the MDRC further to the right (i.e. larger reduction in airway sensitivity) than did the combo. A potential explanation for this difference could be that a uLABA-induced increase in airway cilia beat frequency coupled with the LAMA-induced block on further mucus production temporarily leads to some airflow obstruction (Bennett 2002); the airways may be temporarily obstructed by the mucus layer that is being moved by the airway cilia up the airways towards the oesophagus to be swallowed.

One last potential observation of synergism may be the bronchodilation found post-combination therapy. Of the three treatment arms, the combo produced the largest increase in FEV_1, which improved by 160mL at 48 hours post-combo. This is not clinically significant in this study population, as published guidelines for significant post-bronchodilator FEV_1 improvement is a minimum of 200mL (American Thoracic Society 1995). Despite not being clinically significant, this bronchodilation may be the product of either boosted β_2 receptor activity due to concomitant LAMA use or perhaps due to the mean baseline FEV_1 pre-combo being lower (albeit not significantly) than that prior to the other two treatments.

Although the mean data present interesting findings that allow for the differentiation between each treatment, the individual data shown in Figure 3.2 reflect how variable individual responses to treatments can be. This variability may be related to the various asthma phenotypes and could be the result of several factors such as the specific pathophysiologies involved and their severity as well as potential long-term effects from previous treatments used. These findings illustrate the relevance of modernizing health care to personalized medicine, as asthma treatments are not one-size-fits-all. Future research should aim to identify a set of feasible procedures to perform in patients in order to more accurately determine which drug will be of most benefit for them. The ideal drug is particularly effective at minimizing excessive airway

narrowing (i.e. reducing maximal responsiveness), as this symptom if unmitigated causes asthma-related deaths.

Finally, with regards to the non-asthmatic participants recruited, it was unexpected that so many (32%) of these asymptomatic individuals would demonstrate AHR. The variability in MDRC shape within the non-asthmatic non-hyperresponsive group was also worth noting. For example, not all non-hyperresponsive individuals developed a response plateau, and the closer a participant's PC_{20} was to 16mg/mL, the more likely their MDRC would be exponential in shape. Unfortunately, the sample size of non-asthmatics precluded any statistical analyses, such as a comparison of the MDRC slopes of each subgroup (i.e. plateau vs. near-plateau vs. non-plateau vs. hyperresponsive group).

The characteristic of the MDRC that is typically described as the most serious problem for hyperresponsive individuals is the loss of protective mechanisms that minimize airway narrowing, resulting in an exponential curve (Woolcock et al. 1984; Moore et al. 1996). This is an oversimplification, as AHR is a dynamic process and as such MDRC shapes can change; for example, studies have shown the reversal of hyperresponsiveness over time in previously hyperresponsive non-asthmatics (Rijcken et al. 1993; Van Den Nieuwenhof et al. 2008). AHR also appears to result from several mechanisms, especially since the recruited sample of non-hyperresponsive non-asthmatics did not all show a response plateau.

Interestingly, past studies have linked AHR to factors such as smoking and a family history of respiratory illness (Burney et al. 1987; Woolcock et al. 1987; Gray et al. 2000) and yet neither was present in the hyperresponsive sample. While AHR does not necessarily correlate with asthma development, evidence has shown that atopy is strongly correlated with the condition, which was present in some of the hyperresponsive sample (but only one produced asthma-related symptoms) (Van Den Nieuwenhof et al. 2008). Several suggestions have been put forth to explain asymptomatic AHR, but studies have been largely divided on the issue (Woolcock et al. 1987; Pin et al. 1993; Laprise & Boulet 1997; Salome et al. 1997; Laprise et al. 1999; Boulet 2003; Boulet et al. 2006; Yoshikawa & Kanazawa 2011). We are left wondering if these individuals have a higher risk of developing asthma or if they simply have a "nonnegative" methacholine challenge due to high test sensitivity (Cockcroft & Davis 2010). It is worth noting that the MDRCs of hyperresponsive asymptomatic non-asthmatics resembled those of asthmatics in terms of seemingly excessive airway narrowing. The significant response post-diluent in one

participant and the PC_{20} <8mg/mL in four hyperresponsive non-asthmatics also closely resembles responsiveness observed in mild asthmatics.

Our findings provide important preliminary information on the usefulness of glycopyrronium and indacaterol alone and in combination in mild asthmatics based on their effects on the MDRC. While indacaterol produced little benefit, glycopyrronium and combination therapy both provided significant bronchoprotection with regards to reduced airway sensitivity and reactivity to cholinergic stimuli. Only combination therapy significantly protected against excessive airway narrowing through the formation of a response plateau. This physiological benefit is important for preventing asthma-related deaths. Future studies examining the physiological effects of the study drugs in human *in vivo* models would be beneficial for informing clinical decision-making, particularly given that asthma patients exhibit a wide range of phenotypes.

4.0 GENERAL DISCUSSION

As we move forward in drug development research for the treatment of asthma, it is important that studies maintain pace and explore these new medications for their influence on current research and clinical practices. Knowledge on the duration, degree, and characteristics of bronchoprotection provided by various treatments against methacholine-induced bronchoconstriction is important not only for ensuring continued MCT data accuracy but also for guiding future physiological research.

We focused particularly on LAMAs and uLABAs, both of which are the subjects of ongoing research exploring their use in asthmatics. We first wished to inform MCT standardization guidelines of an appropriate, scientifically confirmed abstinence period from LAMAs. We succeeded in identifying that a single administration of the recommended dose of glycopyrronium or tiotropium each only provides clinically significant bronchoprotection for up to seven days. We were therefore able to make the recommendation that LAMAs should be avoided for at least seven days prior to undergoing MCT.

The first study was not without its limitations. For example, our findings after a single dose of each LAMA may not be equivalent to that found with regular daily dosing. Future studies could thus investigate long-term daily-use of LAMAs to determine if such a regimen significantly influences perceived drug effects through MCT. An inherent limitation to this type of study is the severity of asthma that can be tested through MCT – perhaps the drug effects may

be significantly shorter (or longer) in more severe asthmatics who are more likely to be taking such medications, but MCT is not advisable in that patient population given their more severe airway constriction at rest and their need for daily therapy. To gain a better appreciation of the bronchoprotection provided by LAMAs against real-world constricting stimuli (cholinergic stimuli are highly unlikely in one's every day environment), studies could explore these medications through allergen or indirect bronchoprovocation testing.

Following the unexpected MDRC results from the first study, we sought in the second study to confirm our findings and to explore the newly developed uLABA drug class. The well established drug classes of ICS, SABAs, and LABAs have each been thoroughly studied for their influence on the MDRC of asthmatics through which important information about bronchoprotective mechanisms and long-terms effects has been elucidated. Our study was the first to explore a LAMA and a uLABA for their individual and combined effects on the MDRC of mild asthmatics. With regards to the mean data, we were able to distinguish the LAMA and combination therapies from the uLABA, as the former two produced comparable and significant improvements in airway sensitivity and reactivity while the latter afforded only a mild improvement in airway sensitivity. Interestingly, only the mean combination MDRC at one hour produced a response plateau like the non-asthmatic control MDRC; however, the one-hour LAMA curve most resembled that of the control despite not producing a response plateau. Altogether, the mean data suggest that LAMAs and combination LAMA/uLABAs are worthwhile to consider for use in asthmatics and merit further investigation to identify the asthma phenotypes most likely to benefit from their use.

The individual asthmatic results present more complex findings that reflect how mean data can oversimplify study findings. The high variability in response to each treatment is not surprising, considering how different the study participants can be from each other in terms of pathophysiology, genetics, and environment. The movement towards the practice of more personalized medicine seems highly appropriate for this respiratory condition given our findings.

The non-asthmatic group presented a variety of MDRC shapes and raised questions as to long-standing assumptions regarding "normal" responsiveness to methacholine. The absence of a measurable plateau in some non-hyperresponsive participants agrees with previous findings that a plateau is not necessary in asthmatics for attaining satisfactory control of asthma symptoms (Prieto et al. 1999). AHR and accompanying asthma stem from a combination of

factors, which likely cannot be differentiated fully through MDRCs. Nonetheless, the majority of non-hyperresponsive participants achieved or almost achieved the expected response plateau at a low percent fall in FEV_1 (<15% fall). These data were valuable as control references to better visualize the improvements in lung function provided by the study treatments in the asthmatic participants. The unexpected high prevalence of asymptomatic AHR in the study population presents this phenomenon as a worthy subject for further investigation of its long-term implications, especially since these study participants were devoid of most known causative factors of AHR (e.g. family history of respiratory illness, respiratory symptoms, smoking).

As with the first study, the MDRC investigation possessed several limitations. Although inferences could be made regarding each treatment's physiological mechanisms based on current knowledge of MDRCs, these remain speculative until further studies are performed, preferably in human models. Again, investigations of drug effects after a single dose may not accurately reflect what would be observed with long-term treatment; however, such studies do help in establishing the initial treatment response and are not biased by patient adherence (only one dose). The range of methacholine concentrations available and the safety rules used for determining when to stop a methacholine challenge also limit the extent to which plateau development and overall responsiveness can be examined on the MDRC. Such limitations are difficult to overcome considering the high cost and available doses of methacholine as well as the need to maintain patient comfort and safety during testing. Insufficient information on individual participants' phenotypes and history of asthma was gathered, which precluded any correlation analyses between phenotypes and resulting responses to the treatments.

With the non-asthmatic data, we did not foresee the large percentage of participants with AHR and so, our protocol did not incorporate specific questions for these participants to better understand their past and whether they have in fact experienced similar symptoms as those induced by methacholine. In addition, retrospective statistical analyses of the non-asthmatic slope data could not be performed due to the small sample size. Finally, in the hyperresponsive non-asthmatic participants, MCT was stopped at a 20% fall in FEV_1 per our protocol, which prevented the accurate determination of the maximal response plateau or lack thereof; regardless, this is the typical stopping criterion for MCT and so, asthmatic responsiveness to methacholine is usually affected by the same limitation.

The ideal system for the personalized treatment of asthma would feature a set of objective tests for determining which treatment option is most appropriate for a given patient. Future studies could therefore focus on developing one such system perhaps through the identification and differentiation of different markers of inflammation and/or airway remodelling by phenotype in addition to MCT. Further research into asymptomatic AHR could also prove beneficial, particularly if we were able to distinguish transient AHR from potentially asthma-causing AHR. Whether all uLABAs behave like indacaterol on the MDRC could be an interesting investigation, considering the small degree of bronchoprotection observed in this study in comparison with the significant finding with olodaterol found in a previous study (O'Byrne et al. 2009). LAMAs and LAMA/uLABA combinations could also be examined with ICS co-therapy, given that these combinations are recommended for poorly controlled asthma.

In summary, the LAMAs tiotropium and glycopyrronium were found to produce significant and persistent bronchoprotection against methacholine-induced bronchoconstriction for seven days, which reinforces the importance of such studies considering that standardization guidelines originally recommended only a 48-hour abstinence period from LAMAs (except tiotropium). Glycopyrronium also provided significant reductions in airway sensitivity and reactivity, both of which are important characteristics of AHR. In addition to significant reductions in airway sensitivity and reactivity, combination glycopyrronium/indacaterol was the only study treatment to produce a mean response plateau, an important feature for preventing asthma related deaths through excessive airway closure. With further experimentation, LAMAs and combination LAMA/uLABA therapies may prove to be valuable for the effective and long-term treatment of asthma symptoms and progression.

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6.0 APPENDICES

6.1 Appendix A: Respiratory Medicine Publication of LAMA Study Manuscript

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Duration of bronchoprotection of the long-acting muscarinic antagonists tiotropium & glycopyrronium against methacholine-induced bronchoconstriction in mild asthmatics

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ABSTRACT

The duration of bronchoprotection against methacholine-induced bronchoconstriction by long-acting muscarinic antagonists (LAMA's) in asthmatics and whether these drugs differ in their pharmacodynamic properties remain to be determined. The most recent published guidelines for methacholine challenge testing (MCT) suggest that LAMA's should be abstained from for 48 h prior to testing, perhaps one week in the case of tiotropium.

The objectives were to determine and compare the duration of protection of a single dose of two different LAMA's, tiotropium and glycopyrronium, against methacholine-induced bronchoconstriction.

Thirteen mild-to-moderate asthmatics [with a forced expiratory volume in 1 s (FEV_1) > 65% of predicted and a baseline methacholine provocation concentration causing a 20% reduction in FEV_1 (PC_{20}) ≤ 8 mg/mL] completed this double-blind, double-dummy, crossover study. Methacholine challenges were performed before treatment (5 μg tiotropium or 50 μg glycopyrronium) and at 1, 24, 48, 72, 96 and 168 h post-treatment. The minimum duration between treatment administration was 11 days.

Both drugs provided significant bronchoprotection, each producing greater than a 16-fold increase in mean PC_{20} by 1 h. Tiotropium still provided statistically significant protection at 7 days ($p = 0.0282$) while glycopyrronium provided bronchoprotection until day 7 ($p = 0.0590$). Tiotropium provided statistically superior bronchoprotection at 24 and 72 h compared to glycopyrronium.

To minimize the occurrence of false negatives, MCT guidelines should be updated to recommend a minimum one-week abstinence period from all LAMA's. MCT was also able to statistically differentiate between tiotropium and glycopyrronium with respect to the degree and duration of bronchoprotection provided by each.

Clinical trial registration number: NCT02622243.

1. Introduction

Asthma is a respiratory condition that causes either persistent or sporadic airway hyperresponsiveness (AHR) in response to a variety of triggers [1]. Although the exact cause of asthma remains unknown, it is believed that genetic and environmental factors contribute to its development [2]. In order to treat patients with varying symptoms and severity of asthma, a range of pharmaceutical agents have been developed. Short-acting bronchodilators (e.g. salbutamol) are used to alleviate symptoms in the event of an acute exacerbation. For more severe asthmatics, common drug regimens include inhaled corticosteroids (ICS) for reducing airway inflammation taken as a monotherapy or in combination with long-acting beta agonists (LABA's). Recently, the safety of long-term use of LABA's as a monotherapy has been questioned, as they appear to significantly reduce beta agonist efficacy and may lead to more severe asthma exacerbations [3,4].

Due to increased demand for more effective treatment regimens, research has now begun to examine the use of long-acting

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muscarinic antagonists (LAMA's) in combination with ICS, and as triple therapy with ICS plus LABA [5,6]. LAMA's function by selectively inhibiting muscarinic M_1 and M_3 receptors and slowly dissociating from them, thereby generating a long duration of action [7]. Unlike the short-acting muscarinic antagonist ipratropium bromide (IB), LAMA's are more selective and do not greatly inhibit M_2 receptors, which are key for preventing the excessive presynaptic release of the bronchoconstrictor acetylcholine. A number of LAMA's such as tiotropium and glycopyrronium have been developed and are approved for use in chronic obstructive pulmonary disease (COPD). Currently, the only LAMA included in the Global Initiative for Asthma (GINA) report 'Global Strategy for Asthma Management and Prevention (2016 update)' is tiotropium [8].

Given incorporation of LAMA's into asthma treatment regimens, it is important to know the impact of such medications on diagnostic tests. One of the most common diagnostic tests used in asthma research and clinical practice is the methacholine (MCh) challenge. This test is typically used for confirming or refuting a physician diagnosis of asthma [9]. A number of factors, including concomitant medications, have been found to influence the accuracy of this diagnostic test, thereby contributing to false negative results. To establish a standard testing protocol for accuracy and safety purposes, the American Thoracic Society (ATS) published a set of guidelines for MCT which includes suggested abstinence periods from several classes of pharmaceuticals [9]. Due to the various durations of pharmacological effects, the ATS guidelines suggest withholding long-acting bronchodilators for 48 h and possibly one week for tiotropium [9]. However, the references provided to support these washout periods only investigated the duration of action of single doses of LABA's [10,11]. It may be inappropriate to generalize these results to all long-acting bronchodilators, as LABA's and LAMA's have completely different mechanisms of action.

The two LAMA's examined in this study, tiotropium and glycopyrronium, are both potent antimuscarinic quaternary ammoniums that are widely used, once-daily medications for COPD [12,13]. These treatments are generally well tolerated with a good safety profile [12,14]. The most common side effect reported for both drugs is xerostomia, which is caused by the inhibition of acetylcholine signaling, the neurotransmitter responsible for saliva production via M_1 and M_3 receptor activation [12].

Few studies have examined the duration of effect of single doses of LAMA's in asthmatics and no study has captured the effect in its entirety. We hypothesized that these two drugs would have similar efficacy against methacholine-induced bronchoprotection in asthma and that the duration protection would be greater than 48 h.

2. Methods

2.1. Participants

Eligible participants were at least 19 years of age, had a MCh $PC_{20} \leq 8$ mg/mL and had baseline lung function (% predicted FEV_1) of 65% or greater. None had taken any anticholinergic agent within 30 days nor had they suffered a respiratory tract infection within 4 weeks of study entry. No participants were taking inhaled corticosteroids as a monotherapy. Known contraindications for MCT include pregnant or nursing women, and those suffering from cardiovascular or prostate problems, kidney or urinary retention problems, or glaucoma. Such participants were excluded. Consent was obtained from all participants prior to commencing the study. The study was approved by the University of Saskatchewan Biomedical Research Ethics Board (Bio-REB 15–254).

2.2. Methacholine challenge test

The MCT method used was the 2-min tidal breathing dosing protocol outlined in the ATS guidelines for MCT [9]. The protocol was completed with the Bennett Twin jet nebuliser (Puritan Bennett Corporation, Carlsbad, CA), which was calibrated to produce an output of 0.13 mg/mL prior to beginning the study. For each testing session, spirometry was first performed to obtain a baseline FEV_1 with the highest (peak) FEV_1 being used for further analysis. For the tidal breathing protocol [9], the participant wore nose clips and calmly inhaled nebulised aerosol through a loose-fitting facemask for 2 min. Spirometry was then performed at 30 and 90 s post-inhalation to determine the resulting change in FEV_1. The next inhalation was started 5 min following the start of the previous inhalation. Aerosolized saline (0.9%) was administered first and subsequent inhalations consisted of doubling concentrations of MCh. This procedure was repeated until a fall in FEV_1 of 20% or more occurred. Post-dose, a maximum MCh concentration of 256 mg/mL could be administered to achieve a 20% fall in FEV_1, thereby allowing for interpolation or extrapolation to determine the MCh PC_{20}. If the maximum MCh concentration was administered and the fall in FEV_1 was only between 10 and 20%, the PC_{20} was extrapolated from the log dose vs. response curve algebraically. If the FEV_1 fell by less than 10% at the maximum MCh concentration, the MCh PC_{20} was arbitrarily set at 1024 mg/mL.

2.3. Study design

This was a randomised, double-blind, double-dummy, crossover study. For each treatment arm, participants underwent baseline MCT followed by treatment inhalation and MCT at 1, 24, 48, 72, and 96 h post-treatment. Each MCT was performed at the same time of day and a minimum of 11 days elapsed between doses. Initially, testing on day 7 was only performed in participants who showed persistent bronchoprotection at 96 h. It was subsequently recognized however, that statistical analysis would be weakened if a participant had data until day 7 on one drug, but only data until 96 h post-treatment on the other study drug. Following this, all remaining participants underwent 168 h post-dose MCT for both treatments.

2.4. Study drugs and blinding

Inhalers were pre-loaded with active and placebo capsules (Breezhaler®) or canisters (Respimat®); both participants and the administering investigator were blinded to the treatment. For each treatment, participants took two inhalations from a Respimat and two inhalations (from the same capsule) from a Breezhaler (one active and one placebo), holding their breath for 5 s after each inhalation. The glycopyrronium treatment arm consisted of a dose of 50 μg and the tiotropium treatment arm consisted of a dose of 5 μg. The treatments were administered in random order.

2.5. Statistical analysis

With a sample size of thirteen participants, the power of this study was greater than 85% for detecting a half concentration difference in MCh PC_{20}. PC_{20} data was log-transformed before further analysis. Bronchoprotection was assessed as the dose shift in MCh PC_{20} [dose shift = (ΔlogMCh PC_{20})/0.3]. Paired t-tests were used to compare treatment differences in PC_{20} dose shift and in baseline FEV_1. Repeated measures analysis of variance was performed to compare dose shifts and baseline FEV_1 data between the two drugs. Results are reported as mean data ± standard error of mean (SEM), unless otherwise stated. Significance was set at 0.05.

3. Results

3.1. Participants

Eighteen non-smoking subjects with physician-diagnosed mild-to-moderate asthma were recruited from the local community and thirteen of them (8 males, 5 females) completed the study (see Table 1). Five participants were excluded; four did not have a positive MCh challenge and one did not have an adequate baseline FEV_1. No unexpected serious adverse effects occurred. Reported side effects were mild and included topical dryness, shortness of breath, chest tightness, muscle stiffness, and headaches. All were known side effects of either drug or of MCT, and subsided without the need for intervention.

3.2. Bronchoprotection

Dose shift data and MCh PC_{20} values for both treatments are shown in Fig. 1 and Table 2 respectively. Both treatments significantly increased the amount of MCh required to cause a 20% decrease in FEV_1 by more than 16-fold at 1 h. Tiotropium and glycopyrronium each continued to provide clinically significant bronchoprotection at 96 h (p-0.0010 and p = 0.0122, respectively), as shown in Fig. 2. At 168 h, tiotropium provided statistically significant bronchoprotection (p = 0.0282, n = 10) while glycopyrronium did not (p = 0.0590, n = 9).

Tiotropium appears to be superior to glycopyrronium until the 168 h time-point. Tiotropium provided significantly more bronchoprotection than glycopyrronium at 24 h (p = 0.0058) and 72 h (p = 0.0082) post-treatment, and approached statistical significance at 48 h (p = 0.0503) and 96 h (p = 0.0568). Paired t-tests of the dose shift produced by each drug at 1 h and at 7 days post-treatment showed no statistical difference between the two treatments (p = 0.8651 and p = 0.2123 respectively).

3.3. Bronchodilation

Overall, mean peak FEV_1 data only differed significantly from baseline at 24 h post-glycopyrronium, as shown in Table 3. In terms of mean shift in peak FEV_1 and the 95% confidence intervals, glycopyrronium provided an average increase in volume of 90 mL, from 3.65 L [3.00, 4.29] at baseline to 3.74 L [3.12, 4.37] at 24 h post-treatment. Borderline statistically significant shifts in mean peak FEV_1 from baseline were observed with glycopyrronium at 1 h post-treatment and with tiotropium at 24 h post-treatment. No significant difference in mean FEV_1 was observed between the two treatment arms at any point.

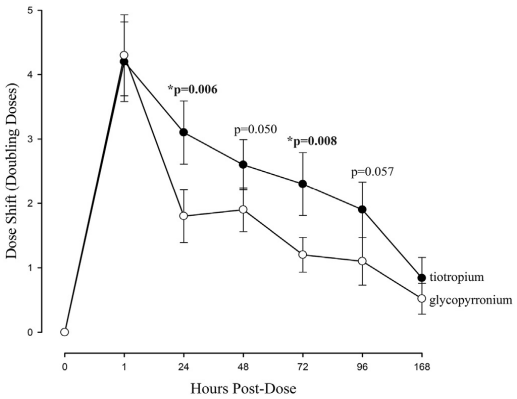

Fig. 1. Mean dose shift and standard error at various time points following treatment with tiotropium (TIO) and glycopyrronium (GLY). The sample size was 13 except for TIO at 96 h (n = 12), TIO at 168 h (n = 10) and GLY at 168 h (n = 9). P values represent the difference between the treatment arms at the given time points.

3.4. Post hoc analysis of MCh dose-response curves

Fig. 3 shows that the mean MCh dose-response curves prior to each treatment arm did not appear to plateau (i.e. produce a maximal response to MCh). At 1 h post-treatment, both drugs shifted the curve to the right and produced a plateau.

4. Discussion

Given the gap of knowledge in current literature on LAMA use in asthmatics, the primary objective of this study was to determine the duration of bronchoprotection provided by tiotropium and glycopyrronium against methacholine-induced bronchoconstriction. Previously, O'Connor et al. found that different dosages of tiotropium provide bronchodilation for up to 24 h and dose-dependent bronchoprotection for at least 48 h in twelve mild-to-moderate asthmatics [15]. With a reduced sample size of four, they also found persistent bronchoprotection at 72 h post-

Table 1
Participant demographics.

Participant	Gender	Age (yrs.)	Height (cm)	Pre-GLY FEV_1 (% predicted)	Pre-TIO FEV_1 (% predicted)	PC_{20} Pre-GLY (mg/mL)	PC_{20} Pre-TIO (mg/mL)
1	M	69	168	72	77	1.0	1.4
2	M	39	178	88	85	1.2	0.64
3	M	22	183	83	82	6.4	7.2
4	F	22	173	96	96	0.98	1.2
5	M	31	196	99	96	1.1	3.4
6	F	27	168	102	96	1.5	2.4
7	M	24	178	100	99	1.1	1.1
8	M	29	185	105	105	10.0	7.0
9	M	44	175	95	95	7.1	7.6
10	F	28	160	89	91	1.4	3.4
11	M	20	185	76	94	0.78	0.73
12	F	21	170	79	80	0.66	1.5
13	F	24	147	68	76	0.36	0.16
Mean:	62% M	31 [13]	174 [12]	89 [12]	90 [10]	2.6[a]	2.9[a]

Standard deviation.
GLY; glycopyrronium, TIO; tiotropium.
[a] Geometric mean.

Table 2
MCh PC_{20} data.

	MCh PC_{20} (mg/mL) at:						
	Baseline	1 h	24 h	48 h	72 h	96 h	168 h
Tiotropium treatment arm:	2.9 (0.74)	199 (104)	74 (35)	57 (38)	97 (78)	44 (31)	9.6 (4.4)
Glycopyrronium treatment arm:	2.6 (0.86)	202 (104)	29 (14)	25 (13)	11 (5.3)	17 (10)	4.7 (1.6)

Baseline and post-treatment PC_{20} data presented as geometric means (SEM).

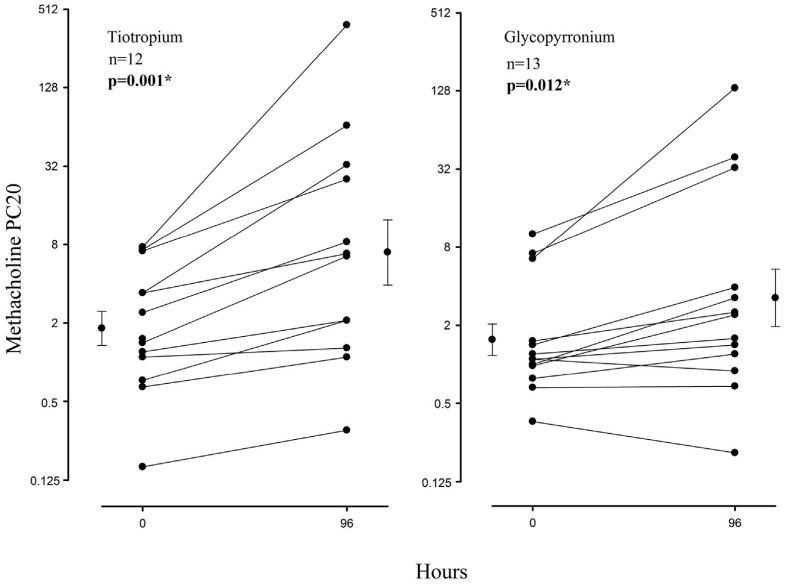

Fig. 2. Individual MCh PC_{20} values at pre-treatment and at 96 h post-dose for each drug. Single data points represent geometric mean and SEM values. P values represent the difference between the PC_{20} values at 96 h versus those values at baseline within the same treatment arm.

Table 3
Mean peak FEV_1 at each time point during both treatment arms.

	Mean peak FEV_1 values at:						
	0 h	1 h	24 h	48 h	72 h	96 h	168 h
Tiotropium	3.70	3.70 (0.091)	3.82 (0.058)	3.71 (0.931)	3.66 (0.506)	3.75 (0.817) n = 12	3.36 (0.158) n = 10
Glycopyrronium	3.65	3.75 (0.053)	3.74 (0.005*)	3.70 (0.174)	3.65 (0.880)	3.66 (0.671)	3.40 (0.697) n = 9

P values in brackets.
Sample sizes are specified if different from n = 13.
*$p < 0.05$.

treatment. Hansel et al. performed a similar study comparing bronchoprotection provided by IB versus three different dosages of glycopyrronium in ten mild-to-moderate asthmatics [16]. They found that while IB provides more bronchoprotection at 2 h post-treatment, bronchoprotection by glycopyrronium is greater at 12, 24 and 30 h post-dose. Unfortunately, in both of these studies, participants were not followed until the drugs had lost all effect and so, the complete duration of bronchoprotection provided by each treatment remains unknown.

The results from this study reflect that bronchoprotection provided by glycopyrronium lasts up to 7 days while tiotropium still provides statistically significant bronchoprotection at 7 days. However, it should be noted that clinically significant bronchoprotection is defined as a shift in MCh PC_{20} that is greater than one doubling concentration of MCh. Therefore, while tiotropium did provide statistically significant bronchoprotection at 7 days compared to pre-treatment, neither tiotropium nor glycopyrronium provided clinically significant bronchoprotection at 7 days. Initially, participants only completed day 7 of testing if they still showed significant bronchoprotection at 96 h. This resulted in reduced sample sizes on day 7 for both treatments and it is possible that neither drug may have been found to be significant at 7 days

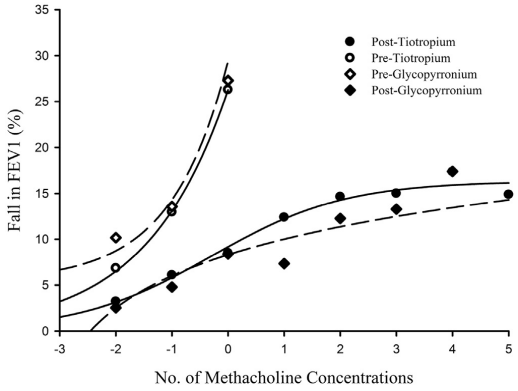

Fig. 3. Comparison of mean MCh dose-response curve pre-treatment versus 1 h. post-treatment with tiotropium and glycopyrronium, respectively. 0 was set as the MCh concentration that caused participants' FEV_1 pre-treatment to drop by ≥20%. Any lower concentrations administered were plotted as negative and any higher concentrations were plotted as positive. Best-fit curves were then generated. Any MCh concentration numbers with less than n = 6 were excluded.

had all participants completed day 7 testing. Nonetheless, these findings suggest that current guidelines for MCT need to be updated to account for the significantly longer duration of bronchoprotection provided by LAMA's than the currently suggested 48 h and "perhaps 1 wk for tiotropium" [9].

The secondary objective of this study was to compare the two LAMA's in order to determine if there is any difference in bronchoprotection between the two treatments. As previously mentioned, the two drugs investigated in this study are typically used in COPD patients and as such have more research data in that patient population. Glycopyrronium has been found to provide a dose-dependent bronchodilator effect, with higher doses (i.e. 50–115.2 μg) producing comparable effects to the recommended tiotropium dosage of 18 μg in COPD patients [17–19]. The main difference observed between the two treatments was that glycopyrronium provides more rapid and significant bronchodilation from 5 min to 4 h post-treatment [18,19]. This presented the hypothesis that these two drugs would exert similar durations of bronchoprotection and bronchodilation in asthmatics undergoing MCT.

Our findings show that while their overall duration is similar in terms of clinical significance, tiotropium provides superior bronchoprotection at almost all time points, and its superiority reached statistical significance at 24 and 72 h post-treatment. Reasons for this difference may be that the dosage of tiotropium administered was that recommended for asthmatics while glycopyrronium was administered per guidelines for COPD patients. Although glycopyrronium is somewhat more selective for M_3 receptors than for M_1 and M_2 types, only tiotropium has kinetic selectivity for M_3 over M_2 receptors, which could lead to a significant difference in perceived bronchoprotection between the two treatments [20]. If glycopyrronium binds M_2 receptors for a longer period of time, this could produce prolonged inhibition of this receptor-type's normal function as a moderator of acetylcholine in the airways. The result could be a reduction in overall bronchoprotection, as elevated acetylcholine levels would promote some bronchoconstriction.

It has also been suggested that differences in receptor binding kinetics between tiotropium and glycopyrronium only explain some of the variation seen in their respective durations of action [21]. Drug rebinding may prolong the duration of action of pharmaceuticals, as freshly dissociated drug molecules could rebind to receptors further down the target tissue rather than being immediately metabolized [22]. Given that some drug molecules would be lost to metabolism with each dissociation and rebinding event, glycopyrronium could lose effect more quickly, as it dissociates more rapidly than tiotropium. Another potential factor contributing to the different effects of each drug could be their respective delivery methods. It has been argued that soft mist inhalers deliver pharmaceuticals more efficiently than do dry powder inhalers, leading to more drug deposition in the airways even in the event of poor inhalation technique [23].

Neither drug produced a strong bronchodilator effect, which is contradictory to results obtained in previous studies in mild asthmatics [15,16]. Only glycopyrronium provided statistically significant bronchodilation at one time point, 24 h post-treatment. However, this effect was not clinically significant and only represented an increase in volume of 97 mL while the ATS guideline for significant postbronchodilator FEV_1 is a 200 mL increase (or 12%) [24] This lack of significant bronchodilation in the current study may be explained by the much greater dosages of the treatments administered in previous studies. It should also be noted that the participants were mildly asthmatic and so, they likely had relatively low airway constriction at rest. Therefore, the airways may not have had much capacity to dilate further. Nonetheless, while this study's findings do not suggest that the two LAMA's are effective bronchodilators in mild asthmatics, they both provide significant bronchoprotection, particularly at 1 h post-treatment where both drugs produced a dose shift of 4 doubling concentrations of MCh (or a 16-fold increase in mean PC_{20}). This is comparable to the duration of bronchoprotection provided by other drug types; at 30 min post-dose, the long acting beta agonist formoterol (24 μg) provides 20-fold bronchoprotection and the ultra long-acting beta agonist olodaterol (20 μg) provides 19-fold protection [25,26].

An unanticipated finding was that both LAMA's produced a reduced maximal response to MCh and shifted the MCh dose-response curve to the right compared to the pre-treatment curve. The reduction in maximal response reflects a decrease in what appeared at baseline to be unlimited airway hyperresponsiveness [27]. The rightward shift reflects a reduction in sensitivity to MCh, which is unsurprising given LAMA's function of binding to M_1 and M_3 receptors, thereby preventing the binding of bronchoconstrictors [3,27]. Potential mechanisms behind the decrease in maximal response to MCh include reduced airway smooth muscle contraction and inhibition of mucus secretion responsible for airway wall swelling [27]. Tiotropium has also been found to trigger anti-inflammatory mechanisms in COPD patients [28]. This function may contribute to both the rightward shift and plateau in the MCh dose-response curve, as the airways are less sensitive to stimuli (i.e. lower release of inflammatory mediators) and swelling is reduced. In comparison, ICS also produce a lower maximal response and a rightward shift in the MCh dose-response curve while beta agonists simply produce a rightward shift [29,30].

Overall, the results from this study suggest that, in the absence of objective data, the current guidelines have underestimated the complete duration of clinically significant bronchoprotection by LAMA's and yet still accurately predicted that tiotropium would have a longer duration of effect. It is now questionable whether all LAMA's act roughly equivalently to tiotropium or to glycopyrronium and so, in order to better account for the length and degree of bronchoprotection of this drug class, it would be recommended that all LAMA's be abstained from for a minimum of one week prior to undergoing MCT to avoid false negative or difficult to interpret test results.

Future studies in this area could compare other LAMA's to those tested in this study. In addition, potential reasons for the

differences in effects between tiotropium and glycopyrronium could be further elucidated in order to then determine which may be more suitable as add-on therapy in the treatment of poorly controlled asthma. Studies could also investigate whether long-term, daily use of LAMA's influences the overall duration and degree of bronchoprotection provided by these drugs, as we only investigated single dose effects. An additional aspect of this study to ponder is the dosage of each drug, as the dose of glycopyrronium administered was based on indications for COPD patients. Perhaps a different dosage would be more advisable for asthmatics and may therefore impact the duration of bronchoprotection provided.

Conflicts of interest

The authors do not have any conflicts of interest to declare.

Acknowledgements

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6.2 Appendix B: Certificate of Ethics Approval for LAMA Study

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	15-254

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)
Christianne Blais

FUNDER(S)
INTERNALLY FUNDED

TITLE
Protocol : Duration of Bronchoprotection of the Long-Acting Muscarinic Antagonists Tiotropium & Glycopyrronium against Methacholine-Induced Bronchoconstriction

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
07-Oct-2015	12-Nov-2015	Application for Human Biomedical Research Ethics Approval Protocol (rec'd Sept 23, 2015) Participant Information and Consent form (November 2015)	11-Nov-2016

Delegated Review ☐ Full Board Meeting ☒ Date of Full Board Meeting: 07-Oct-2015

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

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

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

Co-Chair, Vice-Chair or Delegate
University of Saskatchewan
Biomedical Research Ethics Board

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

6.3 Appendix C: LAMA Study Amendment Approval

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval
Study Amendment

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	15-254

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)
Christianne Blais

FUNDER(S)
INTERNALLY FUNDED

TITLE
: Duration of Bronchoprotection of the Long-Acting Muscarinic Antagonists Tiotropium & Glycopyrronium against Methacholine-Induced Bronchoconstriction

APPROVAL OF	APPROVED ON	CURRENT EXPIRY DATE
Recruitment Poster for PAWs, rec'd 14-Dec-2015	16-Dec-2015	11-Nov-2016

Delegated Review ☒ Full Board Meeting ☐

CERTIFICATION
The study is acceptable on scientific and ethical grounds. 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://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION
In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. This approval and the views of this REB have been documented in writing.

Chair, Vice-Chair or Delegate
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

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

6.4 Appendix D: Application for Ethics Approval for LAMA Study

For administrative use only	
File Number:	Date received:

Application for Biomedical Research Ethics Review

PART 1: Identification		
1.1	**Project Title** Duration of bronchoprotection of the long-acting muscarinic antagonists tiotropium & glycopyrronium against methacholine-induced bronchoconstriction Protocol Number (if applicable): Bio-15-254	
1.2	**Principal Investigator** Full Name: Dr. Donald W. Cockcroft Mailing Address: Division of Respirology, Critical Care and Sleep Medicine, Department of Medicine 5th Floor Ellis Hall 103 Hospital Drive Saskatoon, SK S7N 0W8 Email: don.cockcroft@usask.ca Phone: 306-844-1446 NSID number (U of S faculty only): dwc614	
1.3	**University/Institutional Affiliation of Principal Investigator** Position: Professor Department: Medicine Division: Respirology, Critical Care and Sleep Medicine	
1.4	**Project Personnel (including graduates/post graduates/residents)**	
	Full Name: Dr. Beth Davis Project Position/Role: Sub-Investigator University/Institutional Affiliation: Research Scientist for the U of S Department of Medicine, Division of Respirology, Critical Care and Sleep Medicine Email: Phone:	Full Name: Project Position/Role: University/Institutional Affiliation: Email: Phone:

	beth.davis@usask.ca 306-844-1444	
	Full Name: Project Position/Role: University/Institutional Affiliation: Email: Phone:	Full Name: Project Position/Role: University/Institutional Affiliation: Email: Phone:
	If this is a student/graduate/resident project, please provide the following information:	
	a) Student Name: Christianne Blais	b) Supervisor Name: Dr. Cockcroft & Dr. Davis
1.5	**Primary Contact Person for Correspondence (if different than Section 1.2)** Full Name: Dr. Beth Davis Mailing Address: Same as Principal Investigator Email: beth.davis@usask.ca Phone: 306-844-1444	
1.6	**Research Site(s) where project will be carried out:** Room 346 Ellis Hall, University of Saskatchewan	
1.7	**Proposed Project Period:** From (MM/DD/YY) 10/01/2015 To (MM/DD/YY) 04/01/2016	
	Specify any time considerations the REB should be aware of (e.g. short enrolment period): Student Honour's project for the 2015-16 academic year	
1.8	**Has this project applied for/received ethical approval from any other Saskatchewan REB?** ☐ Yes ☒ No If yes, specify where:	
	Has this project applied for/received ethical approval from another Research Ethics Board outside of Saskatchewan? ☐ Yes ☒ No If yes, specify where (if known):	
1.9	**Do you consider this project to involve:** ☐ Minimal Risk ☒ More than Minimal Risk	
1.10	**Provide name of funding source:**	
	Source of Funds: ☐ Industry ☐ National Institute of Health (NIH) ☐ Not-for-Profit Foundation ☐ Cooperative Group (NCIC, COG, RTOG) ☐ Tri-Council Grant ☒ Internally funded ☐ Grant-in-aid	
	Status of Funds: ☒ Awarded ☐ Pending	
1.11	**Name of Sponsor if different from above funding source:**	

PART 2: REGULATORY REQUIREMENTS	
2.1	**If the project involves an investigational drug, natural product, medical device or**

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

☒ N/A – Proceed to Question 2.2

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

Date of approval (MM/DD/YY):
Please forward the NOL and/or ITA to the Research Ethics Office when available.

2.2 **Is there a requirement for this research to comply with United States (OHRP/FDA) regulations for research ethics?**
☐ Yes ☒ No

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

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

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

2.5 According to Good Clinical Practices Section 3.1.2, the Principal Investigator should submit a current curriculum vitae (CV) providing evidence of qualifications to conduct the project. If a CV has not been submitted within last 5 years, please attach. Is the PI's CV attached? ☐ Yes ☒ Not applicable

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

3.1 **Research Question/Hypothesis**
Specify the research question(s) being evaluated in the project.
What is the duration of bronchoprotection of the long-acting muscarinic antagonists (LAMA's) tiotropium and glycopyrronium against methacholine-induced bronchoconstriction? How do they compare to each other? How do they compare to the time lines suggested in the American Thoracic Society Guidelines for Methacholine Challenge Testing?

3.2	**Academic Validity** Provide evidence (scientific literature, pilot projects, etc.) that the scientific reasoning and design of the project are sufficiently sound to meet the objectives of this project. Methacholine challenge testing (MCT) has long served as a common and reliable procedure for measuring airway responsiveness. As such, it can be utilized to determine the extent and duration of bronchoprotection provided by various drugs. The ATS's guidelines for MCT are quite vague as to the duration for which LAMA's should be withheld before the implementation of this testing method. As they stand, the guidelines indicate that such medications must be abstained from for a minimum period of 48 hours, one week in the case of tiotropium, prior to testing. Data to support these time frames is lacking and no reference data is provided that pertains specifically to the two drugs in question.
3.3	**Research Design/Methods** Provide a description of research design (e.g. parallel group or cross-over design) and methods to be used. Include a justification for the use of a placebo, if applicable. Please note that if the analysis or the interpretation of the research results refers to Aboriginal people, language, culture or history as a primary focus of the project, consultation with the appropriate community is required. Please outline the process to be followed. This will be a randomized, double blind, double dummy study. The MCT method used will be the two-minute tidal breathing dosing protocol. Twelve to fifteen subjects will participate, each of which will undergo a total of twelve methacholine (MCh) challenges. Each MCh challenge will be stopped when the subject's FEV_1 drops by 20%, or if the concentration of MCh reaches 256 mg/mL. On the first day of testing, subjects will undergo a MCh challenge to determine their baseline MCh PC_{20}. They will then self-administer the study treatment from two different inhalation devices. One device will contain active LAMA treatment and the other a placebo. The standard dose of tiotropium is two puffs (5µg) from the Respimat® inhaler and that of glycopyrronium is one tablet (50 µg) inhaled from the Breezehaler® dry powder inhaler. The identity of the active drug inhaled will be unknown to the participants and to the study staff. One hour following drug inhalation, the subjects will undergo a second MCh challenge to determine the initial physiological response to the drug through a variation in their MCh PC_{20} compared to their baseline result. The subjects will then each return to the lab at the same time of day for four consecutive days to undergo MCh challenge testing at 24hrs, 48hrs, 72hrs, and 96hrs post-antimuscarinic. After a rest period of one week to allow for drug washout, the same procedure will be followed with the second treatment.
3.4	**Statistical Analysis** Include a summary of the primary and secondary end-points/outcomes, the planned sample size (with justification) and planned statistical and interim analyses. MCh PC_{20} data will first be log-transformed. Comparison of the shift in dose of MCh required to produce the MCh PC_{20} within and between treatments will be done using Student's t-test with the significance set at 0.05. The sample size of 12-15 participants will power the test by over 80% for detecting a half concentration difference in PC_{20} values.
3.5	**Potential Significance/Justification** Explain the significance of the project in order to support the ethical tenet that the proposed research has value (i.e., what are the anticipated public and scientific benefits of the project?). The results of this study will provide data to either confirm or update the current guidelines on

	MCT with regards to the appropriate period of abstinence from LAMA's required before the performance of a MCh challenge. This study will also provide data on the difference in duration of bronchoprotection between two different medications with the same mechanism of action.

PART 4: PARTICIPANT RECRUITMENT	
4.1	**How many participants will be enrolled in the project:** Globally? 0 Locally? 12-15
4.2	**Describe who will be selected (target population) and the criteria for their inclusion.** Individuals 19 years of age or older who have been diagnosed with asthma with a MCh PC_{20} ≤ 4mg/mL (+10%) and who have a baseline lung function (% predicted FEV_1) ≥65% may participate.
4.3	**Describe who will be excluded from participation.** No individual may participate in this study if they have taken any anticholinergic agent within 30 days prior to the beginning of the study. Several contraindications for MCT are known, such as pregnancy and nursing mothers, and cardiovascular problems. Such individuals will therefore be excluded from this study. In addition, individuals with a prostate, kidney or urinary retention problem, or with glaucoma will also be excluded, as these are contraindications for the two LAMAs being administered.
4.4	**Provide a detailed description of the method of recruitment.** a) How will prospective participants be identified? We will offer the study to individuals who have previously participated in research in our lab and who have indicated their willingness to consider participation in future studies where their asthma diagnosis and demographics match the inclusion criteria. b) Who will contact prospective participants? Any of the named study staff/student c) How will this be done? (Ensure that any letters of initial contact or other recruitment materials are attached to this submission (e.g. advertisements, flyers, verbal or telephone script, etc.). Communication will most likely occur by email and if not, then phone contact will be made. If recruitment is slow, an REB-approved ad will be placed on PAWS

PART 5: CONSENT PROCESS	
5.1	**Describe the consent process.** a) Who will ask for consent? Dr. Cockcroft or Dr. Davis. The student researcher may explain and obtain consent provided either Dr. Cockcroft or Dr. Davis is present. b) Where, and under what circumstances? Signing of the consent form will occur in Room 346 Ellis Hall. c) Describe any situation in which the renewal of consent for this research might be appropriate and how this would take place (e.g. Participant turns 18 or emergency situation). No such situation is anticipated. It is understood that re-consent will be required if such a situation presents itself.
5.2	**How long will the participant have to decide whether or not to participate?** If less than twenty-four hours, provide an explanation. At least 24 hours will be given.
5.3	**Will all participants be able to consent on their own behalf?** ☒ Yes ☐ No

	If No, explain why: a) If a participant is unable to consent, who will consent on his/her behalf? b) Will the participant be able to assent to participate? ☐ Yes ☐ No If yes, explain how assent will be sought:
5.4	**If monetary compensation or reimbursements for expenses will be offered to the participants please provide the details.** The participants will be reimbursed $25 per MCh challenge performed.
5.5	**Describe your plans for providing project results to the participant?** Participants are welcome to contact the lab following the completion of the study and the breaking of the randomization code to obtain personal results. They may request a summary of the results and/or provision of any research article regarding the study when (if) available.

PART 6: PROCEDURES AND RISKS	
6.1	**Identify those procedures that are different from the current standard of care (i.e. unique to the research project).** MCT is a commonly used testing method in clinical practice and as such is a standard procedure in our lab. This is not an everyday procedure and in that sense, it is specific to this study.
6.2	**What are the known risks associated with the procedures outlined in Section 6.1? Also include any risks associated with the placebo or wash out periods, if applicable.** MCT may cause wheezing, coughing, mild shortness of breath, chest tightness, dizziness, and/or headaches. However, most people do not experience any symptoms. There are no known risks with the placebo, and the washout period (at least four days) is sufficient in duration to prevent any reaction between test drugs.
6.3	**What strategies will be put in place to minimize and/or manage the potential risk(s) to participants and other affected individuals?** The lab has standard procedures to help minimize/manage risks (e.g. Ventolin is kept on hand to counteract MCh if the need arises). The student researcher will have expert guidance available at all times.
6.4	**For double blind projects, describe the provisions made to break the code in an emergency situation [24 hour availability], and indicate who has the code.** If it is clearly articulated in the clinical protocol, it is acceptable to append the information or provide the protocol page reference. The randomization code will be maintained in a sealed envelope in the lab until all testing is complete. Should an emergency occur where the code must be broken, the envelope will be opened and the treatment administered identified. ☐ N/A, not a double blind project

PART 7: DATA SECURITY AND STORAGE
The *Saskatchewan Health Information Protection Act (HIPA)* requires an assessment of the risks to privacy and how the risks will be minimized. Accessing existing patient information, such as Health Records, requires consent of the individual which must be addressed in the consent form.

<table>
<tr><td>7.1</td><td colspan="2">Indicate from which sources personal and health information data will be collected:
☒ Participant data collected prospectively for the purpose of this project (e.g. case report form)
☐ Family physician record
☐ Heath Region – please specify Region, Site & Dept. if applicable:
☐ SK Ministry of Health
☐ SK Cancer Agency
☐ Other – please specify:
☐ Not applicable (No personal or health information to be collected). Proceed to Section 8.</td></tr>
<tr><td>7.2</td><td colspan="2">How will the confidentiality of participants and their health information be protected?
Subject data will be de-identified by use of study code and initials.</td></tr>
<tr><td>7.3</td><td colspan="2">Describe the storage arrangements and final disposition of the project data collected. All data will be stored in Room 346 Ellis Hall, which has keyed entry.</td></tr>
<tr><td>7.4</td><td colspan="2">List the project personnel who have access to any identifiable personal health information and who will have access to any list that links participant names to their project ID number, consent form, enrolment log, etc.
Principal Investigator, Sub-Investigator and Student Researcher</td></tr>
<tr><td>7.5</td><td colspan="2">Check all applicable boxes below to provide an assessment of the potential privacy risks and the safeguards/solutions that you will put in place to mitigate the risks.</td></tr>
<tr><td></td><td>Potential Privacy Risks</td><td>Possible Safeguards/Solutions (check all that you will use)</td></tr>
<tr><td></td><td>☒ Unauthorized external or internal access to identifying
information through active use or transmission</td><td>☐ Project personnel screening/agreements
☒ Access authorization procedures
☐ Designated systems administrator
☒ Passwords/screen timeouts
☐ System access audits/disclosure logs
☐ Secure mail/transport
☒ Firewall/virus protect
☐ Encrypted transmission</td></tr>
<tr><td></td><td>☐ Identification through publication or release</td><td>☒ Aggregation levels
☒ Alternate identifiers</td></tr>
<tr><td></td><td>☐ Identification through data-matching</td><td>☐ Use of non-linkable elements or identifiers</td></tr>
<tr><td></td><td>☐ Loss of data control outside jurisdiction</td><td>☐ Confidentiality and security agreements for out-of-
province recipients or storage providers</td></tr>
</table>

PART 8: CONFLICT OF INTEREST

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

- Receive personal benefits in connection with this project over and above the direct costs of conducting the project, such as remuneration or employment?

☐ Yes ☒ No

- Receive significant payments of other sorts from the sponsor such as grants, compensation in the form of equipment or supplies or retainers for ongoing consultation and honoraria?

☐ Yes ☒ No

- Have a non-financial relationship with a sponsor (such as unpaid consultant, board membership, advisor or other non-financial interest?

☐ Yes ☒ No

- Have any direct involvement with the sponsor such as stock ownership, stock options or board membership?

☐ Yes ☒ No

- Hold patents, trademarks, copyrights, licensing agreements or intellectual property rights linked in any way to this project or the sponsor?

☐ Yes ☒ No

- Have any other relationship, financial or non-financial, that if not disclosed, could be construed as a conflict of interest?

☐ Yes ☒ No

If yes, please describe the personal benefits or relationship.

PART 9: Declaration by Principal Investigator
(or Supervisor for student projects)

Project Title:
Duration of bronchoprotection of the long-acting muscarinic antagonists tiotropium & glycopyrronium against methacholine-induced bronchoconstriction

- I confirm that the information provided in this application is complete and correct.
- I accept responsibility for the ethical conduct of this project and for the protection of the rights and welfare of the human participants who are directly or indirectly involved in this project.
- I will comply with all policies and guidelines of the University and Health Region/affiliated institutions where this project will be conducted, as well as with all applicable federal and provincial laws regarding the protection of human participants in research.
- I will ensure that project personnel are qualified, appropriately trained and will adhere to the provisions of the REB-approved application.
- I will ensure that any significant changes to the project, including the proposed method, consent process or recruitment procedures, will be reported to the Research Ethics Board for consideration in advance of its implementation.
- I will ensure that a status report will be submitted to the Research Ethics Board for consideration within one month of the current expiry date each year the project remains open,

and upon project completion.

- If personal health information is requested, I assure that it is the minimum necessary to meet the research objective and will not be reused or disclosed to any parties other than those described in the REB-approved application, except as required by law.
- I confirm that adequate resources to protect participants (i.e., personnel, funding, time, equipment and space) are in place *before* implementing the research project, and that the research will *stop* if adequate resources become unavailable.
- I understand that if the contract or grant related to this research project is being reviewed by the University or Health Region, a copy of the ethics application inclusive of the consent document(s), may be forwarded to the person responsible for the review of the contract or grant.
- I understand that if the project involves Health Region resources or facilities, a copy of the ethics application may be forwarded to the Health Region research coordinator to facilitate operational approval.

_____ _____ _____

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

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

_____ _____ _____

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

PART 11: ATTACHMENTS		
Provide a full and accurate listing of all documents submitted with this application. All projects requiring the use of RQHR resources must complete this section.		
Document	**Included?**	**Comments**
Certificate of Approval from another REB	☐ Yes ☒ N/A	

Peer Review reports	☐ Yes ☒ N/A	
Participant Consent Form	☒ Yes ☐ N/A	
Control Participant Consent Form	☐ Yes ☒ N/A	
Assent Form	☐ Yes ☒ N/A	
Tissue/Blood Banking Consent Form	☐ Yes ☒ N/A	
Letter of Initial Contact	☐ Yes ☒ N/A	
Advertisement to Recruit Participants	☐ Yes ☒ N/A	
Questionnaires, tests, interview scripts, etc.	☐ Yes ☒ N/A	
Other- please specify:	☐ Yes ☐ N/A	
Other- please specify:	☐ Yes ☐ N/A	

6.5 Appendix E: LAMA Study Participant Consent Form

PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Duration of bronchoprotection of the long-acting muscarinic antagonists tiotropium & glycopyrronium against methacholine-induced bronchoconstriction

Principal Investigator: Dr. D.W. Cockcroft,
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1446 don.cockcroft@usask.ca

Sub-Investigator: Dr. Beth Davis,
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1444 beth.davis@usask.ca

Student Researcher: Christianne Blais (PHPY 432 Student)
Department of Physiology
University of Saskatchewan
306-480-8983 cmb831@mail.usask.ca
Supervised by the Principal Investigator and Sub-Investigator

24 hour contact: Dr. Beth Davis 306-229-8709

Introduction: You are invited to participate in this research study, as your asthma diagnosis, age and health status make you a suitable representative of the population of interest.
Your participation is entirely voluntary and you may withdraw from the study at any time without providing a reason for doing so. If you do not wish to participate or if you choose to withdraw from the study, your current health care and/or academic status (if you are a student at the U of S) will not be impacted.
Please take time to read the following information carefully. You may direct any questions or concerns to the study staff prior to and throughout the duration of the study.

Agencies Contributing Funds, Resources, and Drugs to the Study: The study will be internally funded. Novartis Pharmaceuticals Canada Inc. will be providing the active drug Seebri®, placebos, and Breezehaler® inhalation devices. Boehringer Ingelheim Pharmaceuticals Inc. will be providing the active drug Spiriva®, placebos, and Respimat® inhalation devices. The researchers and the University of Saskatchewan are not being paid to conduct this study.

Purpose of Study: Bronchodilators are medications that can improve breathing in those who are experiencing difficulty (e.g. an individual with asthma). Ventolin® is an example of a bronchodilator/inhaler that is commonly used. There are other bronchodilator drugs, including drugs that can block muscarinic receptors. When these receptors are activated, the airway smooth muscle contracts. This results in bronchoconstriction and difficulty breathing. Therefore, by blocking these receptors, breathing is improved. Treatments called "long-acting muscarinic antagonists" (LAMA's), including Spiriva®(tiotropium bromide monohydrate) and Seebri®(glycopyrronium bromide) are two such medications. The main purpose of this study is to determine how long each medication will block the effect of inhaled methacholine (MCh), a known bronchoconstrictor. Methacholine challenge testing is a common procedure performed in clinical practice to confirm or refute an asthma diagnosis. Currently, the American Thoracic Society's (ATS's) Guidelines for Methacholine and Exercise Challenge Testing do not contain data on the duration of bronchoprotection provided by LAMA's, such as tiotropium and glyropyrronium. The guidelines simply recommend 48 hours of abstinence from LAMA's, one week in the case of tiotropium, prior to MCh challenge testing. Our results will provide objective data that may influence the recommended guidelines for conducting MCh challenges. We plan to recruit twelve to fifteen participants.

Eligible Participants: To be eligible to participate in this study, you must be 19 years of age or older. You must also have been diagnosed with asthma and have a current MCh PC_{20} [concentration of MCh causing a 20% reduction in forced expiratory volume in one second (FEV_1)] $\leq$ 4mg/mL (+10%) (explained in more detail on the next page). You must also have sufficient baseline lung function. For this study, the amount of air you can forcefully exhale during the first second of exhalation (FEV_1) must be at least 65% of what is predicted for your age, height and gender. You may not participate in this study if you have taken any anticholinergic agent within 30 days prior to the beginning of the study. If you are taking corticosteroids on a daily basis, you may participate in the study provided you continue to take your usual dose. Bronchodilator therapy (e.g. Ventolin®, bricanyl) is allowed but must be withheld for at least six hours prior to all visits. Use of longer-acting beta agonist bronchodilators will not be allowed. Due to contraindications for the two LAMA's and for the testing method, you may not participate in this study if you are pregnant or nursing, if you have cardiovascular, prostate, kidney or urinary retention problems, and/or if you have glaucoma.

Study Overview/Visit Schedule: You will be required to attend the laboratory on twelve occasions, six for each treatment period. For example, on a Monday, you will be required to undergo MCh challenge testing (the procedure described below), following which you will be provided with two inhalers (one Respimat® and one Breezehaler®). One inhaler will contain an active LAMA (either Spiriva® or Seebri®) and the other will contain a placebo. Neither you nor the study staff will know which inhaler contains the active treatment, although this information will be available if necessary. You will inhale two puffs from the Respimat® inhaler and one puff from the Breezehaler®. You will then wait for one hour before undergoing a second MCh challenge. This visit will take between 2.5 and 3 hours. You will then be required to return to the lab at the same time once a day for four consecutive days (days 2-5). On each day you will undergo a single MCh challenge, which will take between 1 and 2 hours. You will then return to the lab on Monday (day 7 post-treatment) for a final methacholine challenge. During each MCh challenge, we will also ask you to rate your perceived level of airway constriction/discomfort on

a scale of 0-10 (0 being no feeling of constriction and 10 being level of constriction is extremely uncomfortable).
Following one week of rest to allow medication washout, you will be required to follow the same schedule with the other treatment. The order in which you receive the treatments will be random. The data collected will then allow for comparison of the duration of action of each drug in relation to the ATS guidelines.

MCh Challenge: MCh challenge testing involves the inhalation of aerosolized MCh through a loose-fitting mask for a period of two minutes. During this time, your nose will be plugged with a noseclip and you will be asked to breathe normally. MCh is a bronchoconstrictor and may cause your airways to constrict. Following the inhalation of MCh, you will be required to inhale deeply and exhale as forcefully as you can into a spirometer, which is a handheld device connected to a computer that allows for the measurement of your lung capacity. This is referred to as spirometry or lung function testing. We will repeat this procedure with increasing concentrations of MCh until the amount of air you can forcefully exhale is decreased by 30% or the maximal concentration of methacholine (256mg/mL) is inhaled. Ventolin® (albuterol) will be kept on hand in the case of an emergency.

Responsibilities of The Participant: As a participant of this study, you will be expected to (1) follow the directions of the investigators and researchers, and (2) report all medications (prescribed, over-the-counter, and herbal products) being used or that you plan on taking prior to and throughout the duration of the study. This step is important both for your own safety as well for accurate interpretation of the test results. If a situation arises where you must use a medication that will interfere with the study, we ask that you take the medication and contact us to reschedule your appointment.

Benefits of Participating in This Study: If you choose to participate in this study, know there will be no benefit for you personally. However, your participation will help increase knowledge on the effective use of MCT as an asthma diagnostic tool, where current data is lacking.

Honorarium: If you choose to participate in this study, you will not be charged for the study drugs or any research-related procedures. Although you will not be paid for your participation, you will be provided with an honorarium in the amount of $25 per MCh challenge ($350 total) to cover your time and out-of-pocket expenses (e.g. parking, meals). Should you choose to withdraw early from this study, you will be compensated according to the number of MCh challenge tests to which you participated. The University of Saskatchewan will require your social insurance number and will send you a T4A slip at the appropriate time.

Potential Risks and Discomforts: MCh challenge testing may cause the following symptoms: wheezing, coughing, mild shortness of breath, chest tightness, dizziness, and/or headaches. The effects of MCh can be treated with inhaled salbutamol or will subside untreated.
Some common side effects (>1% of patients) of Spiriva® (tiotropium bromide monohydrate) include: dry mouth (4.1%), cold symptoms (2.7-15.9%), and headaches (3.8%). Less common (0.5-1% of patients) include stomach aches, muscle pain, and constipation.
Some side effects of Seebri® (glycopyrronium bromide) experienced in 1-10% of patients include dry mouth, headaches, insomnia, cold symptoms and stomach pains.

Voluntary Withdrawal: Your participation in this study is voluntary and as such, you may withdraw from the study at any time. No reason needs to be given for your withdrawal, and your current health care and/or academic status will not be impacted in any way. The data collected about you until the point of withdrawal will be retained for analysis.
You will be notified if new information regarding the drugs or procedure involved becomes available during the course of the study that may affect your willingness to continue to participate.

Study-Related Injury: In the case of a medical emergency related to the study, you should seek immediate care and, as soon as possible, notify the study staff. Inform the medical staff you are participating in a clinical study. Necessary medical treatment will be made available at no cost to you. By signing this document, you do not waive any of your legal rights against the investigators or anyone else.

Confidentiality: In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. Your name will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team. However, no guarantee can be made for complete confidentiality. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.
Your study records will be kept for at least 5 years in a secured area (Room 346 Ellis Hall). This is a locked room when unattended. After storage, your study records will be shredded in a confidential manner.

Contact Regarding Any Questions/Concerns About The Study: If you have any questions or concerns regarding this study before or during participation, you may contact Dr. Cockcroft at 306-844-1446 or Dr. Davis at 306-844-1444.
If you have any concerns about your rights as a research participant and/or your experiences while participating in this study, contact the Chair of the University of Saskatchewan Research Ethics Board at 306-966-2975. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Consent

- I have read the information in this consent form.
- I understand the purpose, procedure and possible risks of the study.
- I have had the opportunity to ask questions and have received satisfactory answers.
- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care or academic status.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Printed name of participant	Date
Signature of participant	Phone #
Printed name of person obtaining consent	Date
Signature of person obtaining consent	

6.6 Appendix F: Certificate of Ethics Approval for LAMA/uLABA Study

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	16-205

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

STUDENT RESEARCHER(S)
Christianne Blais

FUNDER(S)
INTERNALLY FUNDED

TITLE
The Effect of Glyopyrronium and Indacaterol, as Monotherapy and in Combination, on the Methacholine Dose-Response Curve

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
17-Aug-2016	15-Sep-2016	Master of Science Protocol (rec'd July 25, 2016) Notice of Ethical Review response (rec'd September 13, 2016) Asthmatic Participant Information and Consent Form (revised September 13, 2016) Non-Asthmatic Participant Information and Consent Form (revised September 13, 2016) Participant Recruitment Poster for PAWs (asthmatics) (revised September 13, 2016) For Acknowledgement: Application for Biomedical Research Ethics Review (revised September 13, 2016)	14-Sep-2017

Delegated Review ☐ Full Board Meeting ☒ Date of Full Board Meeting: 17-Aug-2016 (REB 1 & REB 2)

IRB 1 FWA Registration #00001471 ☐ IRB 2 FWA Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The study is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

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

Please send all correspondence to:

Research Services and Ethics Office
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Donald W. Cockcroft	Medicine (Respirology)	16-205

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board has been approved by the Minister of Health, Province of Saskatchewan, to serve as a Research Ethics Board (REB) for research studies involving human participants under section 29 of The Health Information Protection Act (HIPA).

Gordon McKay, PhD., Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

6.7 Appendix G: Application for Ethics Approval for LAMA/uLABA Study

For administrative use only	
File Number:	Date received:

Application for Biomedical Research Ethics Review

PART 1: IDENTIFICATION		
1.1	**Project Title** GN 1.1 The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve Protocol Number (if applicable): Bio 16-205	
1.2	**Principal Investigator** GN 1.2 Full Name: Dr. Donald W. Cockcroft Mailing Address: Division of Respirology, Criticial Care and Sleep Medicine, University of Saskatchewan, 546 Ellis Hall, 103 Hospital Drive, Saskatoon, SK S7N 0W8 Email: don.cockcroft@usask.ca Phone: 306-844-1446 NSID number (U of S faculty only): dwc614	
1.3	**University/Institutional Affiliation of Principal Investigator** GN 1.3 Position: Professor Department: Medicine Division: Respirology, Critical Care and Sleep Medicine	
1.4	**Project Personnel (including graduates/post graduates/residents)** GN 1.4	
	Full Name: Dr. Beth E. Davis Project Position/Role: Research Supervisor/Co-Investigator University/Institutional Affiliation: University of Saskatchewan Email: beth.davis@usask.ca Phone: 306-844-1444 NSID number (U of S only):	Full Name: Christianne Blais Project Position/Role: Master's Student Researcher University/Institutional Affiliation: University of Saskatchewan Email: cmb831@mail.usask.ca Phone: **306-480-8983** NSID number (U of S only): **cmb831**
	Full Name: Project Position/Role: University/Institutional Affiliation: Email: Phone: NSID number (U of S only):	Full Name: Project Position/Role: University/Institutional Affiliation: Email: Phone: NSID number (U of S only):

	If this is a student/graduate/resident project, please provide the following information:	
	a) Student Name: Christianne Blais NSID number (U of S only): cmb831	b) Supervisor Name: Dr. Cockcroft and Dr. Davis
1.5	**Primary Contact Person for Correspondence (if different than Section 1.2)** GN 1.5 Full Name: Christianne Blais Mailing Address: Room 346 Ellis Hall, University of Saskatchewan, 103 Hospital Drive, Saskatoon SK. S7N 0W8 Email: cmb831@mail.usask.ca Phone: 306-480-8983	
1.6	**Research Site(s) where project will be carried out:** GN 1.6 Room 346 Ellis Hall, University of Saskatchewan	
1.7	**Proposed Project Period:** GN 1.7 From (MM/DD/YY) 08/01/2016 To (MM/DD/YY) 08/31/2017	
	Specify any time considerations the REB should be aware of (e.g. short enrolment period): Student One-Year Master's Project (must be completed by Summer 2017)	
1.8	**Has this project applied for/received ethical approval from any other Saskatchewan REB?** GN 1.8 ☐ Yes ☒ No If yes, specify where:	
	Has this project applied for/received ethical approval from another Research Ethics Board outside of Saskatchewan? ☐ Yes ☒ No If yes, specify where (if known):	
1.9	**Do you consider this project to involve:** GN 1.9 ☐ Minimal Risk ☒ More than Minimal Risk	
1.10	**Provide name of funding source:** GN 1.10 Internal Funds	
	Source of Funds: ☐ Industry ☐ National Institute of Health (NIH) ☐ Not-for-Profit Foundation ☐ Cooperative Group (NCIC, COG, RTOG) ☐ Tri-Council Grant ☒ Internally funded ☐ Grant-in-aid	
	Status of Funds: GN 1.11 ☒ Awarded ☐ Pending	
1.11	**Name of Sponsor if different from above funding source:** GN 1. 12	

PART 2: REGULATORY REQUIREMENTS

2.1	**If the project involves an investigational drug, natural product, medical device or marketed drug/device being used outside of the approved indication, check whether or not the No Objection Letter (NOL) or the Investigational Testing Authorization (for devices) has been obtained from the appropriate Health Canada regulatory agency.** GN 2.1 ☒ N/A – Proceed to Question 2.2			
		Yes	**Pending**	**N/A**

Therapeutic Products Directorate (TPD)	☐	☐	☐
Natural Health Products Directorate (NHPD)	☐	☐	☐
Biologics and Genetics Therapies Directorate (BGTD)	☐	☐	☐
Investigational Testing Authorization (ITA)	☐	☐	☐

Date of approval (MM/DD/YY):

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

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

☐ Yes ☒ No

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

2.4 **Academic Validity** GN 2.4

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

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

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

Is the PI's CV attached? ☐ Yes ☒ Not applicable

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

3.1 **Research Question/Hypothesis** GN 3.1

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

How do glycopyrronium and indacaterol, as monotherapy and in combination, shift the methacholine (MCh) dose-response curve (DRC)? What is the duration and degree of bronchoprotection provided by each treatment?

3.2 **Academic Validity** GN 3.2

Provide evidence (scientific literature, pilot projects, etc.) that the scientific reasoning and design of the project are sufficiently sound to meet the objectives of this project.

Methacholine challenge testing (MCT) is a common and reliable procedure for measuring airway responsiveness. The airway hyperresponsiveness component of asthma has been shown to result from increased sensitivity to bronchoconstrictors and an increased maximal constricting response to said stimuli. These effects can be visualized on a MCh DRC; a leftward shift symbolizes increased sensitivity (i.e. less stimuli needed to generate a constricting response due to factors like epithelial damage, increased inflammatory cell activity and abnormal autonomic activity); the loss or elevation of a plateau represents a heightened maximal response (i.e. excessive hyperresponsiveness due to factors like increased smooth muscle contractility, a reduction in mechanical loads, intraluminal secretions or swelling of the airways); a steeper slope results from increased reactivity (i.e. increased risk of severe exacerbation in response to a

	stimulus). The representation of a drug's effect on the MCh DRC could not only help to characterize the impact of the drug on the airways, but could also potentially serve as a tool for determining the best treatment for a patient based on a comparison of their baseline MCh DRC to that of a non-asthmatic. So far, it has been shown that inhaled corticosteroids (ICS) produce a righward shift and often a lower plateau (i.e. reduced maximal response). Beta-2 agonists typically only shift the curve rightward. In one of our previous studies, it was found that long-acting muscarinic antagonists (LAMAs) such as glycopyrronium and tiotropium seem to produce a rightward shift and lower plateau. No MCh DRC has been generated for ultra long-acting beta agonists (uLABAs) such as indacaterol as of yet. Given the widespread use of MCT in clinics and in research, it is important that its accuracy be maintained in order to minimize the likelihood of generating false negative results. The determination of the degree and duration of bronchoprotection against MCh provided by each drug will inform standardization guidelines on the required washout timeframes for these treatments prior to undergoing pulmonary function and MCT.
3.3	**Research Design/Methods** GN 3.3 Provide a description of research design (e.g. parallel group or cross-over design) and methods to be used. Include a justification for the use of a placebo, if applicable. Please note that if the analysis or the interpretation of the research results refers to Aboriginal people, language, culture or history as a primary focus of the project, consultation with the appropriate community is required. Please outline the process to be followed. This will be a randomized, double-blind, three-way crossover study testing glycopyrronium (Seebri®) vs. indacaterol (Onbrez®) vs. glycopyrronium and indacaterol. The MCT method used will be the two-minute tidal breathing dosing protocol. We plan to enroll 30 participants, 15 asthmatic and 15 non-asthmatic participants. Asthmatic Participants Procedure: Each participant will undergo a total of 12 MCh challenges. Each MCh challenge will be stopped when a plateau is reached, when the subject's forced expiratory volume in one second (FEV1) drops 40% from baseline, when post-treatment the concentration of MCh administered reaches 128mg/mL, or if the participant wishes to stop for any reason (e.g. discomfort). For the purpose of this study, a plateau will be defined as the last 3 consecutive data points falling within 5% of each other. The first day of testing will take approx. 3hrs. and will entail participants undergoing a MCh challenge to determine their baseline MCh provocative concentration causing a 20% fall in FEV1 (PC20). They will then inhale the contents of two Breezhaler® devices. For each monotherapy treatment arm, one inhaler will contain one of the two active drugs while the other device will contain a placebo capsule (i.e. inhalation of 50µg glycopyrronium and a placebo, or 75µg indacaterol and a placebo). For the combination therapy arm, each device will contain active treatment (i.e. inhalation of 50µg glycopyrronium and 75µg indacaterol)*. The identity and order of the treatments administered will be unknown to the participants and to the study staff. The blinding of the study treatments will be completed by the local pharmacy. Participants will then undergo MCT at 1 hr., 24 hrs., and 48 hrs. post-treatment. Testing at 24 and 48 hrs. should take approx. 1-1.5 hrs. Therefore, participants must attend the lab at roughly the same time of day (±1 hr.) for 3 consecutive days for each treatment arm. Following a minimum 10-day washout between treatment administrations, the same procedure will be repeated with the second study treatment. Following a second 10-day washout, the procedure will be repeated with the third study treatment. A table summarizing the study schedule for asthmatic participants can be found in the consent form.

	*Protocol in the event of coughing post-dosing: The participant will be advised to cough before undergoing drug inhalation to relieve any potential mucus blockage. It will be noted if the participant coughs following drug inhalation, as this could lead to exhalation of some of the medication. Non-Asthmatic Participant Procedure: Each non-asthmatic participant will undergo a single MCh challenge, which should take approx. 1 hr. This will allow for the generation of a "normal" MCh DRC to compare with the asthmatic data. The MCh challenge will be stopped when the response to MCh reaches a plateau, when the participant's FEV1 drops by 40% from baseline, when the highest concentration of MCh (128mg/mL) has been administered, or if the participant wishes to stop for whatever reason. Methacholine Challenge: MCT involves the inhalation of aerosolized MCh, a known bronchoconstrictor that may cause the airway to constrict. The participant will inhale MCh through a loose-fitting mask for a period of two minutes. During the inhalation period, their nose will be plugged with a nose clip and they will be asked to breathe normally. Following inhalation of MCh, they will be required to inhale sharply and exhale as forcefully as they can into a spirometer in order to measure their lung capacity (i.e. spirometry). We will then repeat this procedure with increasing concentrations of MCh until one of the pre-defined stops is reached (i.e. plateau, 40% reduction in FEV1, max MCh administered, or participant choice). Asthmatic participants will be given salbutamol following each MCh challenge, except after the "baseline" or un-medicated MCh challenge prior to each treatment; following these challenges, they will be given one of the study medications instead. If non-asthmatic participants show a response to MCh, they will be given the option of taking salbutamol for quick reversal of the effects of MCh.
3.4	**Statistical Analysis** GN 3.4 Include a summary of the primary and secondary end-points/outcomes, the planned sample size (with justification) and planned statistical and interim analyses. MCh PC20 data will be log-transformed. Bronchoprotection will be assessed as the shift in MCh PC20 from baseline [dose shift= (ΔlogMCh PC20)/0.3]. Comparison of the shift in dose of MCh required to produce the PC20 within and between treatments will be done using Student's t-test and will be illustrated in the form of MCh DRCs. Significance will be set at 0.05. The sample size of 15 participants will power the test by over 95% for detecting a half concentration difference in PC20 values. (The 15 non-asthmatics are only being used to generate a "normal" MCh DRC, no statistical analysis)
3.5	**Potential Significance/Justification** GN 3.5 Explain the significance of the project in order to support the ethical tenet that the proposed research has value (i.e., what are the anticipated public and scientific benefits of the project?). The results from this study will allow for a better understanding of the mechanisms behind asthma and how a uLABA, a LAMA, and a combo uLABA+LAMA influence the airways of asthmatics. The data will also either confirm or update the current guidelines on MCT with regards to the appropriate period of abstinence from each treatment prior to a MCh challenge.

PART 4: PARTICIPANT RECRUITMENT	
4.1	**How many participants will be enrolled in the project:** GN 4.1 Globally? 0 Locally? 30
4.2	**Describe who will be selected (target population) and the criteria for their inclusion.** GN 4.2 Individuals may participate if they are 18 years of age or older. Eligible asthmatic participants must be diagnosed with mild-

	to-moderate asthma, must have a MCh PC20 less than or equal to 8mg/mL, and must have a baseline lung function (% predicted FEV1) greater than or equal to 65%. If they have not previously undergone testing in the Asthma Research Lab, these criteria will be confirmed during their first study visit with baseline testing. Eligible nonasthmatic participants must have a negative MCh PC20 (greater than 16mg/mL).
4.3	**Describe who will be excluded from participation.** GN 4.3 Individuals may not participate if they have taken any anticholinergic agent, any long-acting beta agonist, or any combination therapy within 30 days prior to beginning the study. Due to contraindications for MCT, pregnant and/or nursing mothers, and those with cardiovascular problems will be excluded. Due to contraindications for the study treatments, individuals with a prostate, kidney or urinary retention problem, hypokalemia, diabetes, or with glaucoma will be excluded. Eligible participants cannot have suffered from a respiratory infection or an asthma attack from allergen exposure within 4 weeks prior to beginning the study.
4.4	**Provide a detailed description of the method of recruitment.** GN 4.4 a) How will prospective participants be identified? Non-asthmatic candidates will be recruited through PAWS (i.e. no other form of advertisement will be used to recruit them). We do not have current clinic research records of non-asthmatics. Asthmatics will first be recruited from our research records (i.e. participants from previous studies that match the inclusion criteria) and, if enrolment is slow, a PAWS announcement will be made to recruit asthmatic participants. b) Who will contact prospective participants? Any of the named study staff/student c) How will this be done? (Ensure that any letters of initial contact or other recruitment materials are attached to this submission (e.g. advertisements, flyers, verbal or telephone script, etc.). Communication will likely occur by email and if not, then phone contact will be made. If recruitment is slow, an REB-approved ad will be placed on PAWS.

PART 5: CONSENT PROCESS	
5.1	**Describe the consent process.** GN 5.1 a) Who will ask for consent? Dr. Davis or the student researcher (provided that Dr. Davis is present) b) Where, and under what circumstances? Signing of the consent form will occur in Room 346 Ellis Hall c) Describe any situation in which the renewal of consent for this research might be appropriate and how this would take place (e.g. Participant turns 18 or emergency situation). No such situation is anticipated. It is understood that re-consent will be required if such a situation presents itself.
5.2	**How long will the participant have to decide whether or not to participate?** If less than twenty-four hours, provide an explanation. GN 5.2 Participants will be allowed as much time as they need to make a decision. Knowledgeable participants will be allowed to consent in less than 24 hours. Knowledgeable participants are those who have conducted several methacholine challenge studies in our lab in the past. Such participants are therefore well acquainted with the procedures performed and the risks involved. These participants will still be given the option of reviewing the consent form over at least 24 hours prior to enrolling if they so desire. The anticipated condensed timeframe would be less than one hour for such participants. Following a discussion of the study purpose and procedure during the first testing visit (which is standard procedure in our lab with each participant for every study), knowledgeable participants will still be able to reschedule if they wish to take more time to consider the study and consent.

5.3	**Will all participants be able to consent on their own behalf?** GN 5.3 ☒ Yes ☐ No If No, explain why: a) If a participant is unable to consent, who will consent on his/her behalf? b) Will the participant be able to assent to participate? ☐ Yes ☐ No If yes, explain how assent will be sought:
5.4	**If monetary compensation or reimbursements for expenses will be offered to the participants please provide the details.** GN 5.4 The participants will be reimbursed $25 per MCh challenge performed.
5.5	**Describe your plans for providing project results to the participant?** GN 5.5 Participants will be instructed to contact the lab following the completion of the study and the breaking of the randomization code to obtain personal results. They may request a summary of the results and/or provision of any research article regarding the study when (if) available. The appropriate correspondance email to make such requests is provided in the consent form.

PART 6: PROCEDURES AND RISKS	
6.1	**Identify those procedures that are different from the current standard of care (i.e. unique to the research project).** GN 6.1 Typically, MCT is performed until a concentration of MCh produces a 20% fall in FEV1. This study proposes performing this procedure until a 40% fall in FEV1 is experienced in order to observe the formation of a plateau in response to the study medications. This is not uncommon for this type of investigation; previous studies that have examined the influence of anti-asthma medications on the MCh DRC have followed participants to a 40-60% fall in FEV1.
6.2	**What are the known risks associated with the procedures outlined in Section 6.1? Also include any risks associated with the placebo or wash out periods, if applicable.** GN 6.2 MCT may cause wheezing, coughing, mild shortness of breath, dizziness, and/or headaches. However, most people do not experience any symptoms. The washout period (at least ten days between treatment administrations) is sufficient in duration to prevent any reaction between test drugs.
6.3	**What strategies will be put in place to minimize and/or manage the potential risk(s) to participants and other affected individuals?** GN 6.3 The lab has standard procedures to help minimize/manage risks (e.g. salbutamol is kept on hand to counteract MCh if the need arises, as the effects of MCh can be quickly reversed with this bronchodilator). The student researcher will have expert guidance available at all times (i.e. Dr. Davis and/or Dr. Cockcroft will be available to help during testing, in case the need arises).
6.4	**For double blind projects, describe the provisions made to break the code in an emergency situation [24 hour availability], and indicate who has the code.** If it is clearly articulated in the clinical protocol, it is acceptable to append the information or provide the protocol page reference. The randomization code will be maintained in a sealed envelope in the lab until all testing is complete. Should an emergency occur where the code must be broken, the envelope will be opened and the treatment administered identified. ☐ N/A, not a double blind project

PART 7: DATA SECURITY AND STORAGE GN 7.1

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

7.1 **Indicate from which sources personal and health information data will be collected:** GN 7.1

- ☒ Participant data collected prospectively for the purpose of this project (e.g. case report form)
- ☐ Family physician record
- ☐ Heath Region – please specify Region, Site & Dept. if applicable:
- ☐ SK Ministry of Health
- ☐ SK Cancer Agency
- ☐ Other – please specify:
- ☐ Not applicable (No personal or health information to be collected). Proceed to Section 8.

7.2 **How will the confidentiality of participants and their health information be protected?** GN 7.2 Subject data will be de-identified by use of study codes (i.e. DRC01 for participant 1, etc.). However, no full guarantee can be made that their confidentiality will be protected.

7.3 **Describe the storage arrangements and final disposition of the project data collected.** GN 7.3 All data will be stored in Room 346 Ellis Hall (which has keyed entry) for five years following the publication of the manuscript and thesis stemming from this study. All data will be shredded after this time.

7.4 **List the project personnel who have access to any identifiable personal health information and who will have access to any list that links participant names to their project ID number, consent form, enrolment log, etc.** GN 7.4 Dr. Cockcroft, Dr. Davis, Christianne Blais, a summer student researcher (Yifan Han, until 08/31/2016) and an undergraduate honours student (Samuel Simonson). The latter two students have access in the sense that they can access the lab, but there is no foreseeable need for either to access the study files or view participant information.

7.5 **Check all applicable boxes below to provide an assessment of the potential privacy risks and the safeguards/solutions that you will put in place to mitigate the risks.** GN 7.5

Potential Privacy Risks	Possible Safeguards/Solutions (check all that you will use)
☒ Unauthorized external or internal access to identifying information through active use or transmission	☐ Project personnel screening/agreements ☒ Access authorization procedures ☐ Designated systems administrator ☒ Passwords/screen timeouts ☐ System access audits/disclosure logs ☐ Secure mail/transport ☒ Firewall/virus protect ☐ Encrypted transmission
☐ Identification through publication or release	☒ Aggregation levels ☒ Alternate identifiers

	☐ Identification through data-matching	☐ Use of non-linkable elements or identifiers
	☐ Loss of data control outside jurisdiction	☐ Confidentiality and security agreements for out-of-province recipients or storage providers

PART 8: CONFLICT OF INTEREST

8.1 **Is there any real or perceived conflict of interest (any personal or financial interest in the conduct or outcome of this project)?** GN 8.1 **Will any of the researcher(s), members of the research team and/or their immediate family members:**

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

  If yes, please describe the personal benefits or relationship.

PART 9: DECLARATION BY PRINCIPAL INVESTIGATOR GN 9.1

(OR SUPERVISOR FOR STUDENT PROJECTS)

Project Title:

The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve

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

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

Department Head: The signature/approval of the Department/Administrative Unit acknowledges that he/she is aware of and supports the research activity described in the proposal.

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

PART 11: ATTACHMENTS

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

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

Document	Included?	Comments
Certificate of Approval from another REB	☐ Yes ☒ N/A	
Peer Review reports	☐ Yes ☒ N/A	
Participant Consent Form	☒ Yes ☐ N/A	Two consent forms are attached, one for asthmatic participants and one for non-asthmatic participants
Control Participant Consent Form	☐ Yes ☒ N/A	
Assent Form	☐ Yes ☒ N/A	
Tissue/Blood Banking Consent Form	☐ Yes ☒ N/A	
Letter of Initial Contact	☐ Yes ☒ N/A	
Advertisement to Recruit Participants	☒ Yes ☐ N/A	
Questionnaires, tests, interview scripts, etc.	☐ Yes ☒ N/A	
Other- please specify: Study Protocol	☒ Yes ☐ N/A	
Other- please specify:	☐ Yes ☐ N/A	

6.8 Appendix H: LAMA/uLABA Study Asthmatic Participants Consent Form

Asthmatic Participant Information and Consent Form

Title of Study: The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve

Principal Investigator: Dr. Donald Cockcroft MD FRCP
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1446

Sub-Investigators: Dr. Beth Davis, PhD
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1444

Master's Student Researcher: Christianne Blais, B.Sc. hon.
Department of Physiology
University of Saskatchewan
306-480-8983 cmb831@mail.usask.ca

24-Hour Emergency Contact: In the event of an emergency (i.e. serious side effects), seek immediate medical attention (which will be provided at no additional cost to you). When able to do so, please notify the study staff of the incident (Christianne Blais at 306-480-8983). If you need to cancel or reschedule a lab visit, please call, text, or email Christianne (cmb831@mail.usask.ca).

Introduction: You are invited to participate in this research study, as your asthma diagnosis (mild-to-moderate asthma), age (at least 18 years of age) and health status (controlled, stable asthma) make you a suitable representative of the population of interest.

Your participation is entirely voluntary and you may withdraw from the study at any time without providing a reason for doing so. If you do not wish to participate or if you choose to withdraw from the study, your current health care and/or academic status (if you are a student at the U of S) will not be impacted.

Please take time to read the following information carefully. You may direct any questions or concerns to the study staff prior to and at any time during the study. Please feel free to discuss

the study and your potential participation with friends, family, and/or your personal healthcare provider.

Agencies Contributing Funds and Resources to the Study: The study will be internally funded (i.e. the asthma research lab will be managing the cost of the study without financial aid from a third party organization). Novartis Pharmaceuticals Inc. will be providing the active drugs Seebri® and Onbrez®, matching placebos, and the Breezhaler® inhalation devices. The researchers and the University of Saskatchewan are not being paid to conduct this study.

Purpose of Study: Bronchodilators are medications that can improve breathing in those who are experiencing difficulty (e.g. an individual with asthma). Ventolin® is an example of a bronchodilator/inhaler that is commonly used. There are other bronchodilator drugs that act through different mechanisms to open the airways and provide relief from asthma symptoms. Three medications are being tested in this study: indacaterol (Onbrez®, an ultra long-acting beta agonist), glycopyrronium (Seebri®, a long-acting muscarinic antagonist), and a combination of indacaterol and glycopyrronium. Indacaterol acts directly on beta-2 receptors in the airways to relax the airway smooth muscle, which facilitates breathing. Glycopyrronium works differently as it blocks airway muscarinic receptors, which when activated produce airway constriction and difficulty breathing.

In order to observe the effects of these medications on the airways as well as how long these effects last, methacholine challenge testing can be performed. Methacholine challenge testing (MCT) is a common diagnostic test performed in patients showing symptoms of asthma in order to rule in/out the condition. It is routinely performed in clinics and in research labs, such as our Asthma Research Lab here at the University of Saskatchewan. A detailed explanation of the procedure can be found later in this document.

The influence of each of the three study drugs has not yet been demonstrated through the generation of a dose-response curve to methacholine (MCh). These dose-response curves will be useful for investigating how each medication affects the airways. Dose-response curves are graphical representations of how the response of interest (in this case airway constriction) changes with increasing concentrations of a stimulus (in this case MCh). In the normal or non-asthmatic airway, the target response to MCh is not achieved and the curve develops a plateau. In the asthmatic airway, less stimulation is needed to generate the response of interest and a plateau does not usually form. The specific factors causing these differences in the dose-response curves of asthmatics versus non-asthmatics have been identified and so, these curves can be used to assess which airway abnormalities may be causing the asthma. Curves generated following medication use will then allow for the observation of the drug's effects on these airway abnormalities. We plan to recruit fifteen asthmatic and fifteen non-asthmatic participants within Saskatoon. We will then be able to compare the MCh dose-response curves of asthmatics to non-asthmatics in order to better understand how the improvement in breathing among asthmatic participants in response to the study medications resembles regular breathing in healthy controls.

Eligible Participants: To be eligible to participate, you must be 18 years of age or older, have been diagnosed with mild-to-moderate asthma, and have sufficient baseline lung function. For this study, the amount of air you can forcefully exhale during the first second of exhalation (FEV_1) must be at least 65% of what is predicted for your age, gender and height. In addition,

your current MCh PC_{20} (concentration of MCh causing a 20% reduction in your FEV_1) must be 8mg/mL or less. If you have not previously participated in a study with the Asthma Research Lab, these criteria will be determined during your first visit to the lab.

You may not participate in this study if you have taken long-acting bronchodilators (e.g. Oxeze®, Serevent®) or long–acting anticholinergics (e.g. Spiriva®) within 30 days prior to beginning the study. You may not participate if you are pregnant, nursing, or suffer from cardiovascular problems. Due to potential negative interactions with the study treatments, individuals with prostate, kidney or urinary retention problems, hypokalemia, diabetes or glaucoma are not eligible to participate. In addition, you may not participate if you have suffered from a respiratory infection or an asthma attack from an allergy exposure within 4 weeks prior to beginning the study.

Inhaled corticosteroids (e.g. Flovent®, Pulmicort®) are allowed provided that you have been taking this medication regularly (daily for at least 30 days). Short-acting bronchodilator therapy (e.g. Ventolin®, Bricanyl®, Airomir®) is allowed but must be withheld for at least six hours prior to all lab visits and short-acting anticholinergics (e.g. Atrovent®) must be withheld for 12 hours.

Study Overview: You will be required to attend the laboratory nine times over a minimum time frame of four weeks. Three treatments are being tested: glycopyrronium, indacaterol, and combination glycopyrronium/indacaterol. The order in which you will receive the treatments will be randomized (i.e. assigned by chance) and will be unknown to you and to the study staff. However, we can determine the order if we need to for your safety. A summary of the study schedule can be found on the next page. On day 1, we will discuss the study procedure and requirements, and information regarding your asthma symptoms, severity, triggers, and medication use will be collected. Basic demographic information such as your age, height, weight, and smoking status will also be collected. You will then be required to undergo a MCh challenge (the procedure is described below) to assess your lung function when un-medicated. If your lung function parameters as found through the MCh challenge do not meet the study criteria, you will be deemed ineligible to continue participating and you will be provided with an honorarium for your time. Provided that your lung function parameters meet the study criteria, we will proceed with treatment 1 administration. You will be provided with two Breezhaler® inhalers from which you will inhale twice. If you are being administered one of the study treatments, then one inhaler will contain glycopyrronium or indacaterol while the other inhaler will contain a placebo (i.e. no active medication, a substance without therapeutic effect). If you are being administered the combination of both study treatments, each inhaler will contain an active drug. You will then wait for one hour before undergoing a second MCh challenge. Overall, this visit will take approximately 3 hours.

You will then be required to return to the lab at the same time once per day for two consecutive days (days 2 and 3). On each day, you will undergo a single MCh challenge, which will take 1-1.5 hours. Following a minimum of 10 days between each drug administration, you will be required to follow the same schedule with the other two treatments.

The order in which you receive each of the three treatments will be random (i.e. assigned by chance using a computer program). The data collected will then allow for comparison of each treatment's dose-response curve as well as their respective duration and degree of effect.

Methacholine Challenge: MCT involves the inhalation of aerosolized MCh, a known bronchoconstrictor that may cause your airway to constrict. You will inhale MCh through a loose-fitting mask for a period of two minutes. During the inhalation period, your nose will be plugged with a nose clip and you will be asked to breathe normally. Following inhalation of MCh, you will be required to inhale sharply and exhale as forcefully as you can into a spirometer, which is a handheld device connected to a computer that allows for the measurement of your lung capacity. This is referred to as spirometry or lung function testing.

We will then repeat this procedure with increasing concentrations of MCh until you no longer show a response to MCh [i.e. the amount of air you can forcefully exhale (FEV_1) only decreases by up to 5% after three consecutive MCh concentrations], your FEV_1 is decreased by 40%, or the maximal concentration of MCh (128mg/mL) is inhaled. If you feel too uncomfortable at any time and/or do not wish to continue, the test will be stopped and you will be given salbutamol (i.e. a rescue bronchodilator that opens up the airways, such as Ventolin®). You will be given salbutamol following each MCh challenge, except after the "baseline" or un-medicated MCh challenge prior to each treatment administration*; Following these 'baseline' MCh challenges, you will be given one of the study medications instead.

Breakdown of study visits:

Visit 1 (≈3 hrs.)	▪ Breakdown of study procedure and requirements ▪ Signing of the consent form ▪ Baseline MCh challenge* (no salbutamol inhalation post-MCh challenge) ▪ Treatment 1 inhalation (provided participant meets study criteria) ▪ MCh challenge at 1 hr. post-treatment
Visit 2 (≈1-1.5 hrs.)	▪ MCh challenge at 24 hrs. post-treatment
Visit 3 (≈1-1.5 hrs.)	▪ MCh challenge at 48 hrs. post-treatment
Washout 1 (minimum 10 days between treatment administrations)	
Visit 4 (≈3 hrs.)	▪ Baseline MCh challenge* (no salbutamol inhalation post-MCh challenge) ▪ Treatment 2 inhalation ▪ MCh challenge at 1 hr. post-treatment
Visit 5 (≈1-1.5 hrs.)	▪ MCh challenge at 24 hrs. post-treatment
Visit 6 (≈1-1.5 hrs.)	▪ MCh challenge at 48 hrs. post-treatment
Washout 2 (minimum 10 days between treatment administrations)	
Visit 7	▪ Baseline MCh challenge* (no salbutamol inhalation post-MCh

(≈3 hrs.)	challenge) ▪ Treatment 3 inhalation ▪ MCh challenge at 1 hr. post-treatment
Visit 8 (≈1-1.5 hrs.)	▪ MCh challenge at 24 hrs. post-treatment
Visit 9 (≈1-1.5 hrs.)	▪ MCh challenge at 48 hrs. post-treatment

Responsibilities of The Participant: As a participant of this study, you will be expected to (1) follow the directions of the researchers, (2) report all medications (prescribed, over-the-counter, and herbal products) being used or that you plan on taking prior to and throughout the duration of the study, and (3) report any changes in your health to the study staff. These steps are important both for your own safety as well as for accurate interpretation of the test results. The study investigator may remove you from the study early in the event of non-compliance with the above responsibilities or if significant side effects are experienced.

Your health and wellbeing are more important than this study and so, if a situation arises where you must use a medication that will interfere with the study, we ask that you take the medication and contact us to reschedule your appointment.

Benefits of Participating in This Study: If you choose to participate in this study, there will be no benefit for you personally. However, your participation will help increase knowledge on three respiratory disease medications and their impact on MCT, where data is currently lacking.

Honorarium: If you choose to participate in this study, you will not be charged for any research-related materials or procedures. Although you will not be paid for your participation, you will be provided with an honorarium in the amount of $25 per MCh challenge ($300 total) to cover your time and out-of-pocket expenses (e.g. parking, meals). Should you choose to withdraw early from this study, you will be compensated according to the number of MCh challenge tests you completed. The University of Saskatchewan will require your social insurance number and will send you a T4A slip at the appropriate time.

Potential Risks and Discomforts: MCT may cause the following symptoms: wheezing (10%), coughing (25%), mild shortness of breath (21%), dizziness (6%), and/or headaches (2%). The effects of MCh are short-lasting and should subside untreated. Nonetheless, you will be given salbutamol following each challenge (except when you will be given one of the study drugs) to ensure complete and prompt recovery from any airway constriction.

Some common side effects (1-10% of patients) of Seebri® are: dry mouth, cold-like symptoms (e.g. cough, sore throat), insomnia, headache, urinary tract infection, and stomach flu.

Some common side effects (1-10% of patients) of Onbrez® are: nausea, upper respiratory tract infection, muscle spasms, headache, and cold-like symptoms

Voluntary Withdrawal: Your participation in this study is voluntary and as such, you may withdraw from the study at any time. No reason needs to be given for your withdrawal, and your current health care and/or academic status (if you are a student at the U of S) will not be impacted in any way. The data collected about you until the point of withdrawal will be retained for analysis. You will be notified if new information regarding the procedure or treatments involved becomes available during the course of the study that may affect your willingness to continue to participate. Early withdrawal from the study bears no safety concerns, as the study medications are each only used once and will naturally wear off.

Study-Related Injury: In the unlikely event of an adverse effect arising related to the study procedures, trained staff will be available throughout the conduct of the study who can respond immediately. Necessary medical treatment will be made available at no additional cost to you. By signing this document, you do not waive any of your legal rights against the investigators or anyone else.

Confidentiality: In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. Your name will not be attached to any information nor mentioned in any study report, nor be made available to anyone except the research team (i.e. non-identifying codes will be used for your data e.g. if you are participant 1, your code will be DRC01). Any identifying information collected from you will remain under lock and key at the lab. However, no guarantee can be made for complete confidentiality. For quality assurance and/or monitoring purposes, the University of Saskatchewan Biomedical Research Ethics Board reserves the right to inspect research records and medical records that may identify you in the presence of the Investigator or his or her designate. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.

Your study records will be kept for at least 5 years in a secured area. After storage, your study records will be shredded in a confidential manner.

Contact Regarding Any Questions/Concerns About The Study: If you have any questions or concerns regarding this study before or during participation, you may contact Christianne at cmb831@mail.usask.ca, Dr. Cockcroft at 306-844-1446 or Dr. Davis at 306-844-1444.

If you have any concerns about your rights as a research participant and/or your experiences while participating in this study, contact the Chair of the University of Saskatchewan Research Ethics Board, at 306-966-2975 (out of town calls 1-888-966-2975). The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board (Bio#16-205).

Following the completion of the study, you may contact the study staff (i.e. student researcher at cmb831@mail.usask.ca) if interested in obtaining a summary of the results and/or provision of any research article regarding the study when (if) available.

Consent:

Study: The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve

- I have read the information in this consent form.
- I understand the purpose, procedure and possible risks of the study.
- I was given sufficient time to think about it.
- I have had the opportunity to ask questions and have received satisfactory answers.
- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care or academic status.
- I agree to follow the study staff's instructions and will tell the study staff at once if I feel I have had any unexpected or unusual symptoms.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document, I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Printed name of participant	Date
Signature of participant	Phone Number
Participant Mailing Address	Social Insurance Number
Printed name of person obtaining consent	Date
Signature of person obtaining consent	

6.9 Appendix I: LAMA/uLABA Study Non-Asthmatic Participants Consent Form

UNIVERSITY OF SASKATCHEWAN

Non-Asthmatic Participant Information and Consent Form

Title of Study: The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve

Principal Investigator: Dr. Donald Cockcroft MD FRCP
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1446

Sub-Investigators: Dr. Beth Davis, PhD
Department of Medicine
Division of Respirology, Critical Care and Sleep Medicine
University of Saskatchewan
306-844-1444

Master's Student Researcher: Christianne Blais, B.Sc. hon.
Department of Physiology
University of Saskatchewan
306-480-8983 cmb831@mail.usask.ca

24-Hour Emergency Contact: In the event of a last minute schedule change, please call or text Christianne Blais (306-480-8983) or email (cmb831@mail.usask.ca). In the event that you feel serious side effects from the methacholine challenge, seek immediate medical attention (which will be provided at no additional cost to you) and, when possible, please notify Christianne.

Introduction: You are invited to participate in this research study, as your age (i.e. at least 18 years of age) and health status (i.e. no asthma or other lung disease) make you a suitable representative of the non-asthmatic population of interest.

Your participation is entirely voluntary and you may withdraw from the study at any time without providing a reason for doing so. If you do not wish to participate or if you choose to withdraw from the study, your current health care and/or academic status (if you are a student at the U of S) will not be impacted.

Please take time to read the following information carefully. You may direct any questions or concerns to the study staff prior to and at any time during the study. Please feel free to discuss the study and your potential participation with friends, family, and/or your personal healthcare provider.

Agencies Contributing Funds and Resources to the Study: The asthma research lab will be managing the cost of the study without financial aid from a third party organization. Novartis Pharmaceuticals Inc. will be donating the drugs and devices (which will only be used with the asthmatic participant group). The researchers and the University of Saskatchewan are not being paid to conduct this study.

Purpose of Study: Bronchodilators are medications that can improve breathing in those who are experiencing difficulty (e.g. an individual with asthma). Ventolin® is an example of a bronchodilator/inhaler that is commonly used to treat respiratory disorders like asthma. There are other bronchodilator drugs that act through different mechanisms to produce bronchodilation and relief of asthma symptoms. Three medications are being tested in this study: indacaterol (Onbrez®, an ultra long-acting beta agonist), glycopyrronium (Seebri®, a long-acting muscarinic antagonist), and a combination indacaterol/glycopyrronium. Indacaterol acts directly on beta-2 receptors in the airways to relax the airway smooth muscle, which facilitates breathing. Glycopyrronium works differently as it blocks airway muscarinic receptors, which when activated produce airway constriction and difficulty breathing.

In order to observe the effects of these medications on the airways as well as how long these effects last, methacholine challenge testing can be performed. Methacholine challenge testing (MCT) is a common diagnostic test performed in patients showing symptoms of asthma in order to rule in/out the condition. It is routinely performed in clinics and in research labs, such as our Asthma Research Lab here at the University of Saskatchewan. A detailed explanation of the procedure can be found later in this document.

The influence in asthmatics of each of the three study drugs has not yet been demonstrated through the generation of a dose-response curve to methacholine (MCh). These dose-response curves will be useful for investigating how each medication affects the airways. Dose-response curves are graphical representations of how the response of interest (in this case airway constriction) changes with increasing concentrations of a stimulus (in this case MCh). In the normal or non-asthmatic airway, the target response to MCh is not achieved and the curve develops a plateau. We are seeking your participation to generate dose-response curves to MCh in the non-asthmatic airway to use as a comparator to the dose-response curves in unmedicated asthmatic airways and in asthmatic airways following each of the three treatments. The specific factors causing these differences in the dose-response curves of asthmatics versus non-asthmatics have been identified and so, these curves can be used to assess which airway abnormalities may be causing the asthma. Curves generated following medication use will then allow for the observation of the drug's effects on these airway abnormalities. We plan to recruit fifteen asthmatic and fifteen non-asthmatic participants within Saskatoon. We will then be able to better understand how the improvement in breathing among asthmatic participants in response to the study medications resembles regular breathing in healthy controls.

Eligible Participants: To be eligible to participate, you must be 18 years of age or older and must meet a specific lung function criterion. This criterion will be determined through spirometry and a MCh challenge, the procedures of which are outlined in the next section. The specific lung function parameter required is a negative (>16mg/mL) MCh PC_{20} [the concentration of MCh bronchoconstrictor that reduces the amount of air you can forcefully exhale during the first second of exhalation (FEV_1) by 20%]. Having a negative MCh PC_{20}

simply means that you do not in fact have current asthma. If the result from the MCh challenge is positive, we will discuss with you the implications of a positive test result and provide you with information on how to follow up a positive test result with your family physician. If such an event occurs, please note that you will not be able to enroll in the study as an asthmatic without a physician diagnosis of asthma.

Due to some potential effects of MCT, you may not participate if you are pregnant, nursing, or suffer from cardiovascular problems. In addition, you may not participate if you have suffered from an upper respiratory tract infection 4 weeks prior to beginning the study.

Study Overview/Visit: You will be required to attend the laboratory on one occasion, lasting between 1-1.5 hours. First, information on your respiratory health (i.e. lack of asthma symptoms), age, height, and weight will be collected. Next, you will undergo one MCh challenge (the procedure is described below). This information will allow for comparison of the MCh dose-response curve in asthmatics versus non-asthmatics.

Methacholine Challenge: MCT involves the inhalation of aerosolized MCh, a known bronchoconstrictor that may cause your airway to narrow. You will inhale MCh through a loose-fitting mask for a period of two minutes. During the inhalation period, your nose will be plugged with a nose clip and you will be asked to breathe normally. Following inhalation of MCh, you will be required to inhale sharply and exhale as forcefully as you can into a spirometer, which is a handheld device connected to a computer that allows for the measurement of your lung capacity. This is referred to as spirometry or lung function testing.

We will then repeat this procedure with increasing concentrations of MCh until you no longer show a response to MCh [i.e. the amount of air you can forcefully exhale (FEV_1) only decreases by up to 5% after three consecutive MCh concentrations], your FEV_1 is decreased by 40%, or the maximal concentration of MCh (128mg/mL) is inhaled. If you feel too uncomfortable at any time, the test will be stopped and you will be given the option of taking salbutamol (a rapid-acting bronchodilator that opens up the airways) to quickly reverse any effects of MCh.

Responsibilities of The Participant: As a participant of this study, you will be expected to (1) follow the directions of the investigators and researchers, and (2) report all medications (prescribed, over-the-counter, and herbal products) being used. These steps are important both for your own safety as well as for accurate interpretation of the test results. The study investigator may remove you from the study early in the event of non-compliance with the above responsibilities

Benefits of Participating in This Study: If you choose to participate in this study, there will be no benefit for you personally. However, your participation will help increase knowledge on the difference in the MCh dose-response curve between asthmatics and non-asthmatics. This could then help to form a better understanding of the airway changes that occur in asthma.

Honorarium: If you choose to participate in this study, you will not be charged for any research-related materials or procedures. Although you will not be paid for your participation, you will be provided with an honorarium in the amount of $25 to cover your time and out-of-pocket expenses (e.g. parking, meal).

Potential Risks and Discomforts: MCT may cause the following symptoms: wheezing (10%), coughing (25%), mild shortness of breath (21%), dizziness (6%), and/or headaches (2%). These effects are short-lasting and should subside untreated. If you do respond to MCh, you will be given the option of receiving salbutamol following the MCh challenge to ensure complete and prompt recovery from any airway constriction.

Voluntary Withdrawal: Your participation in this study is voluntary and as such, you may withdraw from the study at any time. No reason needs to be given for your withdrawal, and your current health care and/or academic status (if you are a student at the U of S) will not be impacted in any way. The data collected about you until the point of withdrawal may be retained for analysis. You will be notified if new information regarding the procedure involved becomes available during the course of the study that may affect your willingness to participate. Early withdrawal from the study poses no safety concerns.

Study-Related Injury: In the unlikely event of an adverse effect arising related to the study procedures, trained staff will be available throughout the conduct of the study who can respond immediately. Necessary medical treatment will be made available at no additional cost to you. By signing this document, you do not waive any of your legal rights against the investigators or anyone else.

Confidentiality: In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. Your name will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team (i.e. non-identifying codes will be used for your data e.g. if you are healthy participant 1, your code will be HDRC01). Any identifying information collected from you will remain under lock and key at the lab. However, no guarantee can be made for complete confidentiality. For quality assurance and/or monitoring purposes, the University of Saskatchewan Biomedical Research Ethics Board reserves the right to inspect research records and medical records that may identify you in the presence of the investigator or his or her designate. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.

Your study records will be kept for at least 5 years in a secured area. After storage, your study records will be shredded in a confidential manner.

Contact Regarding Any Questions/Concerns About The Study: If you have any questions or concerns regarding this study before or during participation, you may contact Christianne at cmb831@mail.usask.ca, Dr. Cockcroft at 306-844-1446 or Dr. Davis at 306-844-1444.

If you have any concerns about your rights as a research participant and/or your experiences while participating in this study, contact the Chair of the University of Saskatchewan Research Ethics Board, at 306-966-2975 (out of town calls 1-888-966-2975). The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Following the completion of the study, you may contact the study staff (i.e. student researcher at cmb831@mail.usask.ca) if interested in obtaining a summary of the results and/or provision of any research article regarding the study when (if) available.

Consent:

Study: The effect of glycopyrronium and indacaterol, as monotherapy and in combination, on the methacholine dose-response curve

- I have read the information in this consent form.
- I understand the purpose, procedure and possible risks of the study.
- I was given sufficient time to think about it.
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- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care or academic status.
- I agree to follow the study staff's instructions and will tell the study staff at once if I feel I have had any unexpected or unusual symptoms.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document, I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Printed name of participant	Date
Signature of participant	Phone Number
Printed name of person obtaining consent	Date
Signature of person obtaining consent	