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## Therapeutic Ketosis Attenuates Asthma Associated Airway Hyperreponsiveness By Targeting Bronchial Smooth Muscle

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THERAPEUTIC KETOSIS ATTENUATES ASTHMA ASSOCIATED  
AIRWAY HYPERREPOSIVENESS BY TARGETING BRONCHIAL  
SMOOTH MUSCLE

A Dissertation presented

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## Abstract

Asthma is a chronic respiratory condition characterized by airflow obstruction and breathing difficulties brought about by factors including airway inflammation and pulmonary remodeling that affect the smooth muscle cells of the airway and oftentimes diagnosed using tools to assess hyperresponsiveness to bronchoconstrictive agonists that trigger the smooth muscle. In allergic asthma, immune responses involving cytokines such as IL-4, IL-5, and IL-13, along with leukocytes including eosinophils and type 2 T-helper cells, drive inflammation and structural changes, including fibrosis and airway thickening. Bronchial smooth muscle (BSM) plays a critical role in asthma pathophysiology by exacerbating airway narrowing through pro-inflammatory cytokine release and increased contractility in response to allergens, microbial products, or inflammatory mediators. While bronchodilators, corticosteroids, and biologics effectively manage asthma for many patients, alternative or complementary therapies are needed for difficult-to-treat cases, particularly in obese and allergic individuals.

Previous research has highlighted the potential for metabolic interventions to modify airway physiology. Obesity-associated asthma, which often presents with heightened airway hyperresponsiveness, is linked to metabolic dysfunction, and weight loss has been shown to improve symptoms and reduce inflammation. Emerging studies suggest that therapeutic ketosis, achieved through dietary interventions or exogenous ketone supplementation, may modulate airway function by altering inflammatory and contractile responses in bronchial smooth muscle. The primary ketone body,  $\beta$ -hydroxybutyrate (BHB), augmented during times of ketosis, has demonstrated beneficial properties across various cell types and disease models. However, its specific effects on bronchial smooth muscle and airway hyperresponsiveness remain incompletely understood.

We examined the effects of BHB on human bronchial smooth muscle cells (HBSMC) *in vitro* and mouse precision-cut lung slices (PCLS) *ex vivo*. In a dose-dependent manner, BHB reduced house dust mite extract (HDM)-induced morphological changes in BSM cells and decreased HDM protease activity. Additionally, BHB suppressed IL-1 $\beta$ -induced pro-inflammatory cytokine production and inhibited histamine-induced contraction and bronchoconstriction, as observed through brightfield microscopy of HBSMC and light microscopy of PCLS, respectively. These findings suggest that BHB mitigates airway narrowing by reducing smooth muscle contraction and inflammatory signaling. Further analyses indicate that these effects may be mediated, at least in part, through the activation of Free Fatty Acid Receptor 3 (FFAR3), a proposed mechanism by which BHB elicits its protective effects on bronchial smooth muscle.

These results highlight bronchial smooth muscle as a key target of therapeutic ketosis and support the potential of BHB as a novel adjunct therapy for asthma, particularly in individuals with difficult-to-treat disease.

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## **Dedication**

This dissertation is dedicated to everyone who has believed in me, supported me, and inspired me along the way. I could not have done this without you.

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## **Chapter 1: Comprehensive Literature Review**

### **1.1. Asthma**

#### **1.1.1. Overview**

Asthma is a chronic, heterogeneous respiratory condition defined by the narrowing of the airways, leading to wheezing, shortness of breath, and difficulty breathing. These symptoms are often episodic and can be triggered by various factors, including allergens, respiratory infections, exercise, cold air, and environmental irritants such as smoke or pollution<sup>1</sup>. While asthma can affect individuals of any age, it often begins in childhood and is influenced by genetic, environmental, and immunological factors, and is increasingly prevalent in individuals who are obese<sup>2</sup>.

Asthma is characterized by airway hyperresponsiveness, or an exaggerated response of the airways to stimuli that induce the aforementioned airway narrowing, which acutely induce excessive bronchoconstriction, promote airway inflammation, and eventually cause airway remodeling. These hallmarks of asthma contribute to the narrowing of the airway lumen and difficulty breathing that asthma patients often experience<sup>3</sup>. Asthma-associated airway hyperresponsiveness can be evaluated using the methacholine challenge test, a diagnostic procedure designed to assess the responsiveness of airway smooth muscle that initiate the subsequent responses. This test involves the inhalation of methacholine, a substance that causes airway constriction in individuals with asthma or other airway hyperreactivity conditions. By monitoring the degree of airway narrowing in response to methacholine, the test helps healthcare providers evaluate the functionality of the airway smooth muscle and plays a crucial role in confirming the diagnosis of asthma<sup>4,5</sup>.

### **1.1.2. Allergic Asthma**

Allergic asthma is a subtype of asthma triggered by immune reactions to environmental triggers or allergens such as pollen, dust mites, pet dander, and mold<sup>3</sup>. The most common asthma phenotype, particularly in children, allergic asthma involves a complex interplay of immune cells associated with the cytokines interleukin(IL)-4, IL-5, and IL-13, inflammatory cells including eosinophils, mast cells, basophils, type 2 innate lymphoid (ILC2), and T helper (Th2) lymphocytes, and immunoglobulin E (IgE)-producing plasma cells<sup>6-9</sup>, all of which contribute to chronic inflammation in the airways. These immune responses release mediators that affect other asthma-relevant cell types, such as airway smooth muscle<sup>10-12</sup> and airway epithelium<sup>6,13</sup>, exacerbating airway hyperresponsiveness and remodeling<sup>12,14-17</sup>. Over time, structural remodeling in the airways, including thickening of the airway walls, fibrosis, and increased mucus production caused by the chronic inflammation present in allergic asthma, can contribute to persistent asthma symptoms<sup>15,18</sup>. Environmental factors, such as exposure to allergens, air pollution, and respiratory infections, as well as genetic predisposition, can further influence the development and severity of allergic asthma<sup>19,20</sup>.

### **1.1.3. Obesity-Associated Asthma**

Obesity has reached epidemic levels, affecting nearly 40% of adults and 19% of children in the United States<sup>21</sup>. A meta-analysis involving over 300,000 adults found a significant association between obesity and asthma, with the risk of asthma increasing as BMI rises<sup>22</sup>. Obesity-associated asthma is a prevalent and complex condition where excess body weight not only contributes to the development of asthma but also exacerbates asthma symptoms. The pathophysiology of obese asthma involves a multifaceted interplay of

mechanical and inflammatory factors. Excess adipose tissue in the thoracic and abdominal cavities can impair lung function mechanically and promote airway hyperresponsiveness. Additionally, adipose tissue releases pro-inflammatory mediators, such as cytokines plasminogen activator inhibitor-1, monocyte chemoattractant factor-1, interleukin (IL)-6 and IL-8, as well as adipokines like leptin and adiponectin<sup>23-25</sup>. While the precise role of these mediators in asthma pathogenesis is not fully understood, it is hypothesized that they contribute to increased airway inflammation<sup>23,24,26</sup>. In our previous studies using a murine model of diet-induced obesity, we demonstrated that visceral adipocyte-conditioned media (Ad-CM) from mice fed a high-fat diet can induce proinflammatory responses in transformed mouse airway epithelial club cells<sup>27</sup>. Using an equivalent human system, we have previously found that weight loss following bariatric surgery reduces the ability of plasma from obese asthmatic individuals, to enhance proinflammatory cytokine secretion from human bronchial epithelial cells<sup>28</sup> however, in human bronchial smooth muscle cells, exposure to Ad-CM alone did not reveal differences among the groups, but when an agonist was added, cells exposed to Ad-CM from non-allergic obese subjects exhibited lower responses than those from obese non-asthmatic subjects (**Appendix A**), suggesting that adipocyte-derived factors in the Ad-CM may not be the primary drivers behind the improvements in asthma management observed following weight loss in obese individuals.

Individuals with both asthma and obesity often experience poorer asthma control and reduced responsiveness to standard therapies, including inhaled corticosteroids, long-acting bronchodilators, and leukotriene receptor antagonists<sup>23,24,29,30</sup>. Weight loss, including through bariatric surgery, can significantly improve asthma control<sup>31</sup>. These benefits are evident in improved lung function, better performance on methacholine

challenge, and a reduction in self-reported asthma symptoms<sup>32</sup>. They also end up using less asthma control medication<sup>33</sup>. Together, these findings highlight the intricate relationship between obesity and asthma, emphasizing the contribution of adipose tissue in driving inflammation and airway dysfunction. Targeting obesity through weight loss interventions offers a promising approach to improving asthma outcomes, particularly for individuals with obesity-associated asthma. Understanding the mechanisms and mediators whereby weight loss elicits these beneficial effects remains an area of intense study.

#### **1.1.4. Bronchial Smooth Muscle**

*Remodeling:* Bronchial smooth muscle (BSM) plays a key role in maintaining normal airway tone and regulating airflow through controlled contraction and relaxation. Remodeling of the airway refers to the pathological structural changes such as increased BSM mass<sup>34,35</sup>. This can refer to increased BSM cell size (hypertrophy) and cell number (hyperplasia), which have both been described to be present in asthma<sup>36,37</sup>. Increased BSM mass results from a combination of smooth muscle cell hypertrophy and hyperplasia, driven by inflammatory mediators like cytokines and growth factors, including IL-13 and transforming growth factor-beta (TGF- $\beta$ )<sup>38-43</sup>. These factors stimulate the proliferation of smooth muscle cells and their excessive accumulation in the airway walls<sup>12,14-17,38-40</sup>. Chronic inflammation further induces BSM hypertrophy and hyperplasia, thickening airway walls and reducing airflow<sup>44</sup>. The hypertrophy of smooth muscle cells also increases their contractile capacity, further amplifying airway constriction during asthma exacerbations<sup>34,39</sup>. Moreover, remodeling involves the deposition of extracellular matrix components, such as collagen and fibronectin, within the airway walls<sup>45-47</sup>. These structural

changes reduce the overall elasticity of the airways, impairing their ability to dilate properly and contributing to long-term airway obstruction<sup>43</sup>.

*Inflammation:* Bronchial smooth muscle (BSM) also contributes to bronchial inflammation, acting as both a target and mediator of inflammatory reactions<sup>11,14</sup>. Key immune cells involved in asthma pathogenesis—such as eosinophils, mast cells, basophils, type 2 innate lymphoid cells (ILC2), T helper (Th2) lymphocytes, and IgE-producing plasma cells<sup>6,7</sup>—release pro-inflammatory mediators like cytokines and chemokines that affect BSM cells<sup>10-12</sup>. These immune cells and mediators trigger complex signaling pathways that alter the function of BSM, enhancing airway inflammation and contributing to the pathophysiology of asthma. Notably, cytokines such as IL-4, IL-5, and IL-13 are central to BSM inflammation, driving the recruitment of inflammatory cells and enhancing the release of additional mediators like histamine, leukotrienes, and prostaglandins. These interactions not only exacerbate airway hyperresponsiveness but also amplify airway remodeling<sup>12,14-17</sup>, as discussed previously.

BSM not only responds to inflammation but also actively contributes to the inflammatory environment. In allergic asthma, mast cells infiltrate smooth muscle tissue, intensifying airway hyperresponsiveness and further aggravating asthma symptoms<sup>14</sup>. The interaction between BSM and mast cells is critical; mast cell activation, often by allergen-induced IgE crosslinking, induces the release of histamine, proteases, and cytokines, all of which contribute to the acute inflammatory response and facilitate BSM contraction<sup>11,12,15,48,49</sup>. This infiltration triggers BSM cells to release pro-inflammatory chemotactic cytokines (chemokines), such as IL-6 and IL-8, which attract additional inflammatory cells, including eosinophils and neutrophils, to the site of

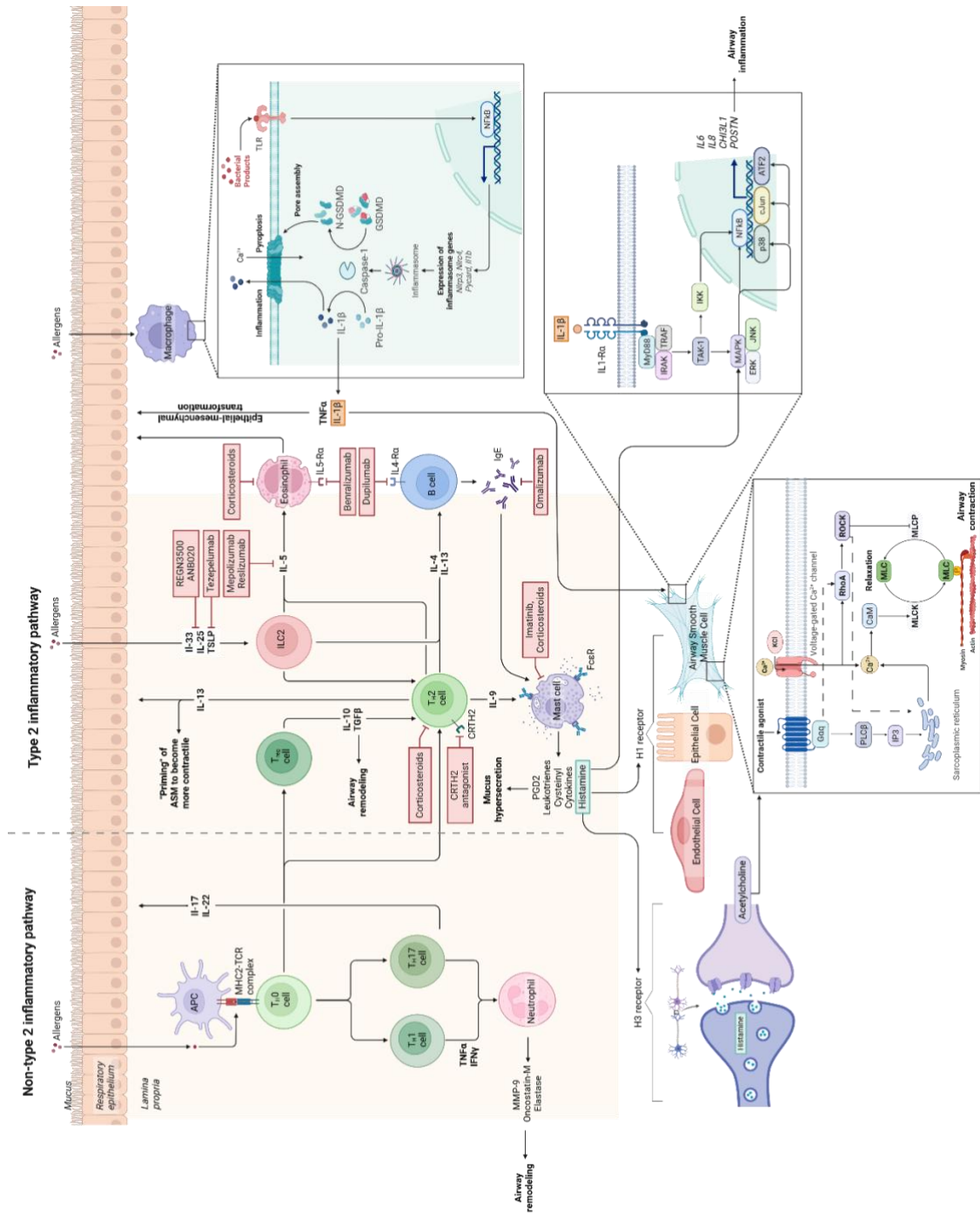
inflammation<sup>34,50,51</sup>. The continued release of these mediators fosters a positive feedback loop of inflammation and BSM activation, further promoting airway narrowing<sup>52</sup>.

*Contraction:* In healthy individuals, BSM contraction is typically a response to stimuli such as neural signals or environmental irritants, allowing for protective airway narrowing<sup>1</sup>. This contraction serves as a defense mechanism to prevent the entry of harmful particles or pathogens into the distal (gas-exchange) regions of the lungs; however, in asthma this process becomes dysregulated<sup>34,53</sup>. The inflammatory response to mast cell infiltration and activation involves the production of mediators like histamine, leukotrienes, and prostaglandins, which independent of causing inflammation, act to induce bronchoconstriction and increase vascular permeability<sup>54,55</sup>. These mediators trigger the activation of intracellular signaling pathways, including calcium mobilization and protein kinase activation, leading to smooth muscle contraction and airway narrowing<sup>34,56-59</sup>. Other inflammatory mediators can also affect the contractile response of BSM. The cytokines IL-13 and IL-17A have been implicated in enhancing the expression of contractile proteins and calcium signaling pathways in BSM cells, further amplifying contraction<sup>60-62</sup>.

BSM contraction is also exacerbated by heightened vagal nerve activity, which activates muscarinic receptors on the smooth muscle. When the vagus nerve, a component of the parasympathetic nervous system, is stimulated, it releases the neurotransmitter acetylcholine (ACh) from its nerve endings in the airways. Acetylcholine binds to muscarinic acetylcholine receptors (M3 receptors) located on the bronchial smooth muscle. These G-protein-coupled receptors trigger intracellular signaling pathways that lead to muscle contraction<sup>63-65</sup>. Activation of the M3 receptor increases intracellular calcium ion

(Ca<sup>2+</sup>) levels via the phospholipase C (PLC) pathway<sup>63,66</sup>. The elevated calcium levels interact with calmodulin, which activates myosin light chain kinase (MLCK), phosphorylating myosin light chain, facilitating the contraction of the smooth muscle fibers<sup>67-69</sup> while inhibiting myosin light chain phosphatase (MLCP) and, thereby, inhibiting the relaxation of the smooth muscle fibers<sup>70,71</sup>.

Bronchial smooth muscle (BSM) is central to the pathophysiology of asthma (**Figure 1.1**), as its contraction causes airway narrowing, resulting in obstruction and breathing difficulties<sup>17</sup>. These complex roles underscore the importance of BSM as a therapeutic target for addressing airway inflammation, hyperresponsiveness, and remodeling in asthma management. In fact, BSM are the target of the asthma control and asthma relief medications based on albuterol (and structurally similar compounds), which stimulates the  $\beta$ 2-adrenergic receptor to promote BSM relaxation and inhibit pro-inflammatory mediator production<sup>72-74</sup>.



**Figure 1.1. The Role of Inflammation in Airway Smooth Muscle Pathology.** The diagram depicts the intricate inflammatory pathways in airway diseases and their profound impact on airway smooth muscle (ASM). A variety of triggers initiate cascades involving immune cells and mediators, ultimately leading to ASM dysfunction. Therapeutic interventions targeting these pathways are highlighted. Inset boxes provide detailed views of cellular interactions and signaling cascades at the ASM level, illustrating the complexity of these inflammatory processes.

### 1.1.5. *In vitro* and *ex vivo* models and their applications

In 1976, British statistician George Box famously stated, “All models are wrong, but some are useful.” This sentiment applies aptly to biological models of disease, including *in vitro*, *ex vivo*, and *in vivo* systems. *In vitro* systems, which simulate biological processes in a controlled laboratory setting using isolated cells, tissues, or biochemical frameworks, play a critical role in advancing biomedical research. These models are invaluable for studying disease mechanisms, testing therapeutic agents, and reducing reliance on *in vivo* animal experiments<sup>75</sup>. By providing controlled environments to manipulate specific variables, *in vitro* systems facilitate detailed mechanistic studies. Additionally, they are often cost-effective, support high-throughput drug screening, and accelerate drug discovery<sup>76,77</sup>. Importantly, models utilizing human cells enhance the translatability of findings, bridging the gap between laboratory research and clinical application, and ensuring greater relevance to human health<sup>78</sup>. Specifically, in studying lung and respiratory health, simplified *in vitro* representations created using human cells in culture can be instrumental for advancing our understanding of respiratory health and diseases such as asthma and that cell culture methodologies can offer control, design flexibility, and scalability to gain human-relevant insight<sup>79</sup>.

The *in vitro* system utilized in the studies presented in this dissertation consists of primary asthmatic human bronchial smooth muscle cells (HBSMC), which are a relevant model to investigate disease-specific mechanisms such as hyperresponsiveness, inflammatory signaling, and structural remodeling, providing insights that are directly translatable to human asthma. This serves as a reductionistic model in which we can focus on a key component of airway hyperresponsiveness without the complexities of cell-to-

cell, whole-organ, or systemic interactions and provides a controlled environment to test hypotheses, examine specific signaling pathways, and evaluate the direct effects of therapeutic agents on HBSMC, offering critical insights into asthma while minimizing confounding variables.

In a less reductionistic modeling approach, an *ex vivo* model or system refers to the study of living tissues or organs outside their natural biological context but in conditions that closely mimic an *in vivo* environment. These models retain the structural, cellular, and functional complexity of intact tissues, enabling researchers to investigate physiological and pathological processes in a controlled setting. For instance, *ex vivo* models of human bronchial tissue are used to study airway remodeling, inflammation, and contractility in diseases like asthma, preserving critical interactions between epithelial cells, smooth muscle, and immune components<sup>80,81</sup>. Precision Cut Lung Slices (PCLS) are a valuable *ex vivo* model for studying airway reactivity and contraction, closely mimicking lung architecture and cellular diversity<sup>82-85</sup>. They bridge the gap between *in vitro* and *in vivo* studies and are particularly useful in asthma research, demonstrating altered responses like hyperresponsiveness and bronchoconstriction when exposed to various agonists<sup>72,82,85,86</sup>. PCLS offer several advantages, including the ability to study tissue responses in a setting that closely mimics *in vivo* conditions, thereby enhancing the translational relevance of research findings. They also reduce the need for animal models, aligning with ethical considerations in research. However, it's important to note that while *ex vivo* models provide a more realistic environment than *in vitro* systems, they may still lack certain systemic factors that are present in living organisms<sup>80,81,85</sup>. In summary, *ex vivo* models like PCLS serve as valuable tools in biomedical research, bridging the gap between *in vitro*

studies and *in vivo* animal models, and offer insights that are more directly applicable to human health.

#### **1.1.6. Current Asthma Therapies**

The management of asthma typically involves avoiding known allergens, using medications such as bronchodilators to open the airways, inhaled corticosteroids to reduce inflammation, or biologic therapies that target type 2 cytokines, their inducers, or their targets<sup>1,3,8</sup>. Relaxation of BSM is mediated by  $\beta_2$ -adrenergic receptors, which activate intracellular signaling pathways to promote smooth muscle relaxation and maintain open airways<sup>87</sup>.  $\beta$ -agonists, a cornerstone of asthma therapy, target BSM contraction to alleviate airway obstruction effectively. These agents activate the  $\beta_2$ -adrenergic receptors, triggering the cyclic adenosine monophosphate (cAMP) signaling pathway to relax BSM, improve airflow, and reduce bronchospasm. Short-acting  $\beta$ -agonists (SABAs), such as albuterol, are commonly prescribed for acute symptom relief, providing rapid bronchodilation during asthma exacerbations<sup>87</sup>.

For long-term asthma management, long-acting  $\beta$ -agonists (LABAs), such as salmeterol and formoterol, are frequently combined with inhaled corticosteroids (ICS) to address both bronchoconstriction and airway inflammation<sup>88</sup>. This combination therapy has demonstrated effectiveness in reducing exacerbations and improving overall asthma control, although it requires careful monitoring to mitigate the potential risk of masking poorly controlled inflammation<sup>1,88-91</sup>. Emerging therapies aimed at modulating immune responses, including monoclonal antibodies against IgE, IL-4, IL-5, IL-13, and TSLP (or their receptors), have shown promise in improving effective disease control<sup>92-94</sup>. For instance, omalizumab targets IgE to reduce allergic inflammation, while dupilumab inhibits

IL-4 and IL-13 signaling to address type 2 inflammation in asthma<sup>95-97</sup>. Similarly, mepolizumab and benralizumab target IL-5 and its receptor to reduce eosinophilic inflammation, which is often present in severe asthma<sup>95,98,99</sup>. Targeting the alarmin, thymic stromal lymphopoietin (TSLP) released by stimulated airway epithelial cells, tezepelumab (Tezspire) is the most recently-approved biological therapy for asthma, and is prescribed to those patients in which other therapies have failed or in which type 2 biomarkers are absent<sup>100,101</sup>. Despite their efficacy, these biologics are not ideal for all patients due to factors such as high costs, potential side effects like injection site reactions or immunosuppression, and the need for frequent administration<sup>102-104</sup>. Additionally, they are typically indicated for patients with specific asthma phenotypes, such as severe eosinophilic or allergic asthma, limiting their utility for individuals whose asthma does not fit these categories<sup>105,106</sup>. For some 'difficult-to-treat' individuals with severe or uncontrolled disease, alternative or adjunctive therapies may be necessary<sup>107</sup>.

## **1.2. Therapeutic Ketosis**

### **1.2.1. Overview**

Under normal conditions, glucose is the primary energy source for the body. It is derived from dietary carbohydrates and stored as glycogen in the liver and muscles. When glucose is plentiful, insulin secretion facilitates its uptake into cells, where it undergoes glycolysis to produce ATP, the body's energy currency<sup>108</sup>. The brain, which consumes about 20% of the body's energy, relies heavily on glucose as its primary fuel<sup>109</sup>. During periods of carbohydrate restriction, fasting, or prolonged exercise, glycogen stores are depleted. The body compensates by shifting to ketogenesis, a process in which the liver converts fatty acids into ketone bodies ( $\beta$ -hydroxybutyrate, acetoacetate, and acetone)<sup>110</sup>. This

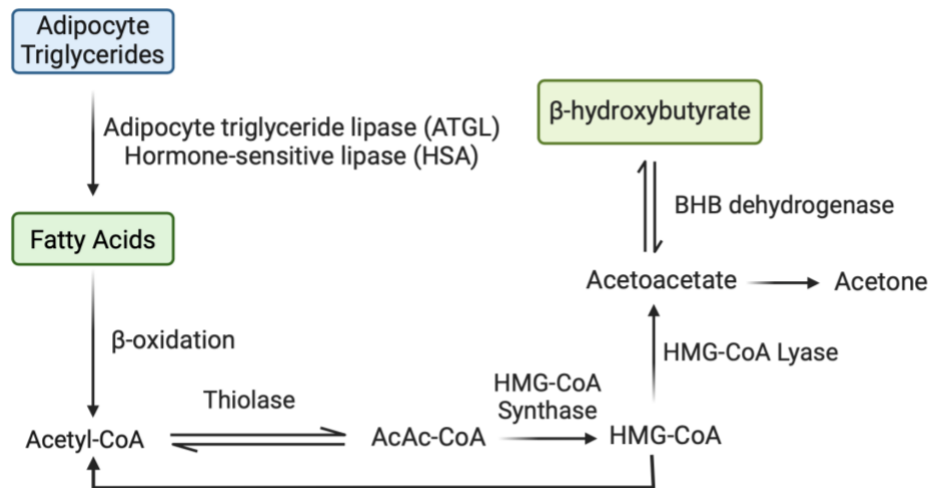
metabolic adaptation ensures a continuous energy supply, especially for the brain, which can utilize ketone bodies in place of glucose during prolonged fasting or carbohydrate deprivation<sup>108-111</sup>. Ketosis, the physiological state characterized by elevated ketone levels, reduces the body's reliance on glucose and promotes fat metabolism as the primary energy source. This metabolic flexibility is crucial for survival during periods of nutrient scarcity and is being studied for therapeutic applications in many disorders<sup>112</sup>.

### **1.2.2. Ketone Metabolism**

*Ketogenesis:* Ketogenesis is a metabolic pathway through which the liver produces molecules called ketone bodies from fatty acids. This process is primarily triggered when glucose levels are low, such as during prolonged fasting, carbohydrate restriction, or vigorous exercise. Ketogenesis serves as a critical adaptive mechanism that provides an alternative energy source, particularly for the brain, when glucose availability is insufficient<sup>110</sup>. Stimulated by hormones such as glucagon, cortisol, and epinephrine, and inhibited by insulin, the process begins with the lipolysis, or the breakdown of adipose tissue into fatty acids<sup>113,114</sup>. Triglycerides, which are the primary form of stored fat in adipocytes (fat cells), are hydrolyzed by the enzymes hormone-sensitive lipase (HSL) and adipocyte triglyceride lipase (ATGL) into glycerol and free fatty acids<sup>115,116</sup>. The free fatty acids are then released into the bloodstream, where they are transported to tissues such as the liver and muscles for oxidation and energy production<sup>114</sup>.

Ketogenesis primarily takes place in the mitochondria of liver cells. Fatty acids are transported into the mitochondria through the action of carnitine palmitoyltransferase 1 (CPT-1) and subsequently broken down into acetyl-CoA through beta-oxidation. Two acetyl-CoA molecules are then combined to form acetoacetyl-CoA via the enzyme thiolase,

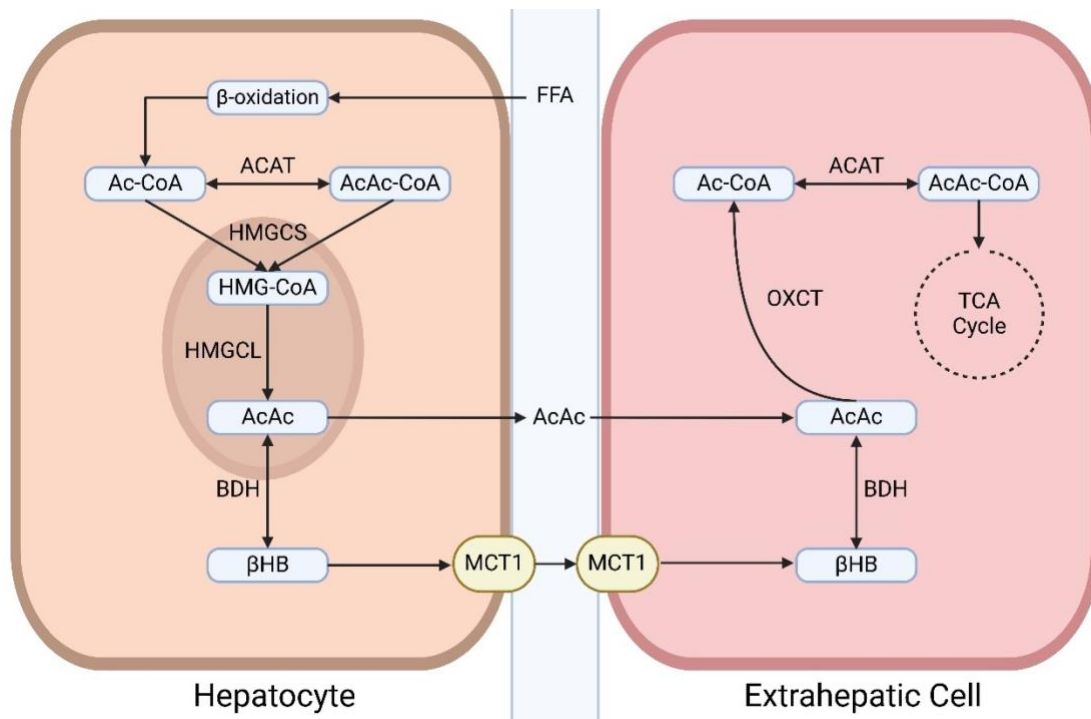
also referred to as acetyl coenzyme A acetyltransferase (ACAT). Acetoacetyl-CoA is further converted into HMG-CoA by the enzyme HMG-CoA synthase (HMGCS2). Next, HMG-CoA lyase catalyzes the conversion of HMG-CoA into acetoacetate. Acetoacetate can either undergo non-enzymatic decarboxylation to form acetone or be reduced into  $\beta$ -hydroxybutyrate by the enzyme  $\beta$ -hydroxybutyrate dehydrogenase (BDH1)<sup>113,114</sup> (**Figure 1.2**). The ketone bodies can then be released into the bloodstream to be utilized by extrahepatic, or peripheral tissues<sup>117</sup>.



**Figure 1.2. Ketogenesis Pathway.** Simplified schematic of ketogenesis, illustrating the breakdown of adipocyte triglycerides into fatty acids and their conversion into  $\beta$ -hydroxybutyrate (BHB) through  $\beta$ -oxidation and key enzymatic steps.

*Ketolysis:* Ketolysis is the metabolic process through which ketone bodies are broken down to generate energy in peripheral tissues. It complements ketogenesis, which produces ketone bodies in the liver, by allowing these energy-rich molecules to be utilized by other cells. Ketolysis occurs primarily in tissues like the brain, skeletal muscles, and heart, which rely on ketone bodies as an alternative fuel source<sup>118</sup>. The process of ketolysis begins with the transport of ketone bodies— $\beta$ -hydroxybutyrate and acetoacetate—from the bloodstream into cells.  $\beta$ -hydroxybutyrate is first oxidized to acetoacetate by the enzyme

$\beta$ -hydroxybutyrate dehydrogenase. Acetoacetate is then activated to acetoacetyl-CoA by the enzyme succinyl-CoA:3-ketoacid-CoA transferase (SCOT), which uses succinyl-CoA as a co-substrate<sup>114,118</sup>. This step is critical because SCOT is absent in liver cells, ensuring that the liver does not utilize the ketone bodies it produces<sup>114,117,119</sup>. Finally, acetoacetyl-CoA is cleaved into two acetyl-CoA molecules by the enzyme thiolase. The acetyl-CoA enters the citric acid cycle (Krebs cycle), where it is oxidized during oxidative phosphorylation to generate 22 ATP per molecule, the cell's primary energy currency<sup>113,120</sup> (Figure 1.3).



**Figure 1.3. Ketolysis and Ketone Utilization.** Overview of ketone metabolism, showing ketone production in hepatocytes via ketogenesis and their subsequent utilization in extrahepatic tissues through ketolysis for energy production. Figure created using Biorender. Adapted from cited<sup>121</sup>.

Ketolysis is essential in maintaining energy homeostasis during periods of limited glucose availability, such as during prolonged fasting or when consuming a ketogenic diet.

The brain, in particular, benefits from ketolysis as it can derive up to 70% of its energy from ketone bodies under such conditions<sup>111</sup>. This process not only conserves glucose for tissues that depend exclusively on it, like red blood cells, but also helps prevent the excessive breakdown of muscle protein, which would otherwise be needed for gluconeogenesis<sup>122</sup>. While ketolysis is a natural and efficient energy pathway, imbalances in this system can contribute to metabolic disorders. For example, in diabetic ketoacidosis (DKA), excessively high levels of ketone bodies overwhelm the body's buffering capacity, leading to acid-base imbalances and severe complications. One of the ketone bodies, acetone, does not convert back to acetyl-CoA and is instead excreted through urine or exhaled<sup>113</sup>. The accumulation of acetone can result in a characteristic "sweet" or fruity odor on the breath, which is often used as a diagnostic indicator of DKA. When properly regulated, as in nutritional ketosis, ketolysis functions as an adaptive mechanism that enhances metabolic flexibility and energy efficiency, offering significant benefits without the risks of ketone over-accumulation.

### **1.2.3. Augmentation of Ketones**

While starvation was historically the primary way our bodies augmented ketones for energy, it is not the only method available to do so today. There are various modern approaches—such as dietary changes, fasting protocols, and supplements—that can stimulate ketone production without relying on prolonged periods of food deprivation<sup>123</sup>.

*Ketogenic Diet:* The ketogenic diet is a high-fat, moderate-protein, and very low-carbohydrate diet that induces ketosis and promotes the production of ketone bodies by limiting glucose availability and shifting the body's metabolism toward fat oxidation<sup>124,125</sup>. Typical macronutrient ratios for this diet are 70–80% fat, 10–20% protein, and 5–10%

carbohydrates<sup>124,126</sup>. In addition to promoting fat loss, the diet has been associated with improvements in insulin sensitivity, reduced inflammation, and enhanced mitochondrial function<sup>126-128</sup>. However, long-term adherence to the ketogenic diet may require careful management of nutrient intake to avoid potential deficiencies in micronutrients<sup>126,129</sup>.

*Intermittent Fasting:* Intermittent fasting (IF) is a dietary approach that alternates between periods of eating and fasting, aiming to induce ketosis. By limiting the window of food intake, IF leads to the depletion of glycogen stores, prompting the body to shift toward fat metabolism and ketone production for energy<sup>130</sup>. Popular methods of IF include the 16/8 method, which involves a 16-hour fast followed by an 8-hour eating window, and alternate-day fasting, where individuals consume very few calories on fasting days<sup>131</sup>. Similar to the ketogenic diet, research has suggested that IF can enhance insulin sensitivity, reduce inflammation, and improve cardiovascular health, while also supporting weight loss and metabolic flexibility<sup>131,132</sup>.

*Prolonged Exercise:* Prolonged exercise is an effective method to augment ketone production by depleting glycogen stores and stimulating fat metabolism. During extended periods of aerobic activity, such as long-distance running or cycling, the body shifts from utilizing glycogen as its primary fuel source to oxidizing fatty acids, leading to the production of ketone bodies in the liver<sup>114</sup>. This metabolic adaptation supports sustained energy supply for both muscle activity and brain function during endurance exercise. Research shows that trained athletes often achieve higher levels of ketogenesis during prolonged exercise compared to sedentary individuals, as their bodies adapt to more efficiently utilize fat as fuel<sup>133</sup>. Additionally, combining prolonged exercise with fasting or a ketogenic diet can further amplify ketone production, promoting enhanced metabolic

flexibility and endurance<sup>134</sup>. However, to optimize the benefits of prolonged exercise, it is crucial to carefully manage intensity, duration, and caloric intake to avoid any negative impact such as ketoacidosis<sup>135</sup>.

*Exogenous Ketogenic Supplementation:* Exogenous ketones, including ketone salts, ketone esters, or ketogenic precursors are dietary supplements that provide an external source of ketone bodies, such as  $\beta$ -hydroxybutyrate (BHB), to rapidly elevate blood ketone levels without a need for fasting or strict dietary modifications. Ketone salts combine BHB with minerals like sodium, potassium, or magnesium, whereas ketone esters, in the form of a monoester or diester, consist of BHB or AcAc bound to an alcohol molecule, enabling efficient absorption and utilization<sup>136,137</sup>. A ketogenic precursor is a molecule or compound that can be metabolized by the body to produce ketone bodies. Medium chain triglycerides and a compound 1,3-butanediol are examples of this. Medium-chain triglycerides (MCTs) are a type of fat composed of medium-length carbon chains, typically ranging from 6 to 12 carbons, which are rapidly absorbed and metabolized by the liver to produce ketones. Found in coconut oil, palm oil, and MCT oil supplements, MCTs bypass normal fat digestion and directly support ketogenesis, making them a popular choice for enhancing ketosis<sup>138,139</sup>. MCT supplementation has been shown to improve energy metabolism, cognitive function, and endurance performance in ketogenic and non-ketogenic states<sup>140-143</sup>. 1,3-Butanediol is a synthetic alcohol compound metabolized in the liver to beta-hydroxybutyrate (BHB), providing a direct source of ketones. Unlike MCTs, 1,3-butanediol does not require fat metabolism for ketone production and has been studied for its potential in enhancing ketone levels for therapeutic and performance purposes,

although promising, its use is primarily experimental, with ongoing research to determine its safety, efficacy, and optimal dosing strategies<sup>143-145</sup>.

*Glucose-lowering Medications:* Glucose-lowering medications can indirectly enhance ketone production by shifting the body's metabolism toward increased fat oxidation. Sodium-glucose cotransporter-2 (SGLT2) inhibitors, such as canagliflozin and dapagliflozin, reduce blood glucose levels by promoting glucose excretion through urine, thereby encouraging lipolysis and ketogenesis as alternative energy pathways<sup>146</sup>. While these drugs are effective for managing type 2 diabetes by lowering blood glucose levels, and increasing ketone levels, they carry a known risk of diabetic ketoacidosis (DKA)<sup>147-149</sup>. Risk factors for DKA in SGLT2 inhibitor users include low carbohydrate intake, prolonged fasting, alcohol consumption, or underlying illness. Because of these risks, SGLT2 inhibitors should be prescribed with caution, particularly in patients with type 1 diabetes or other conditions predisposing them to ketoacidosis<sup>147</sup>. Recently becoming popular as a method of weight loss, glucagon-like peptide-1 (GLP-1) receptor agonists, such as semaglutide and liraglutide, improve glycemic control and promote weight loss by enhancing insulin secretion, reducing appetite, and potentially increasing fat oxidation<sup>150-153</sup>. While these medications may facilitate ketosis, their primary purpose is to manage conditions such as type 2 diabetes and obesity. Additionally, both SGLT2 inhibitors and GLP-1 receptor agonists should be used under medical supervision, as they carry potential side effects, therefore, these medications should not be used as a primary method to augment ketosis without proper clinical guidance<sup>147,149,154-156</sup>.

#### **1.2.4. Current Studies of Ketone Bodies as a Therapeutic**

The concept of therapeutic ketosis refers to a controlled metabolic state in which elevated levels of ketone bodies are utilized for therapeutic potential<sup>112</sup>. The idea of using ketosis to treat epilepsy has its roots in ancient medicine, long before the mechanisms of ketosis or even epilepsy were scientifically understood. As early as 400 BCE, Hippocrates, the "Father of Medicine," documented cases in which fasting alleviated seizure symptoms<sup>157</sup>. Additionally, reference is made in the bible of a patient with epilepsy being cured through "prayer and fasting"<sup>158</sup>. Fasting became a cornerstone of epilepsy treatment in ancient cultures, particularly within religious or spiritual contexts, where it was believed to cleanse the body and spirit. However, the scientific exploration of this approach didn't begin until the early 20th century.

The modern understanding of therapeutic ketosis for epilepsy began in the 1920s, when physicians noticed that fasting was remarkably effective in controlling seizures, especially in patients resistant to available treatments<sup>159</sup>. At the time, fasting was seen as a radical but effective therapy, with case reports documenting patients who experienced significant reductions in seizure frequency or even became seizure-free. However, fasting had obvious limitations—it could not be sustained long-term without serious health risks<sup>159</sup>. This led researchers to search for alternative methods to mimic the metabolic effects of fasting. In 1921, Dr. Russel Wilder at the Mayo Clinic proposed the ketogenic diet as a means to replicate the benefits of fasting without requiring patients to abstain from food. The diet was designed to be high in fat, very low in carbohydrates, and moderate in protein, forcing the body into a state of ketosis. Wilder's work demonstrated that this diet

could control seizures effectively, and the ketogenic diet became a standard treatment for epilepsy, particularly in children<sup>160,161</sup>.

The popularity of the ketogenic diet waned in the mid-20th century with the advent of anti-epileptic drugs (AEDs), which were easier to administer and more socially acceptable than a restrictive diet<sup>159</sup>. However, in the 1990s, interest in the ketogenic diet experienced a resurgence, thanks in part to the advocacy of parents like Jim Abrahams, whose son Charlie had severe, drug-resistant epilepsy<sup>162</sup>. Charlie's dramatic improvement on the diet spurred the creation of the Charlie Foundation, which helped raise awareness and funding for research into the ketogenic diet as a treatment for pediatric drug-resistant refractory epilepsy<sup>163</sup>.

While the exact mechanisms by which ketosis suppresses seizures are not fully understood, it is believed to involve changes in brain energy metabolism, stabilization of neuronal membranes, alterations in neurotransmitter levels, epigenetic modifications, and anti-inflammatory effects<sup>164-167</sup>. The diet's success also inspired the development of modified ketogenic protocols and exogenous ketone supplements to make the therapy more accessible and sustainable<sup>168,169</sup>. Today, the ketogenic diet remains a cornerstone treatment for drug-resistant epilepsy, and the insights gained from its use have opened the door to exploring therapeutic ketosis in a wide range of other neurological and metabolic disorders<sup>168,170 171</sup>.

Therapeutic ketosis is being explored as a treatment strategy for several pathological diseases and conditions, primarily because of its metabolic effects and influence on inflammation, oxidative stress, and cellular energy. Other than epilepsy, neurological or neurodegenerative diseases such as Alzheimer's, Parkinson's, Amyotrophic

Lateral Sclerosis (ALS), and Traumatic Brain Injury (TBI), are currently being investigated in their response to therapeutic ketosis<sup>112,172-176</sup>.

Therapeutic ketosis is being explored as an adjunct treatment for cancer due to its potential to target the unique metabolic vulnerabilities of cancer cells. Many cancer cells rely on aerobic glycolysis for energy production, a phenomenon known as the Warburg effect, which makes cancer cells heavily dependent on glucose<sup>177</sup>. It should be noted that, while foundational in cancer metabolism research, this idea is understood to be more nuanced than originally thought due to cancer heterogeneity and heterogeneity in cancer metabolism<sup>178,179</sup>. While the Warburg effect remains a valuable model, researchers increasingly view cancer metabolism as dynamic and context dependent. This shift has implications for therapeutic strategies like ketogenic diets, which aim to exploit cancer's metabolic vulnerabilities<sup>180</sup>. Such approaches must account for metabolic diversity and tumor adaptability to be effective<sup>181,182</sup>. Preclinical studies have demonstrated that ketogenic diets may slow tumor growth, inhibit metastasis, and enhance the effectiveness of conventional therapies such as chemotherapy and radiation<sup>183-185</sup>. However, there are several reports of cancer cells utilizing ketone bodies as fuels for their malignant transformation, growth, and metastasis<sup>186,187</sup>, making ketone therapies in cancer a topic of controversy.

As previously mentioned, therapeutic ketosis and ketogenic diets (KD) have shown promise in managing various metabolic disorders such as obesity and type 2 diabetes, by addressing underlying issues related to insulin resistance, glucose dysregulation, and mitochondrial dysfunction<sup>114,127,154,188,189</sup>. Emerging evidence also suggests benefits in non-alcoholic fatty liver disease (NAFLD), as the metabolic benefits are thought to result from

the reduction of glucose and insulin spikes, increased fatty acid oxidation, and enhanced mitochondrial efficiency driven by ketone metabolism<sup>190-192</sup>.

Therapeutic Ketosis is being investigated for its potential benefits in autoimmune and inflammatory diseases, primarily due to the anti-inflammatory and immunomodulatory effects. Ketone bodies, such as beta-hydroxybutyrate (BHB), have been shown to inhibit the NLRP3 inflammasome, a key driver of inflammation in many chronic conditions<sup>193</sup>. In diseases like rheumatoid arthritis, ketone bodies have been proposed to reduce inflammation and joint damage by modulating immune responses and reducing oxidative stress<sup>194-196</sup>. Additionally, the anti-inflammatory properties of ketosis have shown promise in inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, by improving gut barrier function and reducing intestinal inflammation<sup>197-199</sup>.

In the context of cardiovascular health, therapeutic ketosis has garnered attention for its potential effects, with evidence suggesting both benefits and potential risks. Positively, ketosis has been shown to improve several cardiovascular risk factors, including reductions in body weight, blood glucose levels, and triglycerides, as well as an increase in high-density lipoprotein (HDL), which is considered to be the "good" cholesterol<sup>128,200,201</sup>. Additionally, the anti-inflammatory effects of ketone bodies, such as beta-hydroxybutyrate, may benefit conditions like atherosclerosis by reducing vascular inflammation and oxidative stress<sup>201,202</sup>. However, concerns persist regarding the long-term safety to cardiovascular health, especially of certain high-fat ketogenic diets. Some studies report increases in low-density lipoprotein (LDL) or "bad" cholesterol, which could raise the risk of cardiovascular events<sup>129,200,203,204</sup>. Given these mixed effects, more research is needed to determine the long-term impact of therapeutic ketosis on cardiovascular diseases,

to identify populations that may benefit most from this dietary intervention, and to devise diets eliciting the positive benefits of ketones without the deleterious effects of long-chain saturated fats.

Despite caveats, therapeutic ketosis shows promise for a wide range of conditions, from neurodegenerative and autoimmune diseases to cancer, though the exact mechanisms behind its benefits remain unclear. However, emerging research has begun to shed light on the potential roles and function of ketone bodies in modulating these pathological conditions and diseases, providing a foundation for understanding their therapeutic effects.

#### **1.2.5. Functions of Ketone Bodies**

Ketone bodies, including  $\beta$ -hydroxybutyrate (BHB), acetoacetate (AcAc), and acetone, are traditionally recognized as alternative energy sources during fasting or carbohydrate restriction. However, their potential to elicit beneficial effects across a wide range of diseases and conditions remains poorly understood. Proposed functions include roles as alternative energy sources, mediators of anti-inflammatory and antioxidant pathways, regulators of mitochondrial and metabolic health, modulators of immune function, and epigenetic regulators, with therapeutic implications for conditions discussed in the previous section. The following sections will explore these functions in detail, providing a comprehensive overview of the evidence supporting their diverse roles.

*Energetic Substrate:* As previously discussed and comprising 78%, 20%, and 2% of circulating levels, respectively<sup>118</sup>, the ketone bodies  $\beta$ -hydroxybutyrate (BHB), acetoacetate (AcAc), and acetone serve as an alternative energy source, especially during fasting or carbohydrate restriction. This adaptation not only ensures survival during nutrient scarcity but also provides a more oxygen-efficient energy source, as BHB and

AcAc generate more ATP per unit of oxygen compared to glucose metabolism<sup>123</sup>. This metabolic strategy highlights the evolutionary importance of ketone bodies as an efficient and adaptive energy source and has been implicated as being closely linked to their ability to mitigate various diseases and conditions by providing efficient, alternative fuel during metabolic stress and improving cellular energy metabolism. In neurodegenerative diseases like Alzheimer's and Parkinson's, impaired glucose metabolism in the brain can be mitigated by BHB, which provides an efficient alternative energy source for neurons. BHB helps restore ATP levels, reduces oxidative stress, and improves cognitive function, as seen in Alzheimer's models<sup>205-207</sup>.

Beyond their metabolic role, ketone bodies, especially BHB since it is the most abundant extracellularly, has been shown to exert several direct signaling effects, including binding to cell-surface receptors, transcriptional regulation, serving as a substrate for protein post-translational modifications, and modulating ion channel activity.

*Recognition by Cell Surface Receptors:* BHB functions as a ligand for G-protein-coupled receptor 109A (GPR109A, also called HCAR2), a receptor expressed in adipose tissue, immune cells, and the colon. Activation of GPR109A by BHB has been linked to anti-inflammatory effects, such as suppressing pro-inflammatory cytokine production and promoting the differentiation of regulatory T cells<sup>193</sup>. While FFAR3, also known as GPR41, is traditionally associated with short-chain fatty acids (*e.g.*, butyrate, acetate, and propionate), emerging evidence suggests that BHB may also modulate FFAR3 activity<sup>208</sup>. FFAR3 is expressed in key tissues, including the gut<sup>209</sup>, nervous system<sup>210,211</sup>, and pancreas<sup>212</sup>, and plays a role in linking metabolic and immune functions, most importantly,

FFAR3 has been shown to be expressed in airway smooth muscle and modulates contraction<sup>213</sup>.

*Transcriptional Regulation:* Ketone bodies, particularly BHB, regulate gene expression through their ability to inhibit key enzymes and serve as substrates for posttranslational modifications of proteins. These mechanisms converge to modulate transcriptional activity, epigenetic states, and cellular responses. BHB directly inhibits histone deacetylases (HDACs), enzymes that remove acetyl groups from histone proteins, thereby influencing chromatin structure and gene transcription. HDAC inhibition generally promotes a more relaxed chromatin state, facilitating the transcription of genes involved in stress resistance, antioxidant defense, and metabolism<sup>214,215</sup>. BHB can covalently modify lysine residues on histones, a process known as  $\beta$ -hydroxybutyrylation. This unique modification alters histone-DNA interactions and influences transcriptional programs distinct from those regulated by acetylation or methylation.  $\beta$ -hydroxybutyrylation has been shown to increase the expression of genes involved in lipid metabolism and oxidative stress, highlighting its role in adapting to metabolic changes during ketosis<sup>216-218</sup>. This adaptive regulation may underline ketone bodies' ability to attenuate disease progression in the aforementioned diseases and conditions.

*Modulation of Ion Channel Activity:* Ketone bodies regulate the activity of various ion channels, impacting cellular excitability and signaling. BHB has been shown to inhibit voltage-gated sodium channels, reducing neuronal excitability and contributing to the anticonvulsant effects of ketogenic diets in epilepsy<sup>219</sup>. Additionally, BHB modulates ATP-sensitive potassium channels, which stabilizes membrane potential in neurons and regulates insulin secretion in pancreatic beta cells during fasting or ketogenic

states<sup>193,220,221</sup>. Emerging evidence also suggests that BHB may influence voltage-gated calcium channels by decreasing calcium influx into cells, thereby reducing excitotoxicity and preventing calcium overload, a critical factor in neurodegenerative diseases<sup>222,223</sup>. The regulation of ion channels by ketone bodies may work in conjunction with their signaling through the aforementioned cell-surface receptors, such as G protein-coupled receptors (GPCRs), to orchestrate a coordinated cellular response. For example, activation of the hydroxycarboxylic acid receptor 2 (HCAR2) by  $\beta$ -hydroxybutyrate (BHB) initiates anti-inflammatory pathways and modulates energy metabolism, potentially influencing ion channel activity indirectly through downstream signaling cascades<sup>223</sup>. Similarly, free fatty acid receptor 3 (FFAR3) activation by short-chain ketones can modulate neuronal excitability and systemic metabolic responses, which may complement the direct effects of BHB on ion channels<sup>222</sup>. Together, these mechanisms integrate extracellular signaling with intracellular excitability, contributing to the many benefits observed.

### **1.3. Therapeutic Ketosis as a Treatment for Asthma**

Asthma is a complex inflammatory disease influenced by immune dysregulation, oxidative stress, metabolic dysfunction, and characterized by airway hyperresponsiveness. Emerging evidence suggests that therapeutic ketosis, primarily through the actions of ketone bodies such as beta-hydroxybutyrate (BHB), may provide significant benefits in asthma management. This section explores the key mechanisms by which ketosis modulates inflammation, oxidative stress, metabolic dysfunction, and airway reactivity in the context of asthma, highlighting its potential as a novel therapeutic.

*Anti-inflammatory Effects:* Ketosis exerts significant anti-inflammatory effects that could be beneficial in managing asthma, particularly through its impact on key immune

pathways. One of the most well-documented mechanisms whereby ketone bodies influence inflammatory pathways is the inhibition of the NLRP3 inflammasome, a protein complex that plays a central role in inflammation and the pathogenesis of asthma<sup>193,224,225</sup>. The NLRP3 inflammasome is responsible for activating pro-inflammatory cytokines including IL-1 $\beta$  and IL-18, which contribute to airway inflammation, hyperresponsiveness, and mucus production in asthma<sup>226-228</sup>. Ketones such as BHB have been shown to inhibit this inflammasome activation, thereby reducing the production of these harmful cytokines<sup>193,229</sup>. Additionally, BHB has been demonstrated to downregulate the release of TNF $\alpha$  and IL-6, two cytokines involved in systemic inflammation and airway remodeling in asthma<sup>229-231</sup>. Ketosis also influences macrophages, which are key players in immune responses, by shifting them from a pro-inflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype. This shift contributes to a reduction in inflammatory mediator production<sup>232,233</sup>. Furthermore, BHB has been shown to mitigate airway inflammation by regulating mast cell function, which in turn reduces ILC2 proliferation and the type 2 cytokine response. These effects suggest that BHB could serve as a promising therapeutic agent for managing many endotypes of asthma<sup>234</sup>.

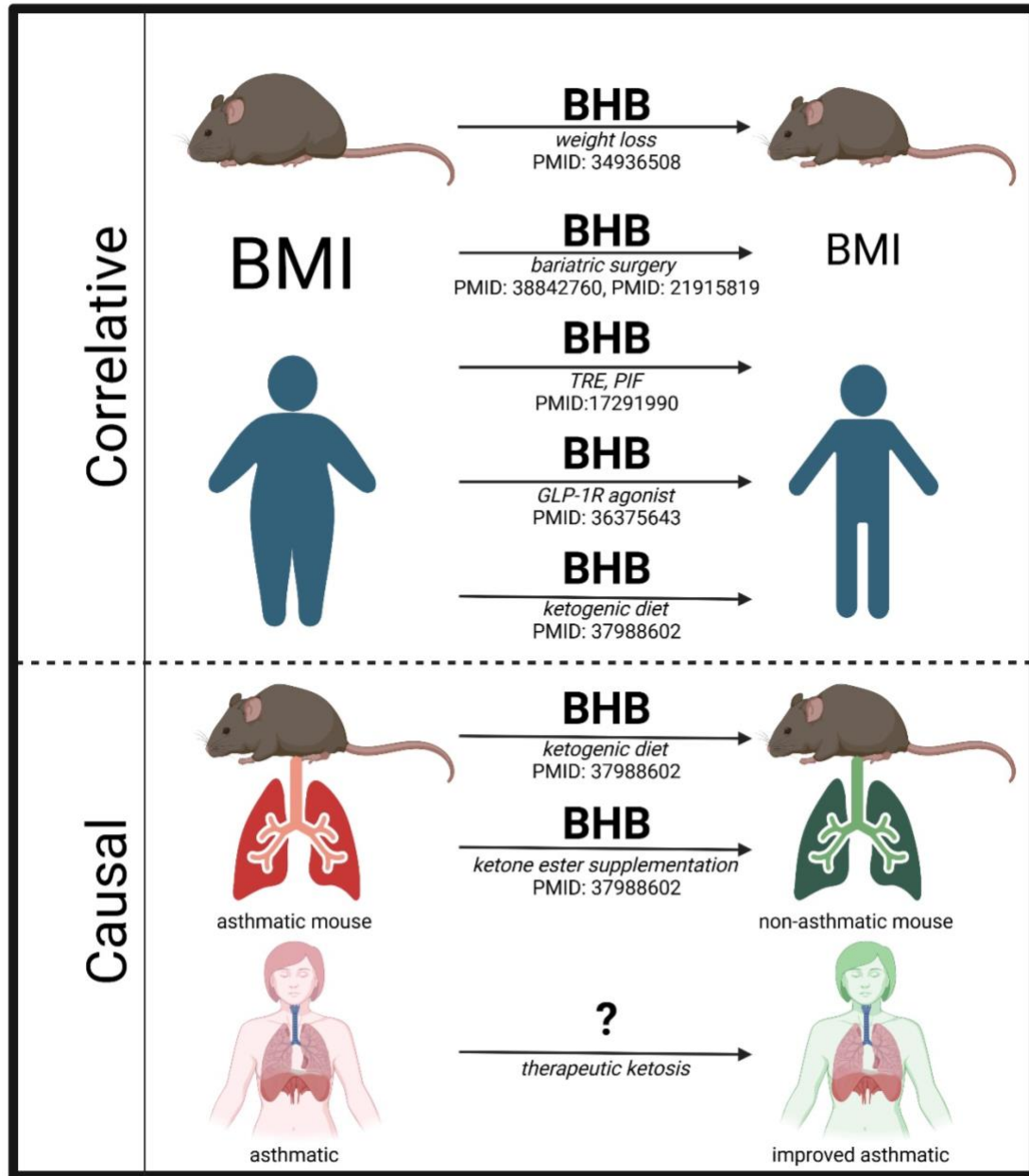
*Anti-oxidant Effects:* Oxidative stress plays a central role in the pathogenesis of asthma, where an imbalance between reactive oxygen species (ROS) and antioxidant defenses contributes to airway inflammation, tissue damage, and bronchoconstriction. Ketosis, by promoting the utilization of ketone bodies like BHB, has been shown to reduce ROS production, particularly in the mitochondria, where electron leakage from the electron transport chain is a major source of ROS. Ketones enhance mitochondrial efficiency by improving electron flow and reducing mitochondrial dysfunction, thus limiting ROS

generation<sup>235,236</sup>. Additionally, ketosis upregulates endogenous antioxidant systems, such as glutathione, which plays a crucial role in neutralizing free radicals and protecting against oxidative damage<sup>237-239</sup>. Studies in animal models have demonstrated that BHB decreases oxidative stress markers and promotes an antioxidant milieu<sup>240</sup> that can protect airway cells from the damaging effects of chronic inflammation and environmental insults<sup>241,242</sup>.

*Metabolic and Obesity-related Factors:* Metabolic dysfunction, particularly obesity, has been recognized as a significant contributor to the exacerbation of asthma symptoms. Obesity is associated with increased airway inflammation, airway hyperresponsiveness, and a greater frequency of asthma attacks<sup>26,243</sup>. The concept of therapeutic ketosis has gained a broader appreciation following observations in patients undergoing bariatric surgery, in which ketosis has been associated with improvements in asthma symptoms and reduced airway inflammation independent of the weight loss, suggesting that similar metabolic changes could benefit asthmatic individuals, particularly those with obesity<sup>28</sup>. This suggests that circulating factors influenced by obesity contribute to airway inflammation in asthma, and that weight loss can mitigate these effects perhaps through the early and transient production of ketone bodies. The study highlights the role of metabolic and obesity-related factors in asthma pathogenesis and supports the potential of therapeutic ketosis, achieved through weight loss interventions like bariatric surgery, to reduce airway inflammation in obese asthmatic individuals. This idea is further supported by *in vivo* murine studies, which demonstrated that weight loss decreased methacholine hyperresponsiveness in mouse models of diet-induced obese asthma, suggesting that similar metabolic changes through ketosis could benefit asthmatic individuals, particularly those with obesity<sup>244</sup>. In murine models of inherent obese asthma, mice with ketone levels

augmented through dietary intervention showed decreases in methacholine hyperresponsiveness<sup>245</sup>. This suggests that ketosis, achieved through weight loss or dietary interventions, may improve airway responsiveness in obese asthmatic individuals.

*Airway Hyperresponsiveness:* Ketosis has been shown to have a beneficial impact on airway hyperresponsiveness, a hallmark of asthma. Studies in animal models suggest that ketone bodies, such as beta-hydroxybutyrate (BHB), can reduce the sensitivity of airway smooth muscles to constrictive stimuli, thereby decreasing airway reactivity<sup>246</sup>. This effect may result from the ability of BHB to modulate key cellular pathways involved in smooth muscle contraction and inflammation. While these mechanisms have been observed in preclinical models, there remains a gap in knowledge regarding how BHB-mediated modulation of these pathways influences the pathogenesis of asthma and whether these effects can be effectively applied in human disease management.



**Figure 1.4. Beta-Hydroxybutyrate (BHB) as a Potential Mediator of Metabolic and Airway Health.** Schematic illustrates the correlative and causal relationships between beta-hydroxybutyrate (BHB), body mass index (BMI), and airway health. The top panel depicts various interventions, including weight loss, bariatric surgery, time-restricted eating (TRE), GLP-1R agonists, and ketogenic diets, which are associated with increased BHB levels and BMI reduction in both rodents and humans. The bottom panel highlights the causal evidence linking ketogenic diets and ketone ester supplementation to improved airway function in asthmatic mice. The potential for therapeutic ketosis to alleviate asthma symptoms in humans remains an open question. Relevant PubMed IDs (PMIDs) are provided for each supporting study. Figure created using Biorender.

## 1.4. Scope of Dissertation

The scope of this dissertation is centered on elucidating the mechanisms by which therapeutic ketosis, particularly through beta-hydroxybutyrate (BHB), influences bronchial smooth muscle function and asthma pathophysiology. The research is anchored in the complex interplay between the key hallmarks of asthmatic airway hyperresponsiveness — remodeling (Chapter 2), inflammation (Chapter 3), and contraction (Chapter 4). By investigating the attenuation of these hallmarks in bronchial smooth muscle, a central component to asthma pathophysiology, this work seeks to bridge gaps in the understanding of how ketosis may mitigate airway hyperresponsiveness in asthma.

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## **Chapter 2: Beta-Hydroxybutyrate Decreases HDM-induced Bronchial Smooth Muscle Morphological Change**

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## 2.1. Abstract

Individuals with allergic asthma exhibit lung inflammation and remodeling accompanied by methacholine hyperresponsiveness manifesting in proximal airway narrowing and distal lung collapsibility, and they can present with a range of mild-to-severe disease amenable or resistant to therapeutic intervention, respectively. There remains a need for alternatives or complements to existing treatments that could control the physiological manifestations of allergic asthma. Our aim was to examine the hypothesis that because ketone bodies elicit anti-inflammatory activity and are effective in mitigating the methacholine hyperresponsiveness associated with obese asthma, increasing systemic concentrations of ketone bodies would diminish the pathological outcomes in asthma-relevant cell types. Therefore, we explored the effects of ketone bodies on bronchial smooth muscle cells *in vitro*. In a dose-dependent manner, the ketone body beta-hydroxybutyrate (BHB) decreased house dust mite extract (HDM) induced morphological change of bronchial smooth muscle cells and decreased HDM protease activity. Increasing systemic BHB concentrations through dietary interventions could provide symptom relief for several endotypes of allergic asthma individuals though the effects on bronchial smooth muscle.

## 2.2. Introduction

Asthma is a common, chronic pulmonary disorder that mechanistically involves a complex interaction of lung inflammation, remodeling, and reactivity<sup>1</sup>. Allergic asthma increases responsiveness to bronchoconstricting agents, making both humans and mice hyperresponsive to the most common clinically used asthma diagnostic, methacholine<sup>2</sup>. As can be effectively modeled in mice<sup>3</sup>, allergic asthma typically manifests in large airway

methacholine hyperresponsiveness (AHR) and additionally involves distal lung compartments that are affected as a consequence of tethering to the airways.

Treatments for allergic asthma include bronchorelaxing beta-agonists that increase airway lumen caliber, anti-inflammatory corticosteroids, and biological therapies targeting causal mediators of the type 2 immune response<sup>4,5</sup>. While affording effective disease control to the majority of patients, there remain those with “difficult-to-treat” allergic asthma for whom alternative or complementary therapies are needed<sup>6-8</sup>. We have recently reported on the beneficial effect of elevating circulating levels of ketone bodies, termed “therapeutic ketosis”, in mouse models of obese asthma<sup>9</sup>, wherein they significantly decrease methacholine hyperresponsiveness. Ketone bodies can become elevated systemically as fatty acids consumed in the diet<sup>10</sup> or mobilized from adipose tissue as a consequence of energetic demand<sup>11-13</sup> are catabolized through  $\beta$ -oxidation in the liver to the ketone bodies acetoacetate (AcAc) and  $\beta$ -hydroxybutyrate (BHB), which are then released into the circulation and can be used as an energy source by cells throughout the body<sup>14</sup>. Consuming a ketone body precursor such as 1,3-butanediol (1,3-BD)<sup>15</sup>, or ketone esters, a dietary supplement approved for human use<sup>16</sup>, can transiently elevate ketone body concentrations.

Ketone bodies can modulate several of the key pathological processes involved in both obese and allergic asthma<sup>16-18</sup>. As an energy source, ketone bodies make cells less reliant on glycolysis<sup>14,19-23</sup> and therefore produce less lactic acid, a catabolite implicated as a causal factor in allergic asthma pathogenesis<sup>24-28</sup>. Ketone bodies have been reported to function through cell surface receptors, including the G protein-coupled receptors hydroxycarboxylic acid receptor 2 (HCAR2/GPR109a) and free fatty acid receptor 3

(FFAR3/GPR41)<sup>11,12,29,30</sup>. Ketone bodies also function as antioxidants<sup>16,17,31,32</sup> and exert anti-inflammatory effects, including suppression of NF-kappaB activation<sup>33</sup> as well as inhibition of the NLRP3 inflammasome and subsequent IL-1 $\beta$  production<sup>18,34-36</sup>, which are also implicated in the pathogenesis of allergic asthma. Interestingly, IL-1 $\beta$ , itself is a cause of elevated glycolysis and accompanying pathology in asthma<sup>25,37,38</sup>. Alternate day caloric restriction elevates BHB levels that are correlated with reductions in oxidative stress and inflammation, along with improved clinical findings in overweight asthmatic subjects, including those with allergic asthma<sup>39</sup>. Importantly, ketone body augmentation in human subjects is well-tolerated<sup>40</sup>.

Despite the strong connections between the mechanisms underlying allergic asthma and the beneficial effects of ketone bodies, their potential to be used therapeutically in allergic asthma has not been evaluated in the modern era<sup>41,42</sup>. We hypothesized that since ketone bodies can exert significant anti-inflammatory, redox-regulating, and metabolic effects, they could be relevant targets and tools in the treatment of allergic asthma. Our objectives were to evaluate the effectiveness of ketone bodies in reducing *in vitro* pathological features of bronchial smooth muscle, a cell type relevant to allergic asthma, caused by exposure to the most common perennial allergens, house dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*)<sup>43</sup>. Additionally, we aimed to identify mechanisms through which ketone bodies may modulate these effects. Further understanding the efficacy and mechanisms of ketone bodies *in vitro* could provide new dietary and pharmacological targets for the treatment of allergic asthma that could in turn be addressed in subsequent clinical trials.

## 2.3. Methods and Materials

### 2.3.1. *In vitro* Bronchial Smooth Muscle Cell Culture

Human bronchial smooth muscle cells (HBSMC) purchased from Lonza (Morristown, NJ) were cultured using the smooth muscle cell growth medium-02 BulletKit according to manufacturer's instructions at 37°C in 95% humidified air containing 5% CO<sub>2</sub>. Cells were used within the first 9 passages to ensure proper smooth muscle phenotype. For experiments, HBSMC were plated at 5x10<sup>4</sup> cells/ml in 500 µl media in 24-well plates and allowed to grow for two days. The media was then removed, fresh media was added, and cells were treated as indicated within the figure legends for each experiment using *Dermatophagoides pteronyssinus* house dust mite (HDM) extract in saline (Part No. XPB70D3A25, Lot No. 343205; Stallergenes Greer, Lenoir, NC) in the absence or presence of BHB for 24 hours. HBSMC were imaged using a Nikon Eclipse TS100 microscope (Melville, NY) at 10x magnification and equipped with a MU1003 AmScope Microscope Digital Camera (Irvine, CA). For quantitative analysis, systemic uniform sampling by independent observers was used to select imaging locations. Images were binarized using ImageJ technology (FIJI)<sup>64</sup> and background was eliminated using the raw images in the Image calculator function. Cellular surface area was quantified using the 'bwarea' function in MATLAB version R2022a (The Mathworks Inc., Natick, MA). All experimental conditions were replicated.

### 2.3.2. Protease Activity Assay

HDM-associated protease activity was measured using a microplate assay in which HDM extract was incubated in the presence of 10 µg/mL of dye/quencher (D/Q)-ovalbumin (catalog # D-12053, Molecular Probes, Eugene, OR) without or with BHB at 37°C for 1 hour. Fluorescence intensity (excitation 485 ± 20 nm and emission 528 ± 20 nm) induced

by the protease-dependent liberation of the quencher (Q) from the BODIPY FL fluorescent dye (D) was read every minute on a Bio-Tek Synergy HTX multi-mode plate reader. End values are presented.

## 2.4. Results

### 2.4.1. Bronchial Smooth Muscle Morphological Change induced by House Dust Mite Extract is inhibited by $\beta$ -hydroxybutyrate.

Results from the animal models of allergic asthma<sup>44</sup> as well as the mouse models of obesity-associated asthma reported previously<sup>9</sup>, suggested prominent effects of ketone bodies on bronchial smooth muscle. Consequently, we exposed human bronchial smooth muscle cells (HBSMCs) to HDM in the absence or presence of a BHB racemic mixture ((*R,S*)-BHB) used in Figures 1 and 2, as well as single (*R*)-BHB and (*S*)-BHB enantiomers, and visualized cell surface area by light microscopy (**Figure 2.1A**). In contrast to unexposed control cells, HBSMCs exposed to HDM displayed a contracted phenotype in which the cell area of the unexposed, confluent cells in culture were markedly condensed. The presence of BHB, whether the mixed enantiomer or single enantiomers (*e.g.*, (*R*)-BHB or (*S*)-BHB), decreased HDM-induced HBSMC contraction. Quantitation of cell pixel density revealed that HBSMC contraction induced by HDM was inhibited in a dose-dependent manner irrespective of the BHB enantiomer (**Figure 2.1B**).

### 2.4.2. House Dust Mite Extract Protease Activity is inhibited by $\beta$ -hydroxybutyrate.

As allergen-associated proteases are an important means of HDM-induced cellular activation<sup>45</sup>, and HDM protease can directly influence bronchial smooth muscle cell morphology<sup>46</sup>, we examined the effect of several BHB enantiomers on HDM protease activity<sup>47</sup>. The assay employed is sensitive to both selective serine (*e.g.*, trypsin) and cysteine (*e.g.*, papain) proteases, as well as to the mixture of serine and cysteine proteases

present in house dust mite extract<sup>48</sup>. Furthermore, the assay employs a substrate optimized for studies of endo-lysosome proteolysis, so is insensitive to acid-mediated inhibition<sup>49</sup>. The BHB racemic mixture as well as the single enantiomers equally inhibited HDM protease activity in a dose-dependent manner (**Figure 2.1C**), implicating an absence of BHB stereoselectivity in this effect.

## 2.5. Discussion

The increasingly prevalent global asthma epidemic<sup>50</sup>, especially of severe asthma<sup>6-8</sup>, has created a pressing need to devise alternative and complementary strategies to limit the impact on patients' lives imposed by the syndrome. "Therapeutic ketosis" is one such approach that has recently gained attention for its potential to provide benefit in a myriad of disease settings and through a number of mechanisms. As reported herein, our studies demonstrate that the ketone body BHB inhibit agonist-induced morphological change in asthma-relevant cell type bronchial smooth muscle, in a dose-dependent manner. Mechanisms whereby these ketones elicit effects *in vitro* remain uncertain, but may include activation of cell surface receptors, providing energetic substrates for utilization through the Krebs cycle, functioning as antioxidants, modulating intracellular signaling regulating cytokine production and cell contraction, and inhibiting allergen protease activity. Moreover, through these or other mechanisms, dietary treatments that increase systemic concentrations of BHB *in vivo*, including ketogenic diet, ketone body precursor feeding, or ketone ester supplementation decrease allergic asthma-associated methacholine hyperresponsiveness, the most relevant pathophysiological manifestation of preclinical asthma models.

Both large airway and peripheral lung dysfunction are present in allergic asthma<sup>51</sup>, and our interventions show that increased BHB concentrations benefit both these anatomical units. In our previously published preclinical obese asthma models presented, and the published manuscript that this data is included in, ketone bodies decreased the parameter  $R_N$ , which provides a measure of the flow resistance of the entire airway tree<sup>9,44</sup>. Ketone body augmentation also decreased tissue damping, G, and tissue elastance, H, which are increased by the development of heterogeneous ventilation to the distal reaches of the lung due to variations in airway narrowing, and also by derecruitment of lung units<sup>52</sup>, both of which are particularly sensitive to contraction of peripheral airways<sup>53</sup>. As the central airways and the lung periphery are not mechanically independent due to the tethering of parenchymal tissues to the airways<sup>54</sup>, we speculate that a mechanism through which ketones restrain AHR may involve affecting bronchial smooth muscle cells affected by HDM challenges, attenuating their capacity to contract in response to methacholine and thereby affecting the changes in all three mechanics parameters.

Our studies using human bronchial smooth muscle cells cultured *in vitro* show that HDM-induced morphological change is attenuated in the presence of BHB and is at least partially a consequence of BHB-mediated inhibition of HDM protease activity. We posit that providing elevated levels of ketone bodies could provide benefit<sup>55</sup> to allergic asthma, and may do so through a number of direct and indirect effects on lung physiology throughout the proximal airways and distal airspaces that modulate inhaled methacholine hyperresponsiveness. Although our studies reveal one mechanism by which ketone bodies may beneficially affect allergic asthma by attenuating HDM-induced effects on bronchial smooth muscle cells, the effects observed *in vitro* may also be evoked if high concentrations

of BHB were achieved *in vivo*, perhaps through pharmacological instead of dietary means. Several studies of *in vivo* anti-inflammatory activities of therapeutic ketosis have been reported, with some directly relevant to lung inflammation<sup>18,34,35</sup>. Intriguingly, it has been shown that ketogenic diet inhibits allergic airway inflammation elicited by the protease papain by decreasing type 2 innate lymphoid cell (ILC2) influx, activation, and accompanying cytokine production, although the mechanism described was related to decreased glucose availability rather than the effects of ketone bodies, per se, and methacholine responsiveness was not assessed<sup>56</sup>.

Beta-hydroxybutyrate has been reported to function as a class-I histone deacetylase (HDAC) inhibitor<sup>11,12,32</sup> and to induce  $\beta$ -hydroxybutyrylation of histone H3 lysines<sup>57,58</sup> to influence gene expression. Histone modifications have been reported in the context of allergic asthma<sup>59,60</sup>.  $\beta$ -hydroxybutyrylation also post-translationally modifies a multitude of additional cellular proteins with both known effects and heretofore unknown consequences<sup>61,62</sup>. Although we speculated that  $\beta$ -hydroxybutyrylation of HDM proteins could account for the ability of BHB to inhibit HDM protease activity, inconsistencies of available reagents did not enable us to provide compelling evidence to support this hypothesis. As we previously reported altered expression of the smooth muscle-associated genes, *Tagln* (transgelin / SM-22 alpha) and *Acta2* (actin alpha 2 / alpha smooth muscle actin), in the lungs of methacholine-hyperresponsive, HFD-fed obese mice, which were decreased in obese mice consuming a ketone ester supplement, perhaps these cells *in vivo* are targets of BHB-induced post-translational modifications affecting gene expression or protein functions related to methacholine hyperresponsiveness. Effects of BHB on methacholine-induced intracellular signaling events in bronchial smooth muscle cells merit

further investigation. Intriguingly, inflammasome activation<sup>18,34-36</sup> and PAD4-regulated netosis<sup>63</sup> are both inhibited by BHB, which decreases the regulated secretion of bioactive products, including IL-1 $\beta$  and neutrophil extracellular traps, respectively. It is possible that in addition to inhibiting bronchial smooth muscle contraction, BHB inhibits mucus secretion from methacholine-stimulated airway goblet cells that could affect airflow and, therefore, the parameters measured in our flexiVent analysis<sup>9,44</sup>.

There are several limitations to our findings. Namely, we conducted *in vitro* studies using primary human bronchial smooth muscle cells instead of human subjects. While informative, human cell studies fail to capture the prolonged and complex nature of human asthma. They offer a reductionist approach by focusing on a single cell type and a specific agonist that readily initiates an inflammatory cascade, allowing us to observe only the inhibitory effects of BHB at that initial step without assessing its role in maintaining suppression or facilitating recovery. Another notable limitation of this study is the lack of assessment of the remodeling response of bronchial smooth muscle to HDM extract stimulation. Overall, these findings support the potential of therapeutic ketosis as a promising complement or alternative to other conventional approaches for asthma treatment in patients.

## 2.6. Figure Legends

### **Figure 2.1. $\beta$ -hydroxybutyrate decreases bronchial smooth muscle morphological change and inhibits protease activity of house dust mite extract.**

Human bronchial smooth muscle cells (HBSMCs) cultured *in vitro* for 24 hours in the absence (control) or presence of house dust mite extract (HDM) and increasing concentrations of a racemic BHB mixture or individual enantiomers of BHB were visualized by light microscopy for a contractive phenotype **(A)**. The pixel count of cells in the images were quantitated **(B)**. Images and values are representative of studies performed twice. HDM was incubated in the absence of or in increasing concentrations of BHB and a fluorogenic protease substrate for 1 hour and fluorescence was measured **(C)**. N = 4 samples/group. \*\*\*\* =  $p \leq 0.0001$  compared to HDM.



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### **Chapter 3: Beta-Hydroxybutyrate Attenuates Bronchial Smooth Muscle Pro-Inflammatory Cytokine Production**

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VAF and MEP designed research studies, conducted experiments, acquired data, analyzed data, prepared figures, and wrote the manuscript. MMM conducted experiments, acquired data, and analyzed data. All authors edited the manuscript and approved the final version.

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### 3.1. Abstract

Asthma is a common airway condition causing breathing difficulties due to reversible airflow obstruction. It often affects obese individuals, with symptoms triggered by environmental factors that induce immune responses, leading to inflammation and bronchoconstriction. Bronchial smooth muscle (BSM) plays a central role in airway narrowing, driven by type 2 immune responses involving cytokines like IL-4, IL-5, and IL-13, along with leukocytes including eosinophils and type 2 T-helper cells. These responses cause structural changes such as fibrosis and airway thickening, while BSM cells worsen asthma by releasing pro-inflammatory cytokines in response to allergens, microbial signals, or inflammatory cytokines from other cells. While current treatments manage asthma in most patients, alternative therapies are needed for difficult-to-treat cases, particularly prevalent in obese, allergic individuals. Emerging research suggests that therapeutic ketosis, induced by dietary changes or ketone supplementation, may reduce airway hyperresponsiveness and inflammation. The primary ketone body,  $\beta$ -hydroxybutyrate (BHB), produced during carbohydrate scarcity, acts via cell-surface receptors and transporters, potentially mitigating asthma symptoms. Weight loss and caloric restriction increase ketone levels, correlating with reduced inflammation and improved asthma outcomes. We hypothesized that  $\beta$ -hydroxybutyrate (BHB) reduces bronchoconstriction and inflammation in asthma by targeting bronchial smooth muscle. Using human bronchial smooth muscle cells (HBSMC) *in vitro*, we demonstrate herein that BHB suppresses IL-1 $\beta$ -induced pro-inflammatory cytokine production through Free Fatty Acid Receptor 3 (FFAR3) activation. These findings suggest that bronchial smooth muscle is a key target of therapeutic ketosis, supporting BHB's potential benefits in preclinical asthma models.

### 3.2. Introduction

Asthma, a condition affecting the airways, is typically provoked by environmental triggers that initiate immune reactions in the lungs, leading to excessive inflammation and bronchoconstriction, and affects approximately 8.9% of adults and 6.7% of children in the United States<sup>1</sup>. Asthma-associated airway hyperresponsiveness can be evaluated using the methacholine challenge test, a diagnostic procedure designed to assess the responsiveness of airway smooth muscle. This test involves the inhalation of methacholine, a substance that causes airway constriction in individuals with asthma or other airway hyperreactivity conditions. By monitoring the degree of airway narrowing in response to methacholine, the test helps healthcare providers evaluate the functionality of the airway smooth muscle and plays a crucial role in confirming the diagnosis of asthma<sup>2,3</sup>. Bronchial smooth muscle is central to the pathophysiology of asthma, as its contraction causes airway narrowing, resulting in obstruction and breathing difficulties<sup>4</sup>.

Bronchial smooth muscle has been implicated in the pathophysiology of bronchial inflammation both as a target as well as a mediator of inflammatory reactions<sup>5,6</sup>. Type 2 inflammation is most common in asthmatics and is associated with the cytokines interleukin(IL)-4, IL-5, and IL-13, inflammatory cells including eosinophils, mast cells, basophils, type 2 innate lymphoid (ILC2) and T helper (Th2) lymphocytes, and immunoglobulin E (IgE)-producing plasma cells<sup>7,8</sup>. These asthma-relevant cell types can release pro-inflammatory mediators such as cytokines and chemokines that can affect bronchial smooth muscle cells<sup>6,9,10</sup>, the products of which further exacerbate airway hyperresponsiveness and remodeling<sup>4,5,10-12</sup>. Over time, this chronic inflammation can lead to structural changes in the airways, including thickening of the airway walls, fibrosis, and

increased mucus production, which in aggregate contribute to the persistent symptoms of asthma<sup>11,13</sup>. We and others have previously reported that bronchial smooth muscle cells secrete pro-inflammatory cytokines in response to immune stimulants, including lipopolysaccharide (LPS) and house dust mite (HDM) extract, as well as cytokines such as IL-1 $\beta$ <sup>10,14</sup> produced from the other asthma-relevant cell types. IL-1 $\beta$  is a potent pro-inflammatory cytokine produced from both immune (leukocytes) and non-immune cells<sup>12,15</sup>. Importantly, IL-1 $\beta$  is elevated in the airways<sup>16</sup> and serum of allergic and non-allergic asthmatic subjects compared to controls<sup>17</sup>.

Current treatments for asthma include broncho-relaxing  $\beta$ -agonists, anti-inflammatory corticosteroids, and biological immunotherapies targeting the type 2 immune response<sup>18</sup>. While these therapies provide effective disease control for most patients, individuals with 'difficult-to-treat' asthma often require alternative or additional approaches, especially as severe asthmatics with type 2 inflammation exhibit poor response to corticosteroids<sup>19</sup>. Recently, we have demonstrated the beneficial effects of increasing circulating ketone bodies, known as therapeutic ketosis, in multiple mouse models of obesity-associated<sup>20</sup> and allergic asthma<sup>21</sup>. Ketone bodies,  $\beta$ -hydroxybutyrate (BHB) and acetoacetate (AcAc), are synthesized in the liver from fatty acids<sup>22,23</sup>, either through dietary modifications<sup>23,24</sup> or the mobilization of adipose tissue during periods of increased energy demand<sup>23</sup>. Once produced, these ketone bodies are transported via the bloodstream to cells throughout the body.

Ketone bodies have been implicated in modulating key pathological processes in asthma. Initially recognized as an energy substrate for ATP production in the Krebs cycle during carbohydrate scarcity, ketone bodies have since been shown to influence cellular

processes through various mechanisms. They can stimulate cell-surface receptors such as the G-protein coupled receptors Hydroxycarboxylic Acid Receptor 2 (HCAR2 or GPR109a) and Free Fatty Acid Receptor 3 (FFAR3 or GPR41)<sup>23,25-29</sup>, or by uptake through transporters such as Monocarboxylate Transporter 1 (MCT1)<sup>27,30</sup>. Beyond these roles, ketone bodies also function as antioxidants<sup>31,32</sup> and exert effects through transcriptional and epigenetic regulation<sup>22,27,33</sup>, including suppression of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation<sup>31,25</sup>. Furthermore, they possess anti-inflammatory properties, notably through inhibition of the NLRP3 inflammasome, which decreases IL-1 $\beta$  production<sup>34-36</sup>. *In vivo*, dietary interventions such as weight loss and alternate-day caloric restriction raise BHB levels, which correlate with reduced asthmatic symptoms, including lower oxidative stress and inflammation in obese asthmatic patients<sup>14,37,38</sup>. Early ketone body elevations are linked to improved asthma symptoms in obese patients following bariatric surgery<sup>39,40</sup>, alternate-day caloric restriction<sup>37</sup>, and during treatment with GLP-1R agonists<sup>41</sup>. Independent of weight loss, therapeutic ketosis can be achieved through providing exogenous ketones or ketogenic precursors (*e.g.*, ketone esters). Therapeutic ketosis can be induced by providing exogenous ketones or ketogenic precursors (*e.g.*, ketone esters). Notably, increasing ketone levels is generally well-tolerated in humans<sup>42,43</sup>, highlighting its potential as a viable therapeutic approach.

Therapeutic ketosis achieved through feeding a high fat/low carbohydrate diet or by supplementing the normal diet with ketone esters, significantly and substantially decreases methacholine hyperresponsiveness in mouse models of asthma<sup>20,21</sup> and also decreases methacholine responsiveness in non-asthmatic mice<sup>20</sup>. As methacholine functions through the activation of bronchial smooth muscle<sup>44-47</sup>, we sought to study these

cells more directly to explore the impact of ketone bodies on the ability of bronchial smooth muscle to contribute to the inflammatory environment in asthma. We have established a well-functioning *in vitro* model using human bronchial smooth muscle cells (HBSMC), allowing us to directly observe the effects of BHB on this cell type<sup>21</sup>. Herein, we used HBSMC as an *in vitro* model of bronchial smooth muscle to address gaps and provide a valuable platform for investigating the therapeutic potential of ketone bodies and their underlying mechanisms of action.

We hypothesized that BHB can mitigate asthma-related pathologies, in part, by inhibiting pro-inflammatory cytokine production from bronchial smooth muscle cells. Our objectives were to assess the effectiveness of BHB in reducing IL-1 $\beta$ -induced pro-inflammatory cytokine secretion *in vitro* and to identify the mechanisms by which BHB may influence these effects. Gaining a deeper understanding of the efficacy and mechanisms by which BHB reduces pro-inflammatory cytokine production by human bronchial smooth muscle *in vitro* could offer valuable insights into potential novel targets for treating asthma and its associated symptoms.

### **3.3. Methods and Materials**

#### **3.3.1. Study Approval**

Studies involving potentially hazardous material were reviewed and approved by the University of Vermont's Institutional Biosafety Committee (REG201900052).

#### **3.3.2. Human Bronchial Smooth Muscle Cell Culture**

Primary human bronchial smooth muscle cells (HBSMC) isolated from a 45-yr-old female patient with asthma (Lonza, Morristown, NJ, Lot No. 00194850, Batch No. 0000195154) were cultured in smooth muscle cell growth medium-02 BulletKit (Lonza,

CC-3182) according to the manufacturer's instructions at 37°C in 95% humidified air containing 5% CO<sub>2</sub>. The cells were used within the first seven passages to ensure proper smooth muscle phenotype. Cell authentication was performed by Lonza (negative Factor VIII-related antigen, positive  $\alpha$ -Actin expression) and cells tested negative for mycoplasma (MycoDect Mycoplasma Detection Kit (Alstem, Richmond, CA)) before being utilized for experiments. For agonist-induced cytokine production experiments, human bronchial smooth muscle cells (HBSMC) were stimulated with 50 ug/mL House Dust Mite extract (HDM) (Stagallery/Greer, Cat No.B70, Lot No.390992), 100 ng/mL ultrapure lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (Invivogen, Cat No.tlrl-3pelps), or 10 ng/mL recombinant human IL-1 $\beta$  (Stem Cell Technologies, Cat No.78034.1).

For simultaneous exposure and stimulation experiments, HBSMC were plated at  $5 \times 10^4$  cells/cm<sup>2</sup> in 1mL of media in a 12-well plate and allowed to grow for 48 hours at 37°C and 5% CO<sub>2</sub>. HBSMC were exposed for 24 hours with vehicle or 1.25-10 mM beta-hydroxybutyric acid (BHBA) (Sigma-Aldrich, St. Louis, MO, Cat No.166898), sodium beta-hydroxybutyrate (NaBHB)(Sigma-Aldrich, Cat No.54965), (R)-beta-hydroxybutyric acid ((R)-BHBA) (Sigma-Aldrich, Cat No.54920), (S)-beta-hydroxybutyric acid ((S)-BHBA) (Sigma-Aldrich, Cat No.54925), FFAR3 agonist, AR420626 (AR) (Caymen, Cat No.17531), or MCT1 inhibitor, AZD3965 (Med Chem Express, Cat No.HY-12750), butyric acid (BA), sodium butyrate (SB), nicotinic acid (NA), and sodium nicotinic acid (NaNA) while being stimulated with 10 ng/mL recombinant human IL-1 $\beta$  (Stem Cell Technologies, Cat No.78034.1).

In pre-exposure experiments with subsequent stimulation, HBSMC were plated at  $5 \times 10^4$  cells/cm<sup>2</sup> in 1mL of media in a 12-well plate and allowed to grow for 24 hours at

37°C and 5% CO<sub>2</sub>. After the initial 24 hours, cells were exposed to vehicle or 1.25-10 mM BHBA, NaBHB, (R)-BHBA, (S)-BHBA, or AR for another 24 hours before being washed and stimulated with 10 ng/mL recombinant human IL-1 $\beta$ .

### **3.3.3. Cytokine Immunoassays**

Conditioned medium from the cell culture studies was collected at the indicated time points, centrifuged for 10 minutes at 3300 x g to eliminate debris, transferred into new tubes or multi-well plates, and frozen at -20°C until analysis. ELISAs to quantitate human IL-8/CXCL8 and IL-6 levels (DuoSets from R&D Systems) were used according to the manufacturer recommendations, with samples diluted to coincide with the range of the standards. A custom human magnetic Luminex assay (R&D Systems, Minneapolis, MN) was performed per manufacturers' instructions to measure cytokines present in the HBSMC supernatants, including CCL2, CCL4, CCL20, CXCL1, CXCL2, and G-CSF. Luminex assays were performed using the Bio-Rad Bio-Plex suspension array system, Bio-Rad Bio-Plex Pro II wash station, and Bio-Plex Manager 6.0 software (Bio-Rad, Hercules, CA). Concentrations were calculated by five-place logistic regression from standards within 70%-130% of the expected values using Bio-Rad Manager 6.0. Studies were conducted with all BHB compounds used in the experiments to validate that they did not interfere substantially with the ability of the ELISAs to accurately quantify the recombinant standards.

### **3.3.4. Statistical Analyses**

All experiments included multiple biological replicates for each condition. Outliers were identified and removed using the ROUT method (Q=1%) and the cleaned data were analyzed using unpaired one-way ANOVA with post-hoc multiple comparisons: Tukey's

test (for comparing all means) or Dunnett's test (for comparing each mean to a control), were performed using GraphPad Prism 10.2.3 (GraphPad Software, Inc., La Jolla, CA). Data are presented as means  $\pm$  SEM from representative experiments. P values below 0.05 are considered statistically significant, and significance levels are indicated in the figure legends.

### **3.4. Results**

#### **3.4.1. Human Bronchial Smooth Muscle Cells (HBSMC) Inducibly Secrete Pro-Inflammatory Cytokines.**

Although known primarily as a contractile cell type, bronchial smooth muscle can produce many chemokines and cytokines in response to various agonists<sup>10,14,48</sup>. A custom human magnetic Luminex assay was performed to measure cytokines present in the HBSMC supernatants following stimulation for 24 hours with House Dust Mite extract (HDM), lipopolysaccharide (LPS), and human recombinant IL-1 $\beta$  to determine which pro-inflammatory cytokines were produced. This analysis revealed that CCL2 (**Figure 3.1A**), CCL4 (**Figure 3.1B**), CCL20 (**Figure 3.1C**), CXCL1 (**Figure 3.1D**), CXCL2 (**Figure 3.1E**), G-CSF (**Figure 3.1F**), IL-6 (**Figure 3.1G**), and CXCL8/IL-8 (**Figure 3.1H**) were produced significantly when HBSMC were stimulated with 10 ng/mL of recombinant human IL-1 $\beta$ .

#### **3.4.2. BHB Attenuates IL-1 $\beta$ -Induced Pro-Inflammatory Cytokine Production From HBSMC**

Ketone bodies, acetoacetate (AcAc) and especially  $\beta$ -hydroxybutyrate (BHB), have been reported to exert anti-inflammatory effects, especially via inhibition of NLRP3 inflammasome activation<sup>34-36</sup>. In our recent studies, we demonstrated the therapeutic benefits of elevating circulating ketone bodies—a state known as therapeutic ketosis—in

multiple mouse models of both obese<sup>20</sup> and allergic asthma<sup>21</sup>, which effectively mitigated methacholine-induced airway hyperresponsiveness and significantly improved lung function *in vivo*<sup>20,21</sup>. We also demonstrated *in vitro* that BHB attenuated pro-inflammatory cytokine secretion from several asthma-relevant cell types, including macrophages, CD4 T lymphocytes, and airway epithelium<sup>21</sup>. Building on these findings, we investigated the effects of ketone bodies on IL-1 $\beta$ -induced pro-inflammatory cytokine production from human bronchial smooth muscle cells (HBSMC). We determined that simultaneous exposure for 24 hours to beta-hydroxybutyric acid (BHBA) or sodium beta-hydroxybutyrate (NaBHB) in the presence of 10ng/mL human recombinant IL-1 $\beta$  caused a dose-dependent decrease in the concentrations of the pro-inflammatory cytokines IL-6 (**Figure 3.2A**) and CXCL8/IL-8 (**Figure 3.2B**) compared to cells stimulated with IL-1 $\beta$  in the absence of BHB or in the presence of appropriate pH controls. BHBA significantly decreased both IL-6 and IL-8 concentrations in a dose-dependent manner, but when neutralized to a pH of 7 the robust effect in attenuating IL-6 secretion at all concentrations and in attenuating IL-8 secretion at a BHBA concentration of 10mM was lost. NaBHB did not exert a robust inhibitory effect on pro-inflammatory cytokine production until the pH of the 10mM working solution was matched to the pH of the working concentration of 10mM BHBA. The acidified NaBHB exerted a more robust inhibitory dose response than the non-acidified NaBHB or the neutralized BHBA. An equivalent concentration of HCl matching the acidity (pKa) of the acidic BHB molecules (10mM) modestly attenuated IL-1 $\beta$ -induced secretion of IL-6 and IL-8, but not to the extent of the BHBA or the acidified NaBHB.

### **3.4.3. Inhibition of Pro-Inflammatory Cytokine Production from HBSMC by BHB Is Not Enantioselective.**

To determine if the inhibitory effect elicited by BHB on IL-1 $\beta$ -induced IL-6 and IL-8 production was through a mechanism dependent on the metabolism of BHB as an energetic substrate, we utilized (R)-beta-hydroxybutyric acid ((R)-BHBA) and (S)-beta-hydroxybutyric acid ((S)-BHBA), the individual enantiomers of the racemic BHBA, as well as included exposure to the (R) enantiomer of NaBHB, and a cocktail representative of circulating ketone bodies *in vivo*. The BHB cocktail consists of 78% BHB, 20% AcAc, and 2% acetone, which are biologically consistent with the concentrations of ketone bodies in circulation during fasting<sup>49</sup>. In this study, with simultaneous exposure to the BHB compounds and 10 ng/mL IL-1 $\beta$  for 24 hours, we determined that both the (R) and (S) enantiomers, the (R) enantiomer of NaBHB, and the BHB cocktail significantly inhibited IL-1 $\beta$ -induced secretion of IL-6 (**Figure 3.3A**) and CXCL8/IL-8 (**Figure 3.3B**) from HBSMC. In this study, all conditions except the (R) enantiomer of NaBHB demonstrated dose-dependent inhibition of pro-inflammatory cytokine production.

### **3.4.4. Short-Chain Carboxylic Acids Inhibit IL-1 $\beta$ -Induced Pro-Inflammatory Cytokine Production From HBSMC.**

As BHB has been proposed to elicit effects through activation of cell surface receptors such as hydroxycarboxylic acid receptor 2 (HCAR2)<sup>22 23 25 26 27</sup>, we sought to determine if a pharmacological HCAR2 agonist would elicit similar beneficial inhibitory effects as BHB. Furthermore, as we have previously shown that small molecules structurally similar to BHB have anti-inflammatory effects<sup>34</sup>, and that short-chain carboxylic acids exert anti-inflammatory functions<sup>50,51</sup>, we wanted to determine if butyric acid and sodium butyrate could elicit the same inhibitory effects as BHB. When HBSMC

were simultaneously exposed to butyric acid, sodium butyrate, nicotinic acid, and sodium nicotinic acid, as well as the HCAR2 agonist, and stimulated with 10ng/mL IL-1 $\beta$ , production of IL-6 (**Figure 3.4A**) and CXCL8/IL-8 (**Figure 3.4B**) was dose-dependently attenuated. Whereas the nicotinic acid and the sodium nicotinic acid both significantly inhibited secretion of the pro-inflammatory cytokines, the butyric acid and sodium butyrate had a much more substantial effect, with the lowest doses of butyric acid and sodium butyrate having a greater impact than the highest dose of exposure, which was 10mM of the nicotinic acid or sodium nicotinic acid.

#### **3.4.5. Pre-Exposure of HBSMC to BHBA Attenuates IL-1 $\beta$ -Induced Pro-Inflammatory Cytokine Production.**

As our previously reported *in vivo* studies have modeled endogenous ketone augmentation through dietary interventions that provide elevated systemic concentrations of these molecules over a protracted period, we sought to model this extended exposure in an *in vitro* system. HBSMC were pre-exposed to biologically relevant concentrations of beta-hydroxybutyric acid (BHBA) for 24 hours, followed by washing and subsequent stimulation with 10 ng/mL human recombinant IL-1 $\beta$ . Pre-exposure to BHBA significantly attenuated IL1 $\beta$ -induced IL-6 (**Figure 3.5A**) and CXCL8/IL-8 (**Figure 3.5B**) production in a dose-dependent manner, consistent with the inhibitory effects observed during simultaneous exposure of BHBA and IL-1 $\beta$ . While not as robust as simultaneous stimulation, pretreatment with the higher concentrations of 5mM and 10mM BHBA significantly attenuated IL-6 and IL-8 production from these cells. Although not statistically significant, a trend towards a dose-responsive effect was observed.

#### **3.4.6. Simultaneous Exposure of BHBA with FFAR3 Agonist Dose-Dependently Attenuates IL-1 $\beta$ -Induced Pro-Inflammatory Cytokine Production.**

Ketone bodies, particularly BHB, have been proposed to mediate their beneficial effects via uptake through Monocarboxylic Transporter 1 (MCT1)<sup>26,43</sup> or through a Free Fatty Acid 3 (FFAR3)-dependent pathway<sup>26-28,52</sup>. To evaluate the involvement of these receptors, we tested the MCT1 inhibitor AZD3965 (AZD) in the presence of the BHB compounds. HBSMCs were exposed to biologically relevant concentrations of BHB to model endogenous ketone augmentation, a combination of both the increasing concentrations of BHB and 50 $\mu$ M of the FFAR3 agonist, or a combination of increasing concentrations of BHB and 40nM MCT1 inhibitor (AZD) while stimulated with 10 ng/mL recombinant human IL-1 $\beta$  for 24 hours. The FFAR3 agonist in combination with BHB dose-dependently attenuated IL-6 (**Figure 3.6A**) and CXCL8/IL-8 (**Figure 3.6B**) production, and to a greater extent than BHBA alone. In contrast, the presence of AZD did not block the inhibitory effects of BHB on IL-1 $\beta$ -induced pro-inflammatory cytokine production, indicating that these effects are independent of MCT1-mediated uptake of BHB.

#### **3.4.7. FFAR3 Activation Is Sufficient to Inhibit IL-1 $\beta$ -Induced HBSMC Pro-Inflammatory Cytokine Production.**

Given that beta-hydroxybutyrate (BHB) has been suggested to act as a ligand for FFAR3 and exert its beneficial effects through a FFAR3-mediated pathway, we investigated whether activating FFAR3 could replicate the inhibitory effects of BHB on IL-1 $\beta$ -induced pro-inflammatory cytokine production from HBSMC. Whereas in our previous study we evaluated the combination of BHBA and FFAR3 agonist, we sought to determine whether FFAR3 activation alone could elicit the same effects. HBSMC were exposed to biologically

relevant concentrations of BHBA, 50 $\mu$ M FFAR3 agonist (AR) alone, or to a combination of the concentrations of BHBA and 50 $\mu$ M FFAR3 agonist. HBSMCs exposed to BHBA and AR individually, as well as those exposed to the combination of BHBA and the FFAR3 agonist, significantly attenuated IL-1 $\beta$ -induced HBSMC production of IL-6 (**Figure 3.7A**) and CXCL8/IL-8 (**Figure 3.7B**), suggesting that the observed inhibitory effects of BHB may indeed be mediated, at least in part, through the activation of FFAR3. Notably, whereas the FFAR3 agonist (AR) had a robust effect alone, the combination of the FFAR3 agonist and BHBA exerted the greatest inhibition, signifying a synergistic effect.

### 3.5. Discussion

The growing asthma epidemic underscores the urgency for new treatment approaches aimed at managing severe and challenging endotypes, ultimately enhancing the well-being of individuals affected by this complex and chronic disorder. One promising strategy that has attracted increasing interest is therapeutic ketosis, recognized for its wide-ranging beneficial effects across various pathological conditions and diseases<sup>53-56</sup>. In our previous studies, we reported that therapeutic ketosis, achieved through dietary interventions such as a ketogenic diet, ketogenic precursor supplementation, or ketone ester intake, augments circulating concentrations of BHB and reduces methacholine hyperresponsiveness, a pathophysiological feature in models of preclinical allergic-associated and obesity-associated asthma<sup>20,21</sup>, decreases airway inflammation, and inhibits a multitude of pathological activities of asthma relevant cell types<sup>20,21</sup>. Several other studies have shown the *in vivo* anti-inflammatory activities of therapeutic ketosis<sup>36,57,58</sup>, with some affecting lung inflammation.

Although we previously demonstrated that exogenous ketones reduce pro-inflammatory cytokine production and exert anti-inflammatory effects *in vitro*<sup>20,21</sup>, their impact on bronchial smooth muscle cells remains unexplored. Given that bronchial smooth muscle cells are key players in bronchial inflammation, acting as both targets and mediators of inflammatory responses<sup>5,6</sup>, investigating their cytokine production represents a critical research area in airway inflammation and asthma management. In preclinical asthma models, distal airway and peripheral lung dysfunction are commonly observed<sup>59</sup>. Our prior work showed that elevated BHB concentrations confer protective effects on these regions<sup>20,21</sup>, including reductions in methacholine-induced hyperresponsiveness, airway resistance, tissue damping, and tissue elastance—physiological markers that increase due to heterogeneous ventilation caused by uneven airway narrowing<sup>59,60</sup> that are closely linked to peripheral airway contraction. These findings suggest that ketones may directly modulate bronchial smooth muscle to reduce airway hyperresponsiveness.

As reported herein, our studies demonstrate that BHB inhibits bronchial smooth muscle pro-inflammatory cytokine production induced by the asthma-relevant agonist, IL-1 $\beta$ <sup>10,12,14-17</sup>. The mechanisms by which BHB can elicit these effects may include activation of cell surface receptors such as free fatty acid receptor 3 (FFAR3)<sup>22,23,27,28</sup> or hydroxycarboxylic acid 2 (HCAR2)<sup>25,26,29</sup>. Given that BHB has been proposed to function across a wide range of applications and through many G-coupled protein receptors (GPCRs)<sup>22,25-29</sup>, including perhaps inducing tachyphylaxis, it likely operates through a fundamental mechanism that is universally relevant to these processes, such as ion exchange<sup>27,36,61</sup>, calcium signaling<sup>27,28</sup>, or altering membrane potential<sup>61</sup>.

Once determining that IL-1 $\beta$  significantly caused pro-inflammatory cytokines to be produced from human bronchial smooth muscle cells (HBSMC), our *in vitro* studies demonstrated that BHB attenuated the production of IL-6 and IL-8 (CXCL8) in a dose-dependent manner. These studies included conditions using beta-hydroxybutyric acid (BHBA) as well as sodium beta hydroxybutyrate (NaBHB). The inclusion of both of these compounds in our studies allowed us to explore the influence of pH by comparing BHBA with a 1M stock pH of 2.09 and the NaBHB, with a 1M stock pH of 9.73. When diluted to biologically relevant concentrations in buffered media, which resulted in near-neutral pH in the cell culture media (*i.e.*, pH 7.2), only BHBA had a significant inhibitory effect on IL-1 $\beta$ -induced production of pro-inflammatory cytokines *in vitro*. These results suggest that the acidic nature of the BHBA influences its ability to inhibit pro-inflammatory cytokine production. The inclusion of pH controls supports this notion in that when the 1M stock pH of the NaBHB was changed to be reminiscent of the 1M stock pH of the BHBA, the inhibitory effects of BHB were then present. Conversely, when the 1M stock pH of the BHBA was adjusted to the 1M stock pH of the NaBHB, the inhibitory effects were lost. The inclusion of a hydrochloric acid (HCl) pH-equivalent control, which did not inhibit pro-inflammatory cytokine production from IL-1 $\beta$  stimulated HBSMCs, implies that although the acid is sufficient to elicit modest inhibitory effects, the inclusion of BHB is necessary to enhance the effect.

BHBA is a racemic mixture containing both enantiomers (R)-BHB and (S)-BHB. Since only (R)-BHB can be efficiently metabolized by  $\beta$ -hydroxybutyrate dehydrogenase to form acetyl-CoA that is subsequently converted into ATP and used as an energy substrate<sup>62</sup>, we confirmed that the attenuation of pro-inflammatory cytokine production

occurs independently of BHB's metabolic function. Additionally, the MCT1 inhibitor, AZD3965 (AZD) failed to block BHB's effects, indicating that MCT1-mediated uptake is not involved in the inhibitory capacity of BHB. The comparison between the (R)-BHB and the (R) enantiomer of the NaBHB (Na-R-BHB) reiterates that the acid allows for a robust pro-inflammatory cytokine production to be inhibited.

The HCAR2 agonists, nicotinic acid and sodium nicotinic acid, elicited similar inhibitory effects, but not to the extent of BHBA or the structurally similar compounds butyric acid and sodium butyrate, suggesting that some inhibitory effects may be mediated through HCAR2. Most likely, BHB does not elicit its attenuating effects on IL-1 $\beta$ -induced pro-inflammatory cytokine production through activation of HCAR2. Furthermore, the FFAR3 agonist AR420626 elicited inhibitory effects similar to and in an additive manner with BHB, suggesting that FFAR3 activation contributes substantially to the inhibitory action of BHB. Confirmation of this action would require an FFAR3 antagonist, a compound that is not currently available. Despite this limitation, these findings support future investigations, including studies using FFAR3 knockdown cells and FFAR3 knockout mice, to clarify the receptor's involvement.

Simultaneous exposure to BHBA during IL-1 $\beta$  stimulation was more effective at reducing IL-1 $\beta$ -induced pro-inflammatory cytokine production in HBSMCs compared to BHBA pre-exposure and subsequent stimulation with IL-1 $\beta$ . This effect may be partly due to greater cytokine induction during simultaneous exposure, likely caused by increased cell confluency from the additional 24 hours in culture. Nonetheless, in a clinical setting, 'therapeutic ketosis' would maintain consistently elevated BHB concentrations, which

could be modeled *in vitro* using the pre-exposure approach without removing BHBA before assessing IL-1 $\beta$ -induced pro-inflammatory cytokine production from HBSMCs.

There are several limitations to our findings. Namely, we conducted *in vitro* studies using primary human bronchial smooth muscle cells instead of *ex vivo* studies or human subjects. While informative, human cell studies fail to capture the prolonged and complex nature of human asthma. They offer a reductionist approach by focusing on a single cell type and a specific agonist that readily initiates an inflammatory cascade, allowing us to observe only the inhibitory effects of BHB at that initial step without assessing its role in maintaining suppression or facilitating recovery. Another notable limitation of this study is the lack of assessment of the fibrotic remodeling response of bronchial smooth muscle to IL-1 $\beta$  stimulation. In our previous work, we used an *in vivo* mouse model of severe asthma characterized by fibrotic remodeling and found that therapeutic ketosis still produced beneficial effects<sup>21</sup>. Overall, these findings support the potential of therapeutic ketosis as a promising treatment strategy for asthma, demonstrating its efficacy in both fibrotic and non-fibrotic contexts and highlighting its broad anti-inflammatory and protective effects. To gain a more comprehensive understanding of BHB's impact on the long-term outcomes of an asthma exacerbation, diverse models, including *in vivo*, *ex vivo*, more advanced *in vitro* systems, and *in clinic* studies could be used to evaluate not only the initiation but also the maintenance and resolution of inflammation.

Therapeutic ketosis is being explored in the clinical treatment of respiratory diseases, including asthma<sup>63,64</sup> and Cystic fibrosis<sup>65</sup>. In asthma trials, medium-chain triglyceride supplementation is used as a substrate to promote ketone body formation *in vivo*, whereas Cystic fibrosis trial uses a ketone ester precursor, which more rapidly and

efficiently increases circulating ketone body concentrations and is the same compound we have employed in mouse models of obese asthma and allergic asthma<sup>20,21</sup>. The inclusion of therapeutic ketosis in clinical trials highlights the increasing scientific interest in its potential benefits. The positive results of the studies reported herein would strengthen the case for investigating therapeutic ketosis into asthma treatment regimens and stimulate further mechanistic and clinical studies into its capacity to serve as a complementary or alternative asthma therapy. This ongoing exploration emphasizes the importance of understanding ketone-based interventions and calls for continued examination of their underlying mechanisms and therapeutic potential.

### 3.6. Figure Legends

#### **Figure 3.1. IL-1 $\beta$ induces pro-inflammatory cytokine secretion from HBSMC.**

HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 50 mg/mL House Dust Mite extract (HDM), 100 ng/mL lipopolysaccharide (LPS), or 10 ng/mL recombinant human IL-1 $\beta$  for 24 hours and (A) CCL2, (B) CCL20, (C) G-CSF, (D) CCL4, (E) CXCL2, (F) CXCL1 (G) IL-6 and (H) CXCL8/IL-8 levels were measured. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  compared to the vehicle.

#### **Figure 3.2. BHB dose-dependently attenuates IL-1 $\beta$ -stimulated pro-inflammatory cytokine production from HBMSC.**

HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 10 ng/mL recombinant human IL-1 $\beta$  in the presence of increasing concentrations of BHBA, NaBHB, or the appropriate pH controls of each for 24 hours and (A) IL-6 or (B) CXCL8/IL-8 were measured. n=4 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq$

0.0001 compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , ### $P \leq 0.001$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

**Figure 3.3. BHB enantiomers dose dependently attenuate IL-1 $\beta$ -stimulated pro-inflammatory cytokine production from HBSMC.**

HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 10 ng/mL recombinant human IL-1 $\beta$  in the presence of increasing concentrations of (R)-BHBA, (S)-BHBA, Na-(R)-BHBA, or a BHB cocktail composed of 78% (R)-BHBA, 20% AcAc, and 2% acetone, for 24 hours and (A) IL-6 or (B) CXCL8/IL-8 were measured. n=4 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , ### $P \leq 0.001$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

**Figure 3.4. Short-chain carboxylic acids inhibit IL-1 $\beta$ -induced pro-inflammatory cytokine secretion.**

HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 10 ng/mL recombinant human IL-1 $\beta$  in the presence of increasing concentrations of butyric acid (BA), sodium butyrate (SB), nicotinic acid (NA), or sodium nicotinic acid (NaNA) for 24 hours and (A) IL-6 or (B) CXCL8/IL-8 were measured. n=4 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , ### $P \leq 0.001$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

**Figure 3.5. Pre-exposure to BHBA dose-dependently decreases IL-1 $\beta$ -induced pro-inflammatory cytokine secretion.**

HBSMCs remained unexposed or were exposed *in vitro* to increasing concentrations of beta hydroxybutyric acid (BHBA) for 24 hours, washed and then stimulated with 10 ng/mL recombinant human IL-1 $\beta$  for another 24 hours. (A) IL-6 or (B) CXCL8/IL-8 were then measured. n=4 per group; values are inclusive of studies performed three times. \*\*\* $P \leq 0.001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , ### $P \leq 0.001$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

**Figure 3.6. Simultaneous exposure to BHBA with a FFAR3 agonist or MCT1 inhibitor dose-dependently attenuates IL-1 $\beta$  induced pro-inflammatory cytokine secretion.**

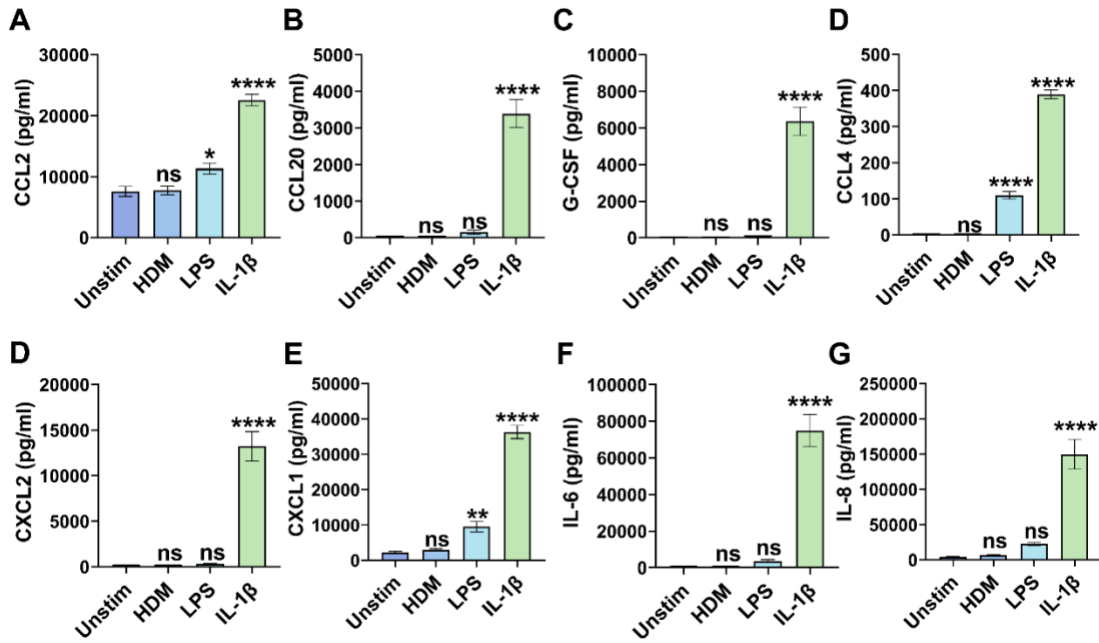
HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 10 ng/mL recombinant human IL-1 $\beta$  in the presence of increasing concentrations of beta hydroxybutyric acid (BHBA), a combination of FFAR3 agonist (AR) and BHBA, or a combination of MCT1 inhibitor (AZD) and BHBA for 24 hours and (A) IL-6 or (B) CXCL8/IL-8 were measured. n=4 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

**Figure 3.7. FFAR3 activation is sufficient to inhibit IL-1 $\beta$  induced pro-inflammatory cytokine secretion.**

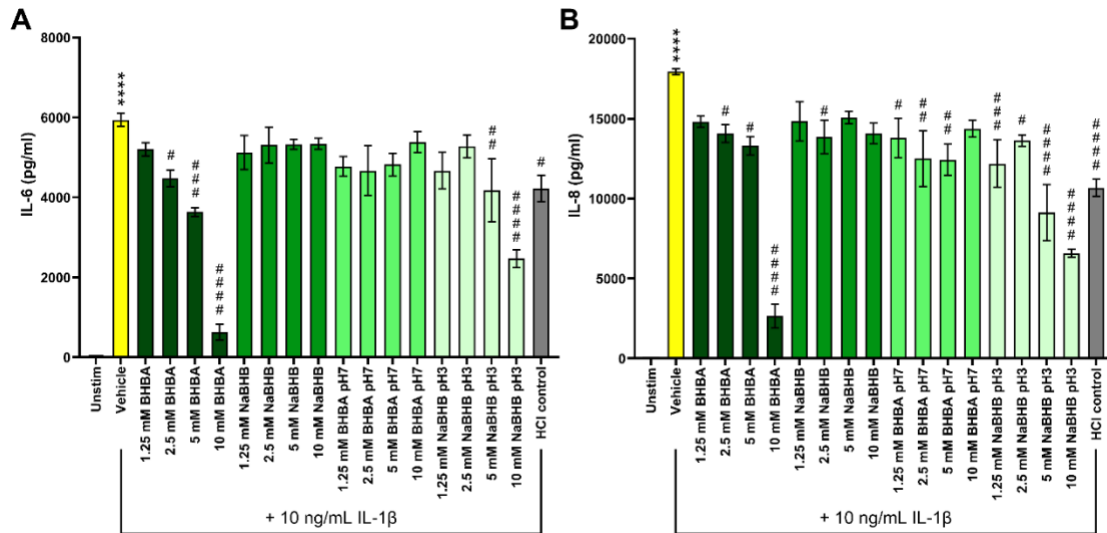
HBSMCs were unstimulated (Unstim) or stimulated *in vitro* with 10 ng/mL recombinant human IL-1 $\beta$  in the presence of increasing concentrations of beta hydroxybutyric acid (BHBA), a combination of FFAR3 agonist (AR) and BHBA, or a combination of MCT1 inhibitor (AZD) and BHBA for 24 hours and (A) IL-6 or (B) CXCL8/IL-8 were measured. n=4 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , #### $P \leq 0.0001$  compared to IL-1 $\beta$ .

### 3.7. Figures

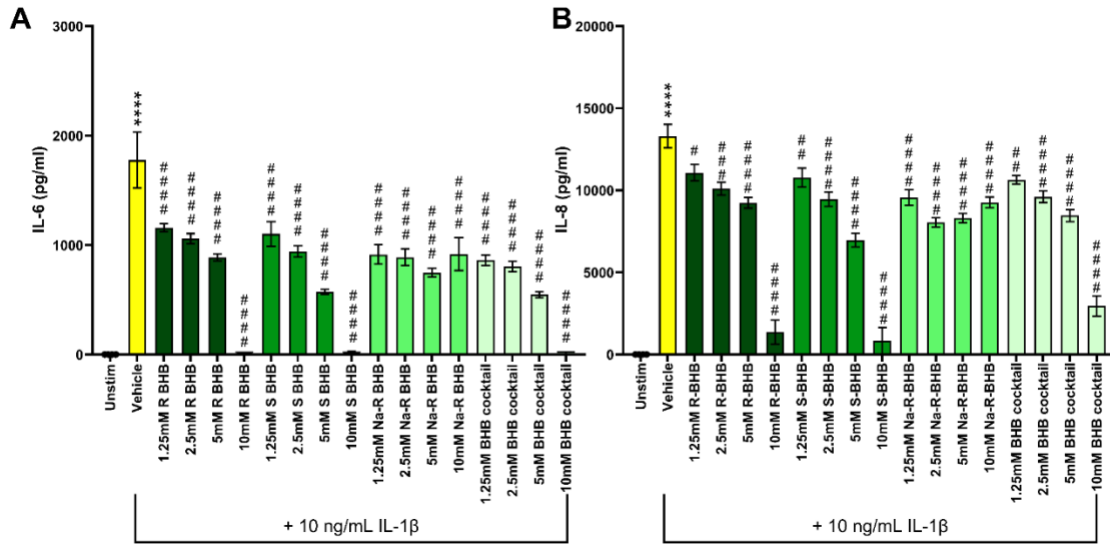
Figure 3.1. IL-1 $\beta$  induces pro-inflammatory cytokine secretion from HBSMC.



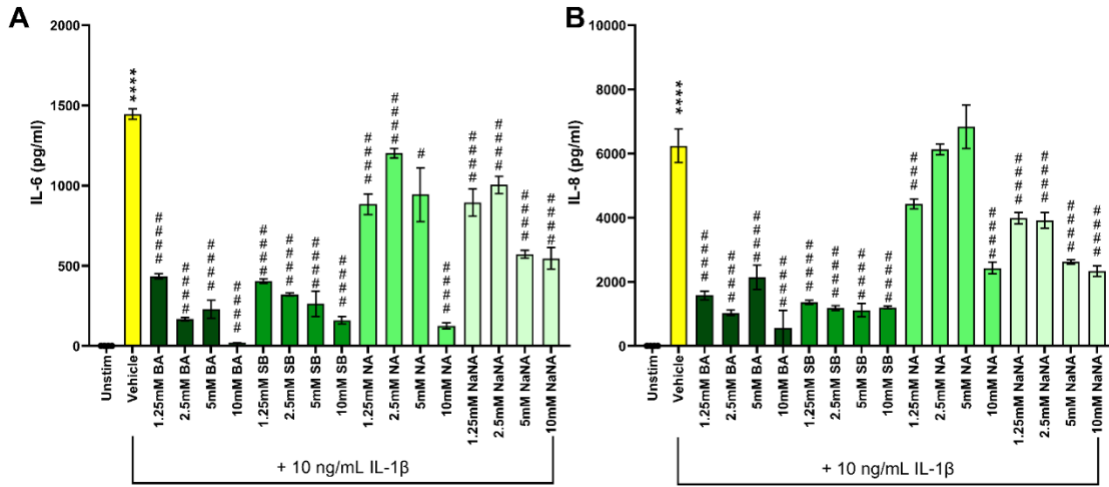
**Figure 3.2. BHB dose-dependently attenuates IL-1 $\beta$ -stimulated pro-inflammatory cytokine production from HBMSC.**



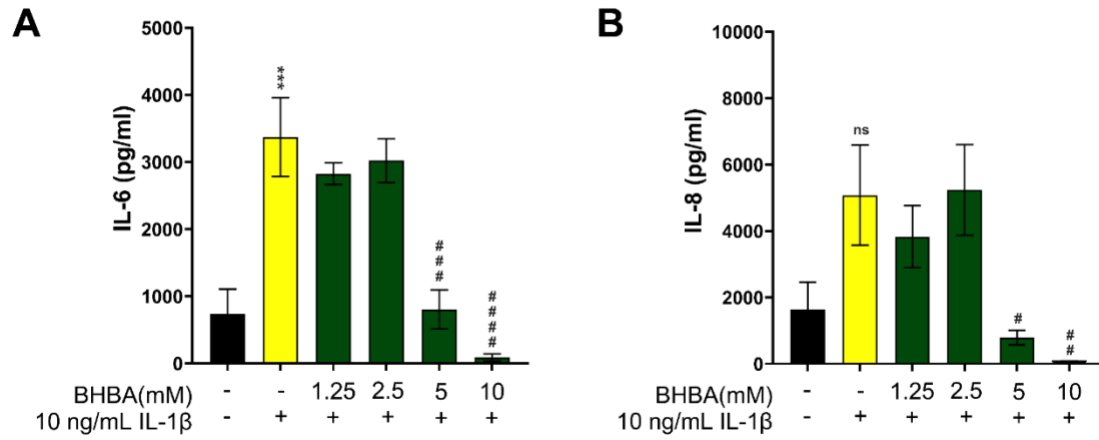
**Figure 3.3. BHB enantiomers dose dependently attenuate IL-1 $\beta$ -stimulated pro-inflammatory cytokine production from HBSMC.**



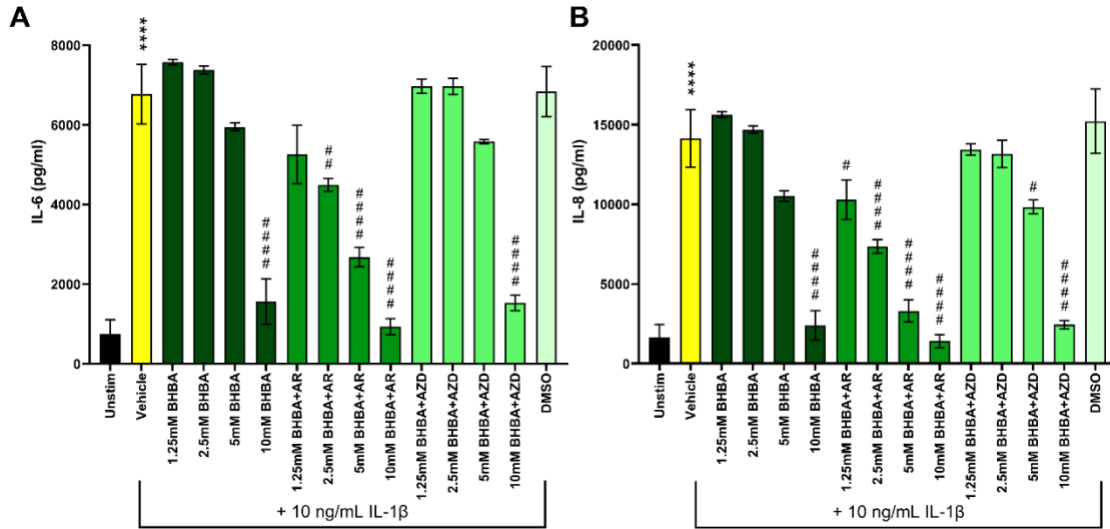
**Figure 3.4. Short-chain carboxylic acids inhibit IL-1 $\beta$ -induced pro-inflammatory cytokine secretion.**



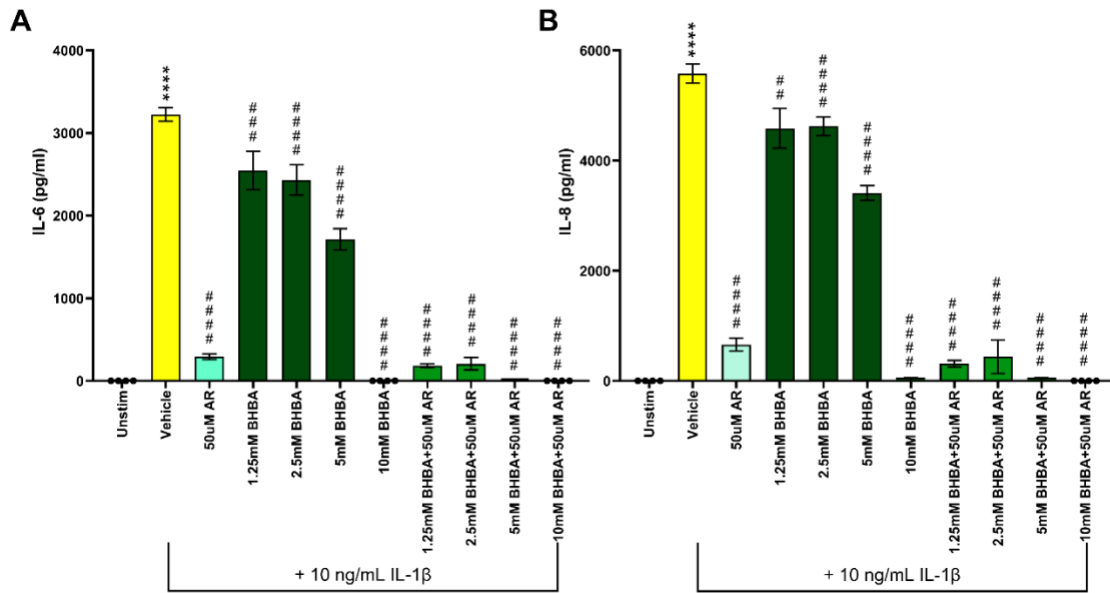
**Figure 3.5. Pre-exposure to BHBA dose-dependently decreases IL-1 $\beta$ -induced pro-inflammatory cytokine secretion.**



**Figure 3.6. Simultaneous exposure to BHBA with a FFAR3 agonist or MCT1 inhibitor dose-dependently attenuates IL-1 $\beta$  induced pro-inflammatory cytokine secretion.**



**Figure 3.7. FFAR3 activation is sufficient to inhibit IL-1 $\beta$  induced pro-inflammatory cytokine secretion.**



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## **Chapter 4: Beta-Hydroxybutyrate Inhibits Bronchial Smooth Muscle Contraction**

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#### 4.1. Abstract

Asthma is a chronic respiratory condition characterized by airway inflammation, remodeling, and hyperresponsiveness to triggers causing airway constriction. Bronchial smooth muscle plays a critical role by narrowing airways, leading to obstruction and breathing difficulties, often exacerbated by mast cell infiltration and histamine release. Whereas current treatments, including bronchodilators, corticosteroids, and biologics provide effective management for most patients, alternative therapies are needed for difficult-to-treat asthma. Recent research highlights the potential of therapeutic ketosis, achieved through dietary interventions or supplementation with exogenous ketones, to reduce airway hyperresponsiveness and inflammation. Ketone bodies, known for providing energy during carbohydrate scarcity, also influence asthma by activating cell-surface receptors and transporters. *In vivo*, interventions like weight loss and caloric restriction increase ketone body levels, correlating with improved asthma symptoms, reduced oxidative stress, and inflammation. These effects suggest ketone bodies, particularly  $\beta$ -hydroxybutyrate, may play a therapeutic role in mitigating bronchoconstriction and smooth muscle contraction in asthma. We utilize human bronchial smooth muscle cells (*in vitro*) and mouse precision-cut lung slices (PCLS) (*ex vivo*) to assess the effects of BHB on histamine-induced bronchoconstriction. Brightfield microscopy showed that BHB reduces contraction in human bronchial smooth muscle cells, an effect involving free fatty acid receptor 3 (FFAR3) activation. Light microscopy of PCLS revealed that BHB inhibits airway narrowing and cellular extrusion, demonstrating its ability to mitigate bronchoconstriction by suppressing smooth muscle contraction. These results implicate bronchial smooth muscle as a cellular target of therapeutic ketosis, an important contributor to the beneficial effects of BHB in preclinical models of asthma.

## 4.2. Introduction

Asthma is a chronic, heterogenous, and widespread respiratory syndrome affecting patients' breathing that is characterized by hyperresponsiveness to broncho-constrictive triggers, inflammation, and remodeling of the airway, and with which more than a quarter of a billion people are diagnosed worldwide<sup>1</sup>. Airway hyperresponsiveness can be assessed through the methacholine challenge test, which serves as a diagnostic tool to evaluate airway smooth muscle function and aid in the clinical diagnosis of asthma<sup>2,3</sup>. Bronchial smooth muscle plays a key role in the pathophysiology of asthma by causing airway narrowing through cellular contraction, leading to obstruction and difficulty breathing<sup>4</sup>. Stimulation of bronchial smooth muscle can also contribute to lung inflammation through the production of pro-inflammatory cytokines<sup>5</sup>.

Bronchial smooth muscle can be both affected by the inflammatory environment as well as contribute to it. Mast cells can infiltrate into the smooth muscle tissue in allergic asthma and thereby induce or augment airway hyperresponsiveness<sup>6</sup>. This infiltration can cause bronchial smooth muscle cells to release pro-inflammatory chemotactic cytokines (chemokines)<sup>7,8</sup>, and the mast cells release mediators such as histamine<sup>8,9</sup> which contributes to airway obstruction by causing smooth muscle contraction, increasing bronchial secretions, and provoking mucosal edema<sup>9</sup>. Bronchoconstriction was amongst the first biological effects described for histamine<sup>10</sup> and can cause bronchial smooth muscle contraction to the same extent as M1 muscarinic receptor agonists (*e.g.*, methacholine) and has been suggested to generate more contraction in peripheral tissue<sup>11</sup>.

Current treatments for asthma include broncho-relaxing  $\beta$ -agonists, anti-inflammatory corticosteroids, and biological immunotherapies targeting the innate and adaptive immune

response<sup>12</sup>. While most patients achieve effective disease control, some with 'difficult-to-treat' asthma require alternative or additional therapies. Our lab<sup>13,14</sup> and others<sup>15</sup> have reported the efficacy of augmenting circulating ketone body concentrations, known as “therapeutic ketosis”, to mitigate the pathophysiological manifestations in mouse models of obese and allergic asthma. Ketone bodies,  $\beta$ -hydroxybutyrate (BHB) and acetoacetate (AcAc), are endogenously produced in the liver from fatty acids<sup>16,17</sup>, either through diet<sup>17,18</sup> or adipose tissue mobilization during energy demand<sup>17</sup>, and are then circulated to cells throughout the body.

Ketone bodies have been implicated in modulating key pathological processes in asthma. Originally described as an energetic substrate for the production of ATP in the Krebs Cycle during time of carbohydrate scarcity<sup>19</sup>, ketone bodies have also been reported to exert their effects through the stimulation of cell-surface receptors, such as G-protein coupled receptors HCAR2 (GPR109a) and FFAR3 (GPR41)<sup>15,17,20-23</sup>, or by uptake through transporters such MCT1<sup>22,24</sup>. Additionally, ketone bodies act as antioxidants<sup>25,26</sup> and have anti-inflammatory properties, including the suppression of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation<sup>25,25</sup> and inhibition of the NLRP3 inflammasome, which reduces IL-1 $\beta$  production<sup>27-29</sup>. *In vivo*, dietary interventions such as weight loss and alternate-day caloric restriction raise BHB levels, which correlate with reduced asthmatic symptoms, including lower oxidative stress and inflammation in obese asthmatics<sup>30-32</sup>.

Notably, ketone augmentation or therapeutic ketosis is well-tolerated in human subjects<sup>33,34</sup>. Early ketone body elevations are associated with decreased asthma symptoms in obese asthmatic subjects following bariatric surgery<sup>35,36</sup>, undergoing alternate-day caloric restriction<sup>30</sup>, and during treatment with GLP-1R agonists<sup>37</sup>. Therapeutic ketosis can

also be achieved through providing exogenous ketones or ketogenic precursors (*e.g.*, ketone esters). Therapeutic ketosis is achieved through feeding a high fat/ low carbohydrate diet or by supplementing the normal diet with ketones esters, significantly and substantially decreases asthma associated methacholine responsiveness<sup>13,14</sup> and also decreases methacholine responsiveness in non-asthmatic mice<sup>13</sup>. As methacholine functions through the activation of bronchial smooth muscle<sup>38-41</sup>, we sought to study these cells more directly to explore the impact of ketone bodies on their activity. We developed a reliable *in vitro* model using human bronchial smooth muscle cells (HBSMC), enabling direct observation of BHB's effects on morphological change in this cell type<sup>14</sup>. Herein, we used HBSMC as an *in vitro* model of bronchial smooth muscle along with precision cut lung slices (PCLS) as an *ex vivo* model to more thoroughly assess the effects of ketone bodies on these cells and the mechanisms whereby they function.

PCLS are a valuable *ex vivo* model for studying airway reactivity and contraction, closely mimicking lung architecture and cellular diversity<sup>42-45</sup>. They bridge the gap between *in vitro* and *in vivo* studies and are particularly useful in asthma research, demonstrating altered responses like hyperresponsiveness and bronchoconstriction when exposed to various agonists<sup>42,45-47</sup>. Building on the utility of PCLS in asthma research, this study explores the potential of ketone bodies, particularly BHB, to directly influence bronchial smooth muscle contraction, addressing gaps in our understanding of their therapeutic potential.

Although connections between the underlying mechanisms of asthma and the benefits of ketone body augmentation are increasing, further evaluations are still needed, specifically studies on the capacity of ketone bodies to affect bronchial smooth muscle

directly<sup>13,14</sup>. We hypothesized that BHB can mitigate bronchoconstriction by inhibiting contraction of bronchial smooth muscle. Our objectives were to assess the effectiveness of BHB in reducing histamine-induced bronchial smooth muscle contraction *in vitro* and *ex vivo* to identify the mechanisms by which BHB may influence these effects. Further understanding of the efficacy and mechanisms of BHB attenuation of contraction in bronchial smooth muscle could provide insight into novel targets for the treatment of asthma and asthmatic symptoms.

### **4.3. Methods and Materials**

#### **4.3.1. Study Approval**

The animal experiments were reviewed and approved by the University of Vermont's Institutional Animal Care and Use Committee (PROTO202000195), in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 2011). Studies involving potentially hazardous materials were reviewed and approved by the University of Vermont's Institutional Biosafety Committee (REG201900052).

#### **4.3.2. Human Bronchial Smooth Muscle Cell Culture**

Primary human bronchial smooth muscle cells (HBSMC) isolated from a 45-yr-old female patient with asthma (Lonza, Morristown, NJ, Lot No. 00194850, Batch No. 0000195154) were cultured in smooth muscle cell growth medium-02 BulletKit (Lonza, CC-3182) according to the manufacturer's instructions at 37°C in 95% humidified air containing 5% CO<sub>2</sub>. The cells were used within the first seven passages to ensure proper smooth muscle phenotype. Cell authentication was performed by Lonza (negative Factor

VIII-related antigen, positive  $\alpha$ -Actin expression) and cells tested negative for mycoplasma (MycoDect Mycoplasma Detection Kit (Alstem, Richmond, CA)) before being utilized for experiments.

#### **4.3.3. Microscopy-Based Contraction Assay**

For simultaneous exposure and stimulation experiments, human bronchial smooth muscle cells (HBSMC) were plated at  $5 \times 10^4$  cells/cm<sup>2</sup> in 1mL of media in a 12-well plate and allowed to grow for 24 hours at 37°C and 5% CO<sub>2</sub> to ensure sub-confluency for better visualization. HBSMC were exposed for 5 minutes with vehicle or 1.25-10 mM beta-hydroxybutyric acid (BHBA) (Sigma-Aldrich, St. Louis, MO, Cat No.166898), sodium beta-hydroxybutyrate (NaBHB) (Sigma Aldrich, Cat No.54965), (R)-beta-hydroxybutyrate ((R)-BHBA) (Sigma Aldrich, Cat No.54920), (S)-beta-hydroxybutyrate ((S)-BHBA) (Sigma Aldrich, Cat No.54925), FFAR3 agonist AR420626 (AR) (Caymen, Ann Arbor, MI, Cat No. 17531 ), or a combination of MCT1 inhibitor AZD3965 (AZD) (MedChemExpress, Monmouth Junction, NJ, Cat No. HY12750) and BHBA, before being stimulated with 0.1–10 mM histamine (Sigma Aldrich, Cat No.H7125).

In pre-exposure experiments with subsequent stimulation, HBSMC were plated at  $5 \times 10^4$  cells/cm<sup>2</sup> in 1mL of media in a 12-well plate and allowed to grow for 24 hours at 37°C and 5% CO<sub>2</sub>. After the initial 24 hours, cells were exposed to vehicle or 1.25-10 mM BHBA, NaBHB, (R)-BHBA, or (S)-BHBA for another 24 hours before being washed and stimulated with 2.5 mM histamine.

Cells were imaged with an EVOSxl system (Thermo Fisher, Waltham, MA) to acquire brightfield microscopy images at 20X magnification captured at 10 second intervals for 5 minutes. For experiments, through time-lapse brightfield microscopy, the

contraction of individual cells was identified by visual cellular shape change and the reduction of intercellular space. Cellular area changes were quantified using the polygon tool in ImageJ (FIJI) to outline the perimeter of individual cells. The enclosed area was measured with ImageJ software both before and after agonist stimulation. The pre-stimulation value was normalized to 100%, and percent change was calculated. Percent contraction is presented as the reciprocal of the percent change, in which a negative percent change represents a decrease in cellular area, indicating contraction. Each individual cell analyzed represents n=1.

#### **4.3.4. Precision Cut Lung Slices (PCLS)**

6–12-week-old female and male C57BL/6J mice were euthanized, and their whole lungs were inflated with 40°C 1.5% low-melting point agarose in phosphate buffered saline (PBS) through the cannulated trachea and cooled in ice-cold PBS. The right murine lobe was uniformly cut into 150 $\mu$ M thick slices using a 7000smz-2 Vibratome (Campden Instruments, Lafayette, IN) maintained at 4°C, at a speed of 0.7mm/s. The freshly cut PCLS were placed in sterile, room temperature 1X PBS for imaging or into submerged tissue culture plates for culture with DMEM-F12 medium supplemented with 1X penicillin/streptomycin, 1 $\mu$ g/mL insulin, and 1X Primocin for continued incubation of the PCLS in 5% CO<sub>2</sub> at 37°C. Cultured PCLS were imaged within 4 days.

#### **4.3.5. *Ex vivo* Live Tissue Imaging**

For simultaneous exposure and stimulation experiments, PCLS were exposed for 10 minutes with vehicle or 10mM BHBA or NaBHB while images were acquired and were subsequently stimulated with 10mM histamine. In pre-exposure with subsequent stimulation experiments, PCLS were exposed to 10mM BHBA or NaBHB for 24 hours at

37°C and 5% CO<sub>2</sub>, and then placed in PBS to be imaged and stimulated with 10mM histamine. Live tissue contraction of the PCLS was imaged using the Zeiss Airyscan 2 confocal microscope imaging system in conjunction with the ImageJ Micro-Manager 2.0.0 Multi-Dimensional Acquisition (FIJI). Images were acquired at a frame rate of 1 frame/second and were analyzed using ImageJ (FIJI) software measuring relative diameter change to calculate percent airway narrowing. A 3D-printed PLA wafer was used during imaging to weigh down the PCLS to increase efficiency and reproducibility of imaging. Each individual airway represents n=1.

#### **4.3.6. Statistical Analyses**

All experiments included multiple biological replicates for each condition. Outliers were identified and removed using the ROUT method (Q=1%) and the cleaned data were analyzed using unpaired one-way ANOVA with post-hoc multiple comparisons: Tukey's test (for comparing all means) or Dunnett's test (for comparing each mean to a control), were performed using GraphPad Prism 10.2.3 (GraphPad Software, Inc., La Jolla, CA). Data are presented as means ± SEM from representative experiments. P values below 0.05 are considered statistically significant, and significance levels are indicated in the figure legends.

### **4.4. Results**

#### **4.4.1. Histamine induces human bronchial smooth muscle cell contraction.**

Histamine is well-documented as a potent inducer of airway smooth muscle contraction that causes bronchoconstriction<sup>10</sup>. Using human bronchial smooth muscle cells (HBSMC) as an *in vitro* model, we confirmed that histamine provokes a dose-dependent contraction of these cells (**Figure 4.1A**). The contraction induced by 2.5 mM histamine

was visualized using light microscopy, with comparative images captured before stimulation and 5 minutes after stimulation (**Figure 4.1B**). Post-stimulation images clearly show contracted cells, highlighted by red arrows, emphasizing the effect of histamine exposure. Quantitative analysis revealed that histamine elicited significant contraction compared to the vehicle control. Additionally, dose-dependent increases in contraction were statistically significant across all comparisons, except for the transition from 1 mM to 2.5 mM histamine. These results demonstrate the robust and measurable effect of histamine on bronchial smooth muscle contraction, enabling the examination of ketone bodies on this asthma-related phenotype.

#### **4.4.2. Beta-hydroxybutyrate (BHB) attenuates histamine induced human bronchial smooth muscle cell contraction.**

In our recent studies, we demonstrated the therapeutic benefits of elevating circulating ketone bodies—a state known as therapeutic ketosis—in mouse models of both obese<sup>13</sup> and allergic asthma<sup>14</sup>. This intervention effectively mitigated methacholine-induced airway hyperresponsiveness and significantly improved lung function *in vivo*<sup>13,14</sup>. Building on these findings, we investigated the effects of ketone bodies on histamine-induced contraction in human bronchial smooth muscle cells (HBSMC) using our validated *in vitro* system. Whereas exposure to 2.5 mM histamine elicited pronounced HBSMC contraction, cells that were co-treated with 2.5 mM beta-hydroxybutyric acid (BHBA), (R)-beta-hydroxybutyric acid ((R)-BHBA), (S)-beta-hydroxybutyric acid ((S)-BHBA), or sodium beta-hydroxybutyrate (NaBHB) exhibited significantly attenuated histamine-induced contraction (**Figure 4.2**). Notably, none of the BHB forms tested induced contraction in the absence of histamine, indicating their specific role in modulating histamine-evoked responses.

Interestingly, the attenuation of contraction was more pronounced with the racemic BHBA and the individual enantiomers (R)-BHBA and (S)-BHBA compared to sodium beta-hydroxybutyrate (NaBHB). These findings suggest that the pH of the beta-hydroxybutyrate compound contributes to its efficacy, highlighting the potential for targeted therapeutic strategies leveraging specific BHB formulations.

#### **4.4.3. Beta-hydroxybutyric acid dose-dependently inhibits histamine-induced HBSMC contraction.**

Building on the observed inhibitory effects of various forms of BHB on histamine-induced contraction, we next investigated whether these effects were dose-dependent. Human bronchial smooth muscle cells (HBSMC) were simultaneously stimulated with 2.5 mM histamine and exposed to increasing, biologically relevant concentrations of beta-hydroxybutyric acid (BHBA) for 5 minutes *in vitro*. By quantifying the percentage of cell contraction, we found that BHBA effectively attenuated histamine-induced contraction in a dose-dependent manner (**Figure 4.3**). Notably, all tested concentrations of BHBA significantly reduced contraction compared to the histamine control, further supporting its potential to modulate airway smooth muscle reactivity.

#### **4.4.4. Pre-exposure of HBSMC to BHBA attenuates histamine-induced contraction.**

As our previously reported *in vivo* studies have modeled endogenous ketone augmentation through dietary interventions that provide elevated systemic concentrations of these molecules over a protracted period, we sought to replicate this exposure in an *in vitro* system. To mimic endogenous ketone elevation, human bronchial smooth muscle cells (HBSMC) were pre-exposed to biologically relevant concentrations of beta-hydroxybutyric acid (BHBA) for 24 hours, followed by washing and subsequent

stimulation with histamine. Pre-exposure to BHBA significantly attenuated histamine-induced contraction in a dose-dependent manner (**Figure 4.4**), consistent with the inhibitory effects observed during simultaneous exposure of BHBA and histamine. Notably, all pre-exposure concentrations demonstrated significant reductions in contraction compared to the histamine control, which received an appropriate vehicle pre-exposure. Interestingly, when comparing the two exposure methods, the simultaneous exposure of BHBA with histamine proved more effective at reducing contraction than the pre-exposure approach.

#### **4.4.5. Pre-exposure to BHB or a Free Fatty Acid Receptor (FFAR)3 agonist inhibits histamine-induced HBSMC contraction.**

Given that beta-hydroxybutyrate (BHB) has been suggested to act as a ligand for FFAR3 and exert its beneficial effects through an FFAR3-dependent pathway<sup>21-23,48</sup>, we investigated whether activating FFAR3 could replicate the inhibitory effects of BHB on histamine-induced contraction in HBSMC. To test this possibility, cells were pre-exposed to biologically relevant concentrations of BHB to model endogenous ketone augmentation, as well as to the FFAR3 agonist AR420626 (AR). Both pre-exposure to BHB and AR significantly attenuated histamine-induced HBSMC contraction (**Figure 4.5**), suggesting that the observed inhibitory effects of BHB may indeed be mediated, at least in part, through the activation of FFAR3.

#### **4.4.6. FFAR3 activation is sufficient to attenuate histamine-induced HBSMC contraction.**

The ability of the FFAR3 agonist AR420626 (AR) to attenuate histamine-induced contraction in a simultaneous stimulation mirrors the effects observed with pre-exposure to BHBA, (R)-BHBA, (S)-BHBA, or NaBHB. This similarity suggests that these

compounds may exert their inhibitory effects through a shared mechanism or pathway. In our model of exogenous ketone augmentation, simultaneous stimulation with histamine in the absence or presence of BHB compounds or AR once again attenuated histamine-induced HBSMC contraction (**Figure 4.6**).

Ketone bodies, particularly BHB, have been proposed to mediate their beneficial effects via uptake through Monocarboxylic Transporter 1 (MCT1) (21, 37). To evaluate the involvement of this pathway, we tested the MCT1 inhibitor AZD3965 (AZD) in the presence of the BHB compounds. Notably, the presence of AZD did not block the inhibitory effects of BHB on histamine-induced contraction, indicating that these effects are independent of MCT1-mediated uptake. Interestingly, the use of DMSO as a vehicle for both AR and AZD also resulted in unexpected inhibition of histamine-induced contraction. This observation underscores the need for further investigation into the potential off-target effects of DMSO in this context.

#### **4.4.7. Histamine induces airway narrowing in murine PCLS.**

It has been reported that in murine models of allergic asthma, methacholine (Mch) exposure causes pronounced bronchoconstriction of mouse lungs that were immune-primed with either ovalbumin (OVA) or house dust mite (HDM)<sup>14,49</sup> and that treatment of human and guinea pig PCLS with histamine causes a decrease in airway lumen area<sup>50</sup>. It was also recently reported that agonist-induced bronchoconstriction in the airways can cause pathological airway epithelial crowding, triggering cellular extrusion into the airway lumen<sup>49</sup>. Using live tissue imaging of murine PCLS, we observed that histamine causes dose-dependent narrowing of the airway by causing cellular extrusion during bronchoconstriction (**Figure 4.7A**). The cellular extrusion induced by 10 mM histamine

was visualized using light microscopy, with comparative images captured before stimulation and 10 minutes after stimulation (**Figure 4.7B**). Post-stimulation images clearly show extruded cells, highlighted by red arrows, emphasizing the effect of histamine exposure. Quantitative analysis revealed that histamine elicited significant airway extrusion compared to the vehicle control (**Figure 4.7C**). Interestingly, we did not observe the same robust airway narrowing in our sequential dose-response studies in which the 10mM histamine condition only induced a mean value of about 5% airway narrowing, whereas in the studies in which a single stimulation of 10mM histamine was used, a mean value of about 55% airway narrowing or closure was observed.

#### **4.4.8. Exogenous BHB stimulation is sufficient to inhibit airway cellular extrusion in PCLS.**

We have previously reported that BHB has inhibitory effects on allergen-induced pro-inflammatory cytokine secretion<sup>14</sup> in human bronchial epithelial cells, providing some evidence that BHB already has effects directly on this relevant cell type. Using *in vitro* model systems, histamine can disrupt barrier integrity through adverse effects on tight junction integrity<sup>51-53</sup> and fluid hypersecretion<sup>52,54</sup>. Building on our findings *in vitro* of the inhibitory effects of BHB on histamine-induced HBSMC contraction, we investigated the effects of ketone bodies on histamine-induced airway narrowing *ex vivo*.

Exposure to 10 mM histamine elicited a pronounced phenotype of cellular extrusion and a significant increase in the percentage of airway narrowing occurring, as expected (**Figure 4.8A**). However, in PCLS exposed to 10 mM beta-hydroxybutyric acid (BHBA), or sodium beta-hydroxybutyrate (NaBHB), histamine-induced airway narrowing, and cellular extrusion visualized using light microscopy, was significantly attenuated, with

comparative images captured before stimulation and 10 minutes after stimulation (**Figure 4.8B**) in the presence of BHBA or NaBHB. Post-stimulation images clearly show extruded cells, highlighted by red arrows, emphasizing the effect of histamine exposure compared to the histamine positive control.

#### **4.4.9. Pre-exposure of PCLS with BHB attenuates histamine-induced cellular extrusion.**

As our previously reported *in vivo* studies have modeled endogenous ketone augmentation through dietary interventions, we sought to replicate this exposure in an *ex vivo* system. To model endogenous ketone elevation over a protracted period, PCLS were pre-exposed to biologically relevant concentrations of 10mM beta-hydroxybutyric acid (BHBA) or sodium beta-hydroxybutyrate (NaBHB) for 24 hours, followed by subsequent stimulation with histamine in the imaging medium containing no BHB. Pre-exposure to BHBA significantly attenuated histamine-induced cellular extrusion (**Figure 4.9**), consistent with the inhibitory effects observed during simultaneous exposure of BHBA and histamine. Notably, all pre-exposure conditions demonstrated reductions in cellular extrusion, although the effect of NaBHB only trended towards inhibition and was not statistically significant compared to the histamine control that received an appropriate vehicle pre-exposure. Interestingly, when comparing the two methods of exposure, the simultaneous exposure of BHBA with histamine proved more effective at reducing cellular extrusion than pre-exposure.

## **4.5. Discussion**

The increasingly prevalent asthma epidemic requires novel alternative or additional therapies to treat severe or ‘difficult-to-treat’ endotypes and improve quality of life for

those with this chronic and heterogeneous syndrome. Therapeutic ketosis is one such approach that has been gaining attention as a potential therapy due to its many beneficial effects in a myriad of pathological conditions and diseases<sup>55-59</sup>. Pertaining to asthma, therapeutic ketosis achieved through dietary interventions—such as a ketogenic diet, ketogenic precursor supplementation, or ketone ester intake—reduces methacholine hyperresponsiveness, a key pathophysiological feature of preclinical obese associated and allergic-associated asthma models<sup>13,14</sup> and inhibits pathological activities of several associated cells<sup>13-15</sup>. In models of asthma, distal airway and peripheral lung dysfunction occur<sup>60</sup>, and our previous work has shown that augmented BHB concentrations provide beneficial effects on both<sup>13,14</sup>. Ketone body augmentation decreased markers of methacholine hyperresponsiveness, airway resistance, tissue damping, and tissue elastance, physiological variables which are markedly increased by heterogeneous ventilation to the distal airways commonly due to variations in airway narrowing<sup>60,61</sup>, and sensitive to contraction of the peripheral airways. Therefore, we speculated that the mechanism through which ketones attenuate airway hyperresponsiveness may directly involve the bronchial smooth muscle. As reported herein, our studies demonstrate that BHB inhibits bronchial smooth muscle contraction.

The mechanisms by which BHB can elicit these effects remain uncertain but may include activation of various cell surface receptors such as free fatty acid receptor 3 (FFAR3)<sup>17,22,23</sup>, or through modulation of intracellular signaling regulating cellular contraction and extrusion. Given that BHB has been proposed to demonstrate efficacy across a wide range of applications and activation of many G-protein coupled receptors (GCPRs)<sup>15,16,20-23</sup>, perhaps including the induction of tachyphylaxis, it likely operates

through a fundamental mechanism that is universally relevant to these processes such as ion exchange<sup>22,29,62</sup> through calcium signaling<sup>22,23</sup> or adjusting membrane potential<sup>62</sup>.

Our *in vitro* studies using human bronchial smooth muscle cells (HBSMCs) demonstrate that histamine-induced contraction is attenuated by BHB, including its racemic mixture and the individual enantiomers (R)-BHB and (S)-BHB. Since only (R)-BHB can be efficiently metabolized by  $\beta$ -hydroxybutyrate dehydrogenase to form acetyl-CoA that is subsequently converted into ATP and used as an energy substrate<sup>63</sup>, we confirmed that the attenuation of contraction occurs independently of BHB's metabolic role. Additionally, the MCT1 inhibitor AZD3965 failed to block BHB's effects, indicating that MCT1-mediated uptake is not involved.

We further explored the influence of pH by comparing sodium BHB (NaBHB, pH 9.73) and beta-hydroxybutyric acid (BHBA, pH 2.09). When diluted to biologically relevant concentrations in buffered media, both BHBA and NaBHB attenuated histamine-induced contraction *in vitro*, although BHBA was more effective suggesting that while BHB itself is sufficient, acidity enhances its effect. This mechanism may also explain differences in histamine-induced cellular extrusion *ex vivo*. Additionally, the FFAR3 agonist AR420626 produced similar inhibitory effects, implying that FFAR3 activation may mediate the inhibitory effects of BHB. Confirmation would require an FFAR3 antagonist, although a compound with this activity is not yet commercially available. Nevertheless, these findings provide the rationale for future studies, such as examining the effects of BHB in FFAR3 knockdown cells and FFAR3 knockout mice.

The simultaneous exposure to BHBA with histamine was more effective at reducing histamine-induced HBSMC contraction and PCLS cell extrusion than the BHBA pre-exposure approach. However, it is likely that the application of ‘therapeutic ketosis’ in a clinical setting would provide consistently elevated BHB concentrations that could be modeled *in vitro* and *ex vivo* using the pre-exposure approach without removing the BHB before assessment of histamine-induced contraction and cell extrusion.

Cellular extrusion occurs when the epithelial cell lining the airway becomes too crowded and start to extrude into the airway lumen<sup>49,64</sup>. In previous work in this field, it was determined that pathological crowding due to bronchoconstriction causes epithelial cells to extrude into the airway lumen resulting in inflammation and mucus secretion in models of both human and mouse PCLS<sup>49</sup>. Unlike our mouse PCLS model, the previously mentioned studies used an HDM-primed allergic asthma model in which bronchoconstriction was induced with methacholine. In contrast, our model did not involve an *in vivo* allergic asthma setup but instead used histamine as an immune-relevant agonist to induce bronchoconstriction *ex vivo*. Notably, we found that histamine elicited the same cellular extrusion effects as those observed in the HDM-primed mouse PCLS stimulated with methacholine. Additional future studies include augmenting ketone body concentrations *in vivo* in preclinical asthma models and determining whether the attenuation of histamine-induced effects on PCLS are retained *ex vivo*.

There are several limitations to our findings. In our previous *in vivo* murine studies, methacholine was used as an agonist to assess the asthma-associated lung function phenotype of methacholine hyperresponsiveness, whereas in our *in vitro* and *ex vivo* studies, we utilized histamine as an airway smooth muscle contraction-inducing agonist.

Histamine contributes to asthma by triggering inflammation and airway constriction<sup>6,8,9</sup>. During an asthma attack, histamine is released from mast cells as part of an IgE-mediated immune response to allergens<sup>65</sup> or via their IgE-independent stimulation by irritants<sup>9</sup>, making it a more relevant agonist for asthma studies. In contrast, methacholine is a synthetic compound modeling endogenous acetylcholine that is used in clinical diagnostics to induce airway constriction without triggering the inflammatory response characteristic of asthma<sup>2</sup>. Thus, histamine effectively models relevant mechanisms of asthma exacerbations and is appropriate for the studies reported herein.

There are several limitations to our findings. Namely, we conducted *in vitro* studies using primary human bronchial smooth muscle cells and *ex vivo* studies with mouse precision cut lung slices (PCLS) instead of human subjects. While informative, human cell studies fail to capture the prolonged and complex nature of human asthma. They offer a reductionist approach by focusing on a single cell type and a specific agonist. In our previous *in vivo* murine studies, methacholine was used as an agonist to assess the asthma-associated lung function phenotype of methacholine hyperresponsiveness, whereas in our *in vitro* and *ex vivo* studies, we utilized histamine as an airway smooth muscle contraction-inducing agonist. Histamine contributes to asthma by triggering inflammation and airway constriction<sup>6,8,9</sup>. During an asthma attack, histamine is released from mast cells as part of an IgE-mediated immune response to allergens<sup>65</sup> or via their IgE-independent stimulation by irritants<sup>9</sup>, making it a more relevant agonist for asthma studies. In contrast, methacholine is a synthetic compound modeling endogenous acetylcholine that is used in clinical diagnostics to induce airway constriction without triggering the inflammatory response

characteristic of asthma<sup>2</sup>. Thus, histamine effectively models relevant mechanisms of asthma exacerbations and is appropriate for the studies reported herein.

Therapeutic ketosis is being applied to respiratory diseases in the clinical setting, including asthma<sup>66,67</sup> and Cystic fibrosis<sup>68</sup>. Whereas the asthma trials are providing medium-chain triglyceride supplementation as a substrate for ketone body formation *in vivo*, the Cystic fibrosis trial is providing the ketone ester precursor that more rapidly and efficiently boosts circulating ketone body concentrations and is the same compound we have employed in mouse models of obese asthma and allergic asthma<sup>13,14</sup>. The inclusion of therapeutic ketosis in clinical trials reflects growing scientific interest and recognition of its potential. Providing ketone ester supplementation in asthmatic subjects merits future study. Positive outcomes from these studies would provide robust support for incorporating therapeutic ketosis into treatment protocols, encouraging further exploration of its use as a complementary or alternative asthma therapy. This ongoing research underscores the significance of studying ketone-based interventions, emphasizing the need for continued investigation into their mechanisms and therapeutic potential.

#### 4.6. Figure Legends

##### **Figure 4.1. Histamine dose-dependently provokes HBSMC contraction.**

HBSMCs were sequentially stimulated *in vitro* with increasing concentrations of histamine for 5 minutes each and percent contraction of individual cells was quantitated. n=44-89 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  compared to the vehicle, # $P \leq 0.05$  ##### $P \leq 0.0001$  compared to the previous concentration of histamine (A). Visualization of contracting cells through sample movie stills of 2.5mM

histamine response of HBSMC (minutes: seconds), with red arrows pointing to individual contracting cells (scale bars, 200 $\mu$ m) **(B)**.

**Figure 4.2. BHB decreases histamine-induced HBSMC contraction.**

HBSMCs were simultaneously stimulated *in vitro* for 5 minutes with 2.5mM histamine in the presence of 2.5mM BHBA, (R)-BHBA, (S)-BHBA, or NaBHB and percent contraction of individual cells was quantitated. n=24-100 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to histamine.

**Figure 4.3. BHBA dose-dependently inhibits histamine-induced HBSMC contraction.**

HBSMCs were simultaneously stimulated *in vitro* for 5 minutes with 2.5mM histamine in the presence of increasing concentrations of beta-hydroxybutyric acid (BHBA) and percent contraction of individual cells was quantitated. n=40-114 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to histamine.

**Figure 4.4. BHBA pretreatment attenuates histamine-induced HBSMC contraction.**

HBSMCs were untreated (Vehicle) or exposed *in vitro* to increasing concentrations of beta-hydroxybutyric acid (BHBA) for 24 hours, washed, and then stimulated with 2.5mM histamine for 5 minutes and percent contraction of individual cells was quantitated. n=40-89 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to histamine.

**Figure 4.5. Pretreatment with BHB compounds or FFAR3 agonist attenuates histamine-induced HBSMC contraction.**

HBSMCs were untreated (Vehicle) or exposed *in vitro* with beta-hydroxybutyric acid (BHBA), (R)-BHBA, (S)-BHBA, NaBHB, or FFAR3 agonist (AR) for 24 hours, washed, and then stimulated with 2.5mM histamine for 5 minutes and percent contraction of individual cells was quantitated. n=51-216 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to histamine.

**Figure 4.6. FFAR3 activation is sufficient to inhibit histamine-induced HBSMC contraction.**

HBSMCs were untreated (Vehicle) or exposed *in vitro* with 2.5mM histamine in the presence of 2.5mM beta-hydroxybutyric acid (BHBA), 50 $\mu$ M FFAR3 agonist (AR), or a combination of 40nM MCT1 inhibitor (AZD) and 2.5mM BHBA for 5 minutes and percent contraction of individual cells was quantitated. DMSO was used as a vehicle control for the AR and AZD groups. n= 42-291 per group; values are inclusive of studies performed three times. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to histamine.

**Figure 4.7. Histamine induces cellular extrusion in murine PCLS.**

Murine PCLS were sequentially stimulated *ex vivo* with increasing concentrations of histamine for 10 minutes each and percent airway narrowing of individual airways was quantitated. n=6 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\* $P \leq 0.001$  compared to the vehicle, # $P \leq 0.05$ , ## $P \leq 0.01$ , ### $P \leq 0.001$  compared to 10mM histamine (A). Visualization of cellular extrusion through sample movie stills of 2.5mM histamine response of PCLS (minutes: seconds), with red arrows pointing to cellular extrusions (scale bars, 100 $\mu$ m) (B). Quantification of percent airway narrowing PCLS stimulated with 10mM histamine. n=27-30 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\*\* $P \leq 0.0001$  compared to the vehicle (C).

**Figure 4.8. BHB attenuates histamine-induced cellular extrusion in PCLS.** Murine PCLS were simultaneously stimulated *ex vivo* with 10mM histamine for 10 minutes each for 5 minutes in the presence of 10mM BHBA or NaBHB and percent airway narrowing of individual airways was quantitated. and percent airway narrowing of individual airways was quantitated. n=22-32 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \* $P \leq 0.05$ , \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ##### $P \leq 0.0001$  compared to 10mM histamine (A). Visualization of cellular extrusion through sample movie stills of 10mM histamine response of PCLS in the presence of BHB (minutes: seconds), with red arrows pointing to cellular extrusions (scale bars, 100 $\mu$ M) (B).

**Figure 4.9. Pre-exposure of PCLS to BHB attenuates histamine-induced cellular extrusion.** HBSMCs were untreated (Vehicle) or exposed *ex vivo* with 10mM BHBA or NaBHB for 24 hours, placed in PBS for imaging, and then stimulated with 10mM histamine for 10 minutes and percent airway narrowing of individual airways was quantitated. n=12-22 per group; the values presented represent a normalized dataset created by combining results from three individual studies. \*\*\*\* $P \leq 0.0001$  compared to the vehicle, ### $P \leq 0.001$  compared to histamine.

## 4.7. Figures

Figure 4.1. Histamine dose-dependently provokes HBSMC contraction.

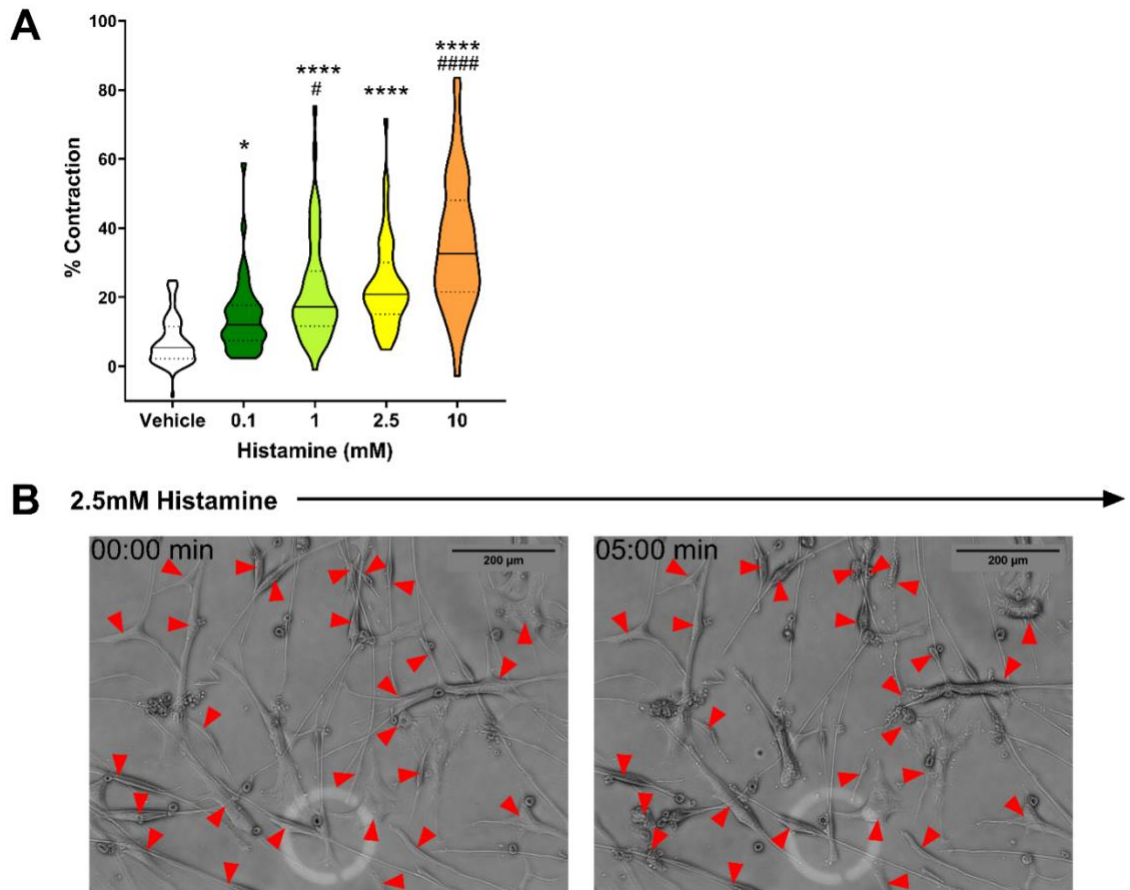
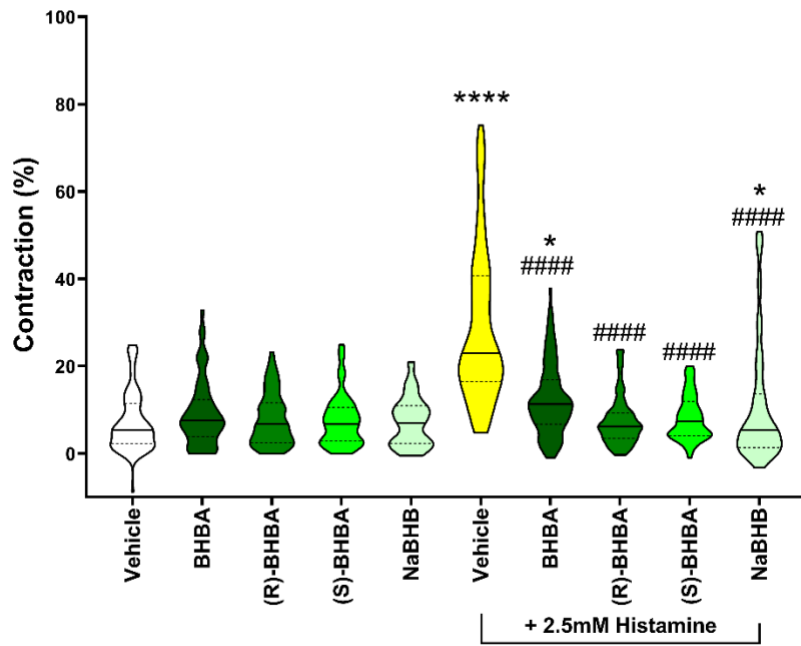


Figure 4.2. BHB decreases histamine-induced HBSMC contraction.



**Figure 4.3. BHBA dose-dependently inhibits histamine-induced HBSMC contraction.**

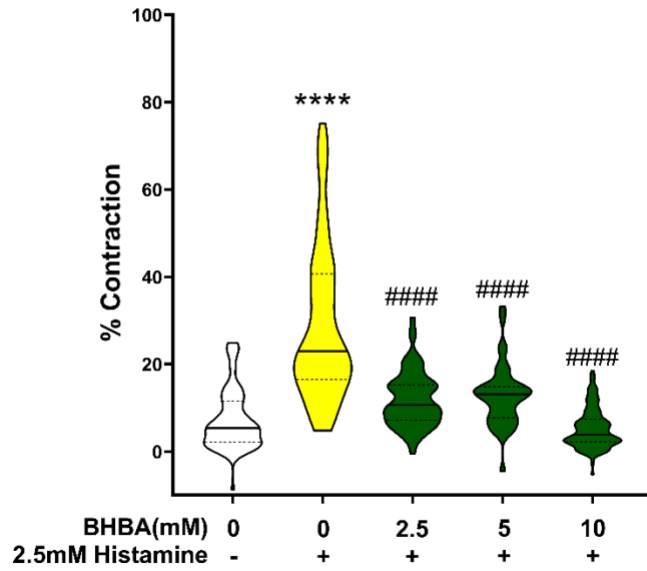
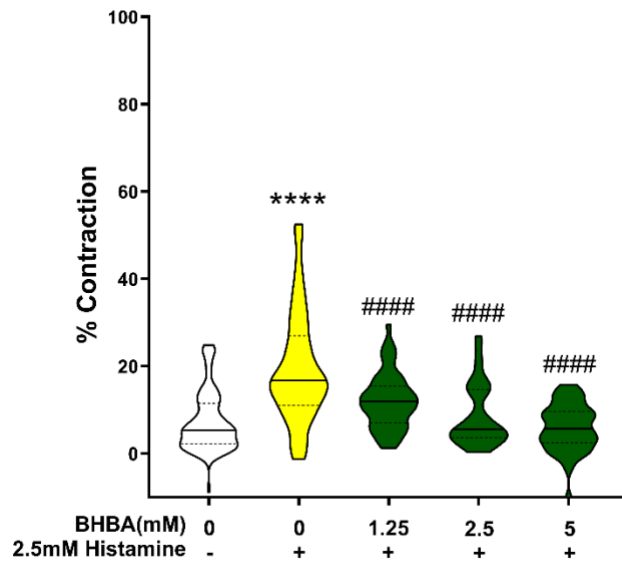


Figure 4.4. BHBA pretreatment attenuates histamine-induced HBSMC contraction.



**Figure 4.5. Pretreatment with BHB compounds or FFAR3 agonist attenuates histamine-induced HBSMC contraction.**

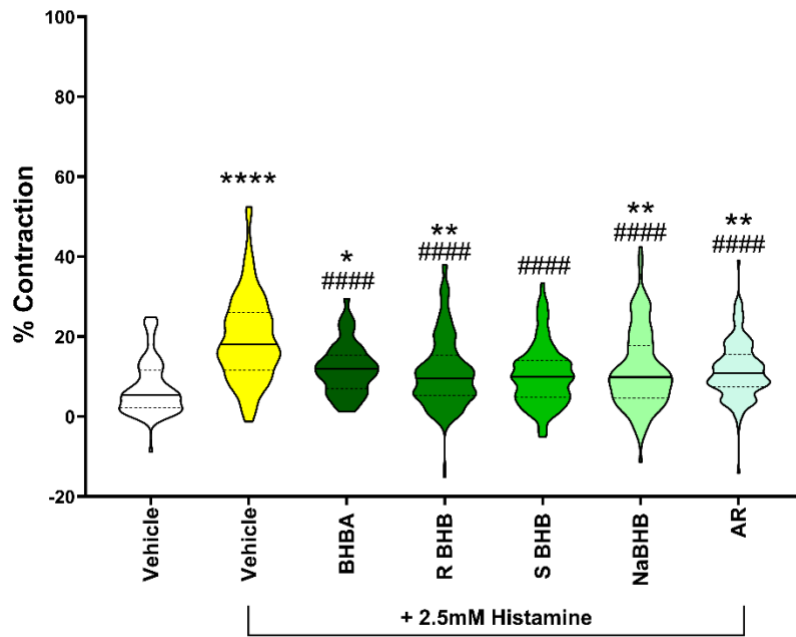


Figure 4.6. FFAR3 activation is sufficient to inhibit histamine-induced HBSMC contraction.

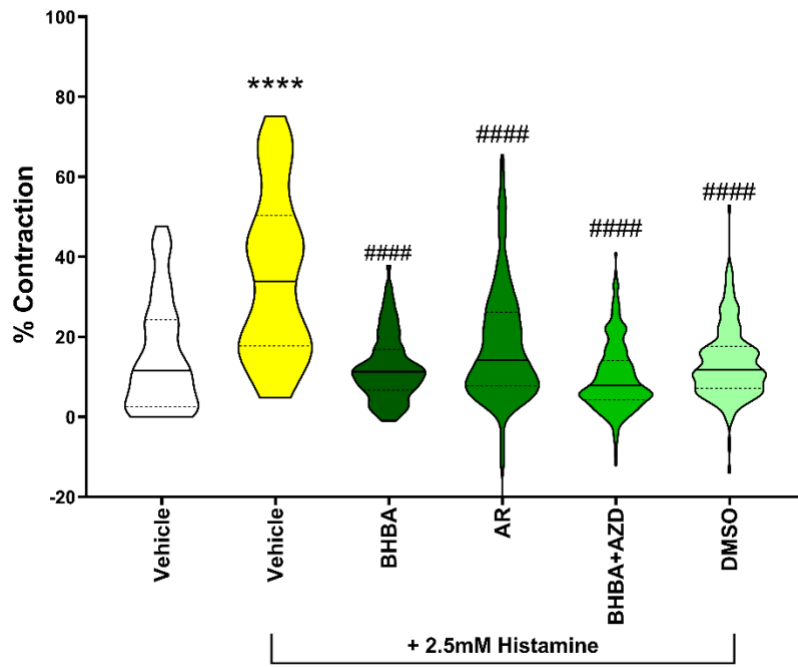


Figure 4.7. Histamine induces cellular extrusion in murine PCLS.

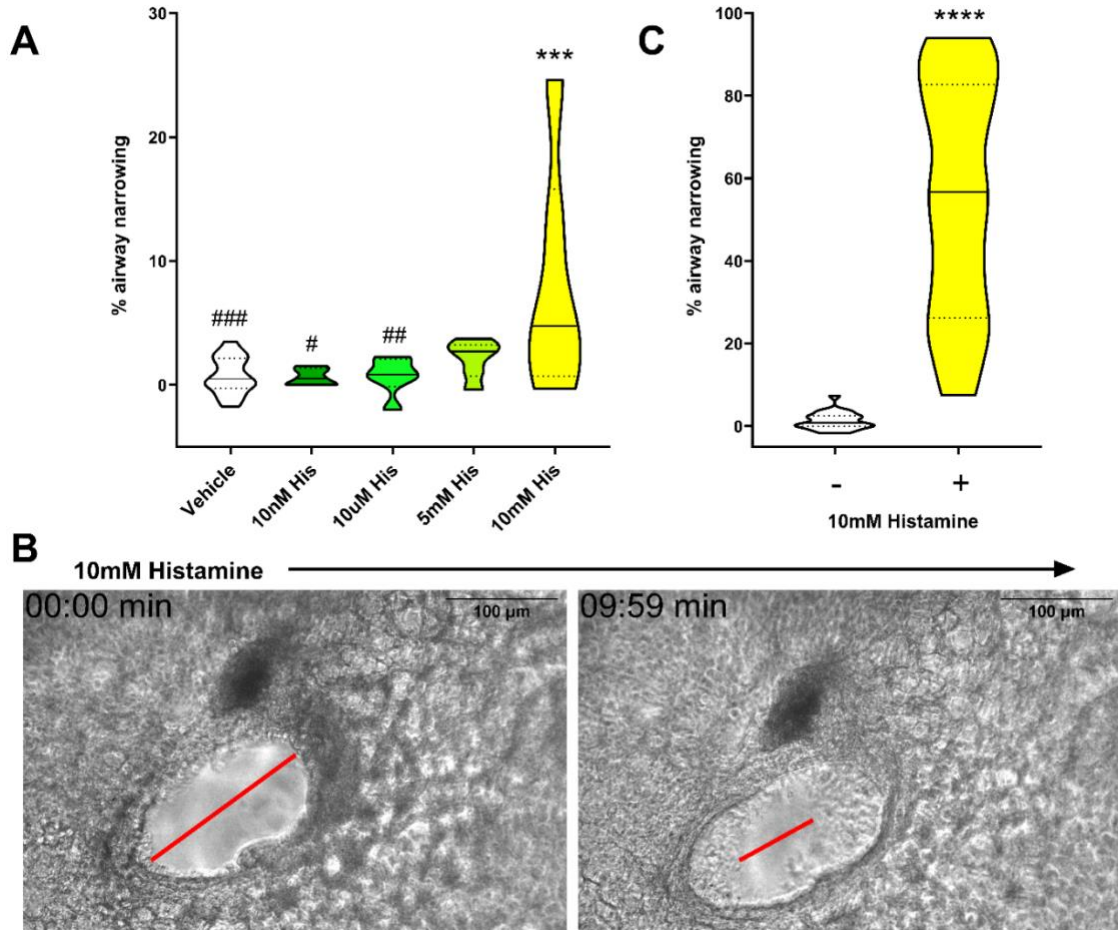


Figure 4.8. BHB attenuates histamine-induced cellular extrusion in PCLS.

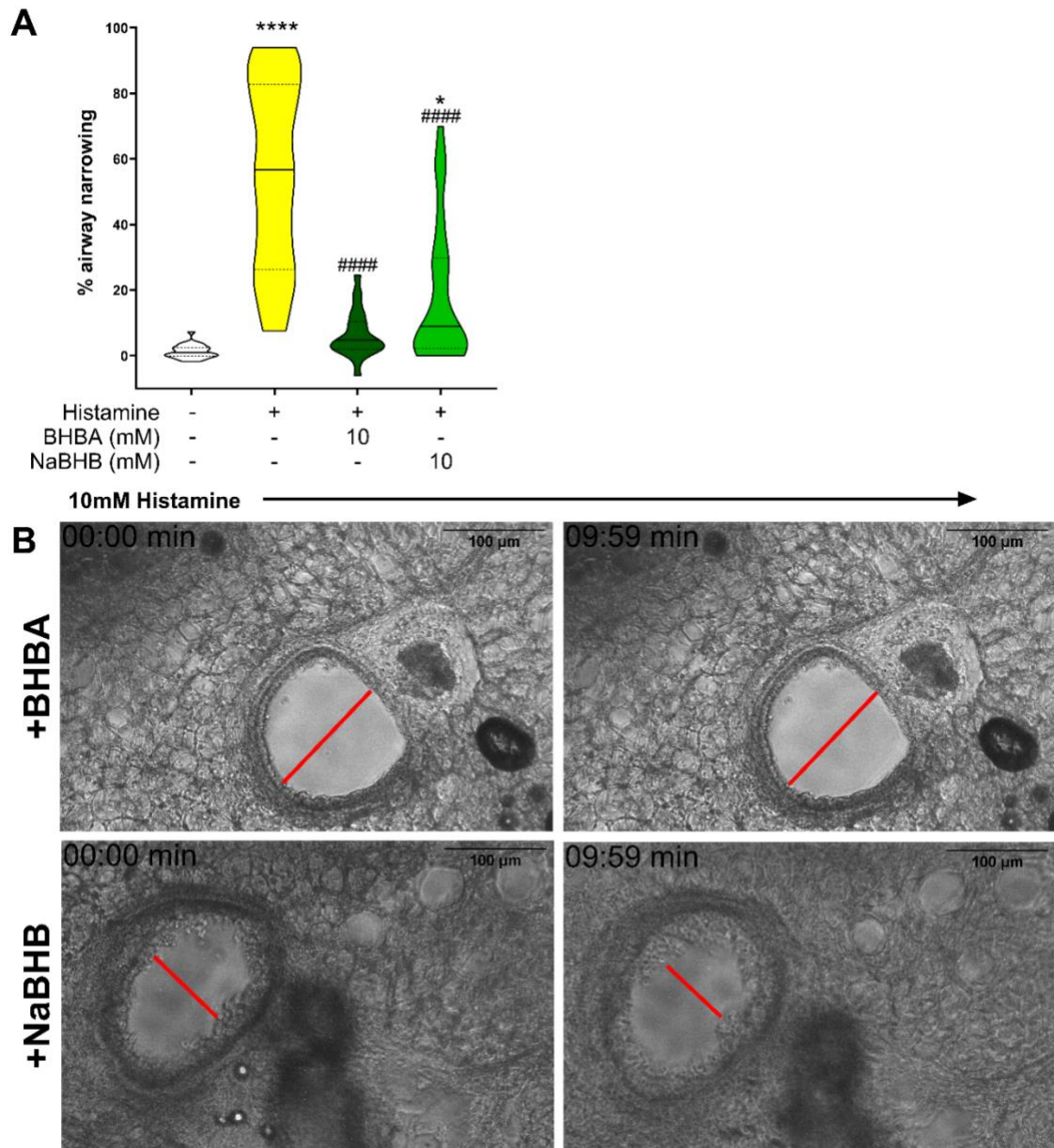
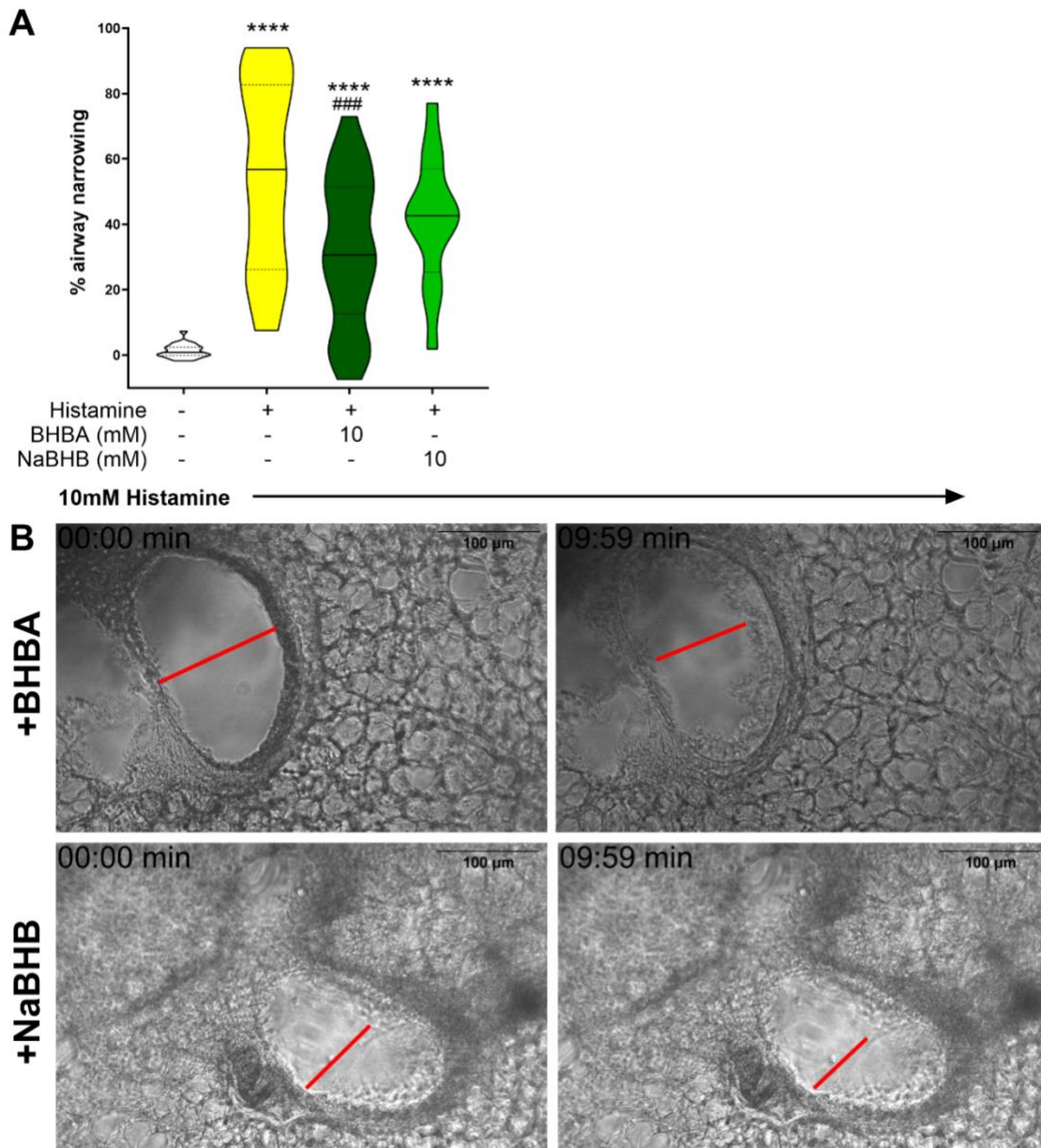


Figure 4.9. BHB attenuates histamine-induced cellular extrusion in PCLS.



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## Chapter 5: Discussion

### 5.1. Summary

Bronchial smooth muscle (BSM) plays a well-established role in airway hyperresponsiveness in asthma by contributing to remodeling, inflammation, and contraction. While therapeutic ketosis has emerged as a potential intervention to alleviate asthma symptoms, the mechanisms whereby it affects the activities of BSM remain largely undefined. Studies have demonstrated that ketone bodies, particularly  $\beta$ -hydroxybutyrate (BHB), exert anti-inflammatory and antioxidant effects, and weight loss-induced ketosis has been associated with improved asthma outcomes. However, the direct impact of ketone bodies on BSM functions, specifically their capacity to modulate airway remodeling, inflammation, and contraction, has not been systematically investigated. This dissertation addresses this critical gap by exploring how therapeutic ketosis influences BSM to attenuate airway hyperresponsiveness. Using *in vitro* and *ex vivo* models, we aim to define the cellular and molecular mechanisms by which BHB modulates BSM remodeling, inflammatory signaling, and contractile responses. By elucidating these pathways, this work seeks to provide a mechanistic foundation for leveraging therapeutic ketosis as a novel strategy for asthma treatment.

We have now demonstrated that BHB suppresses bronchial smooth muscle remodeling and airway hyperresponsiveness (AHR) in response to allergen exposure. House dust mite (HDM) extract induces morphological changes in human bronchial smooth muscle cells (HBSMCs), and we have shown that BHB inhibits these changes in a dose-dependent manner. Notably, BHB also suppresses HDM protease activity in a similar dose-dependent fashion, suggesting that its ability to attenuate HDM-induced

morphological changes may be mediated through protease inhibition. In *in vivo* preclinical models of asthma, it has been shown that dietary interventions that elevate systemic BHB levels—such as ketogenic diets, ketone precursor feeding, or ketone ester supplementation—reduce methacholine-induced AHR, a key pathophysiological feature of asthma<sup>1,2</sup>. BHB also improves both large airway and peripheral lung function by decreasing airway resistance, tissue damping, and tissue elastance, parameters that reflect heterogeneous ventilation and airway constriction<sup>1,2</sup>. These findings, along with the results reported in this dissertation, suggest that BHB directly modulates bronchial smooth muscle cell function to attenuate airway constriction and hyperresponsiveness, providing further support for therapeutic ketosis as a strategy for asthma management (Chapter 2).

We have also provided evidence that BHB suppresses the pro-inflammatory response of human bronchial smooth muscle cells (HBSMC) to IL-1 $\beta$ . IL-1 $\beta$  induces robust production of the cytokines IL-6 and IL-8 from HBSMC, and we have shown that BHB attenuates this response in a dose-dependent manner. The inhibitory effect of BHB is influenced by pH, as beta-hydroxybutyric acid (BHBA) more effectively suppresses cytokine production compared to sodium beta-hydroxybutyrate (NaBHB), suggesting that acidity enhances BHB's action. While the precise mechanism for the enhanced inhibition by BHB in an acidic form or an acidic environment remains uncertain, our data suggest that BHB exerts its effects independently of metabolic conversion, as both the (R)-BHB and (S)-BHB enantiomers provide attenuation, and the effects of BHB do not require its uptake via the monocarboxylate transporter, MCT1. Instead, the activation of the free fatty acid receptor 3 (FFAR3) may contribute to its anti-inflammatory function, as the FFAR3 agonist AR420626 elicits similar inhibitory effects as BHB. These findings suggest that

BHB functions as a modulator of bronchial smooth muscle-initiated inflammation, limiting cytokine-mediated airway inflammation and potentially attenuating the progression of airway hyperresponsiveness. This suppression of pro-inflammatory signaling may be relevant to the broader anti-inflammatory effects of therapeutic ketosis in airway disease (Chapter 3).

We have also demonstrated that BHB suppresses bronchial smooth muscle contraction in response to histamine, a key contributor to airway narrowing in asthma. Histamine stimulation induces robust contraction of human bronchial smooth muscle cells (HBSMCs), and we have shown that BHB attenuates this contraction in a dose-dependent manner. Both (R)-BHB and (S)-BHB enantiomers equivalently exert this effect, indicating that BHB's inhibition of contraction is largely independent of its role as a metabolic substrate. Additionally, the inhibitory effects of BHB are enhanced under acidic conditions, as beta-hydroxybutyric acid (BHBA) is more effective than sodium beta-hydroxybutyrate (NaBHB), suggesting that pH modulates BHB's action to attenuate agonist-induced contraction. The involvement of cell surface receptors is also implicated, as the FFAR3 agonist AR420626 produces similar inhibitory effects on contraction as BHB. *Ex vivo* studies using precision-cut lung slices (PCLS) further support BHB's ability to reduce histamine-induced cellular extrusion, a process initiated by BSM contraction and linked to airway inflammation and mucus secretion. These findings suggest that BHB functions as a regulator of bronchial smooth muscle tone, limiting airway constriction and potentially mitigating asthma symptoms. This suppression of bronchial smooth muscle contraction may contribute to the broader protective effects of therapeutic ketosis in airway disease (Chapter 4).

The studies reported in this dissertation, in conjunction with those of others<sup>1-3</sup> have elucidated an important contribution of therapeutic ketosis in modulating airway hyperresponsiveness in asthma. These effects are achieved through multiple mechanisms, including inhibition of protease activity, attenuation of inflammatory signaling, and activation of cell surface receptors such as FFAR3. The ability of BHB to influence both bronchial smooth muscle-mediated airway inflammation and contractile function highlights its potential as a therapeutic intervention for asthma, a disease characterized by chronic inflammation and airway hyperreactivity.

## **5.2. Discussion**

### **5.2.1. Asthma**

Chapters 2, 3, and 4 of this dissertation collectively explore the role of therapeutic ketosis in mitigating key pathophysiological features of asthmatic airway hyperresponsiveness (AHR) in bronchial smooth muscle, including remodeling, inflammation, and contraction. Asthma is a chronic respiratory disease characterized by persistent airway inflammation, bronchial hyperreactivity, and structural remodeling, all of which contribute to airflow obstruction and respiratory distress<sup>4,5</sup>. While current therapies primarily focus on bronchodilation and inflammation suppression, they are often insufficient for severe or treatment-resistant cases<sup>6,7</sup>. This research suggests that the ketone body beta-hydroxybutyrate (BHB) has direct effects on bronchial smooth muscle and inflammation, offering a potential complementary or alternative approach to traditional asthma treatments.

Allergic asthma is driven by an exaggerated immune response to inhaled allergens, such as house dust mite (HDM), a common, recurrent, environmental trigger that contains

potent proteases capable of disrupting airway epithelial integrity and activating innate immune pathways<sup>5,8-11</sup>. HDM exposure leads to airway inflammation, increased mucus production, and bronchial smooth muscle (BSM) morphological change<sup>8,12-20</sup>. This remodeling thickens airway walls, reduces elasticity, and contributes to obstruction by increasing contractile capacity<sup>21-27</sup>. BSM also acts as both a target and mediator of inflammation, responding to cytokines and chemokines from immune cells such as eosinophils, mast cells, and type 2 lymphocytes<sup>8,9,12-14,16</sup>. Mast cell infiltration exacerbates airway hyperresponsiveness by releasing histamine and other mediators, creating a feedback loop of inflammation and muscle activation<sup>13,14,16,17,28-32</sup>. Contraction of BSM, normally a protective mechanism, becomes dysregulated in asthma due to inflammatory mediators like histamine, leukotrienes, and prostaglandins, which enhance calcium signaling pathways and muscle contraction<sup>21,32-38</sup>. Vagal nerve activity further contributes by stimulating muscarinic receptors, increasing intracellular calcium, and activating myosin light chain kinase, leading to sustained contraction<sup>39-47</sup>. Given its role in inflammation, remodeling, and hyperresponsiveness, BSM is a key effector cell type for asthma therapeutics<sup>48-50</sup>.

Asthma management routinely targets bronchial smooth muscle (BSM) to alleviate airway obstruction, primarily through  $\beta$ 2-adrenergic agonists, which activate intracellular signaling to promote relaxation<sup>51</sup>. Short-acting  $\beta$ -agonists (SABAs) like albuterol provide rapid bronchodilation, while long-acting  $\beta$ -agonists (LABAs) are combined with inhaled corticosteroids (ICS) for sustained control<sup>4,6,51-54</sup>. Biologic therapies, including monoclonal antibodies targeting IgE, IL-4, IL-5, IL-13 (or their receptors), and TSLP, indirectly modulate BSM function by reducing inflammation-driven hyperresponsiveness<sup>55-64</sup>.

Despite their efficacy, biologics are limited by high costs, side effects, and phenotype-specific indications, necessitating alternative approaches for severe or uncontrolled asthma<sup>7,65-69</sup>. This background underscores the need for alternative therapeutic strategies, such as those explored in Chapters 2, 3, and 4, by highlighting the central role of bronchial smooth muscle in asthma pathophysiology.

### **5.2.2. Therapeutic Ketosis**

The studies presented in this dissertation, as well as other published literature, suggest the therapeutic potential of ketone bodies in various physiological contexts. Traditionally, ketone bodies have been recognized as an alternative energy source during glucose scarcity, such as fasting or carbohydrate restriction, with BHB serving as a primary fuel for the brain and peripheral tissues<sup>70-73</sup>. However, recent research highlights BHB's contributions beyond metabolism, showing that it functions as a signaling molecule that can modulate cellular responses through receptor activation, epigenetic regulation, and ion channel modulation<sup>74-85</sup>. One emerging area of interest is the potential for BHB to activate free fatty acid receptor 3 (FFAR3), a G-protein-coupled receptor traditionally associated with short-chain fatty acids like acetate, propionate, and butyrate<sup>86</sup>. FFAR3 is expressed in various tissues, including the gut<sup>87</sup>, nervous system<sup>88,89</sup>, pancreas<sup>90</sup>, and airway smooth muscle<sup>91</sup>, where it regulates metabolic and inflammatory responses. Evidence demonstrated in Chapters 3 and 4, suggest that BHB may interact with FFAR3, modulating airway smooth muscle contraction and pro-inflammatory signaling. In asthma, where bronchial smooth muscle (BSM) plays a key role in airway hyperresponsiveness, FFAR3 activation by BHB could provide a novel mechanism for reducing excessive airway constriction and inflammation.

Given that FFAR3 has been implicated in modulating sympathetic and parasympathetic pathways, its activation by BHB could inhibit excessive bronchoconstriction by regulating calcium signaling and smooth muscle excitability<sup>84,85,91</sup>. Furthermore, the potential immune-modulatory effects of FFAR3 activation could help suppress cytokine production and immune cell recruitment, reducing airway inflammation and fibrosis<sup>75,91</sup>. Future studies should focus on determining whether BHB's effects on airway smooth muscle and inflammation are directly mediated through FFAR3. This could be achieved using FFAR3-specific agonists and antagonists, gene knockdown models, or knockout mice to assess whether the absence of FFAR3 alters BHB's ability to regulate smooth muscle contraction and cytokine production.

### **5.2.3. Research Limitations**

The research described in this dissertation presents several limitations across the *in vitro*, and *ex vivo* preclinical studies conducted to evaluate the effects of therapeutic ketosis in asthma. One major limitation is the reliance on *in vitro* studies using primary human bronchial smooth muscle cells (HBSMCs). While these studies provide valuable mechanistic insights, they fail to capture the complexity of human asthma, which involves multiple interacting cell types, immune responses, and long-term disease progression. The experiments conducted focus exclusively on the initial inhibitory effects of BHB on pro-inflammatory signaling and bronchial smooth muscle contraction, without assessing its long-term impact on inflammation resolution, airway remodeling, or disease progression.

Another limitation is the lack of *ex vivo* or *in vivo* confirmation for key mechanisms. While *ex vivo* studies using precision-cut lung slices (PCLS) from mice provided some evidence of BHB's effects on airway contraction and cell extrusion, the research did not

fully replicate allergic asthma conditions, such as those induced by house dust mite (HDM) exposure *in vivo*. The studies also relied on histamine as an airway smooth muscle agonist, rather than methacholine, which is more commonly used in asthma diagnostics and clinical studies. Since histamine triggers both airway constriction and immune activation, while methacholine primarily stimulates airway smooth muscle contraction, future studies should compare both agonists to better model different aspects of asthma pathology.

A further limitation is the uncertainty surrounding the mechanisms of BHB action. The studies performed suggest that BHB may act through multiple pathways, including protease inhibition, receptor activation (FFAR3, HCAR2), and modulation of intracellular signaling. However, definitive evidence for receptor involvement is lacking, as FFAR3 antagonists and knockout models are not available for validation. Without these tools, it remains unclear whether BHB's effects are directly mediated through FFAR3 activation or if alternative pathways contribute. Additionally, BHB's effects on histone modifications and post-translational protein modifications, such as  $\beta$ -hydroxybutyrylation, remain speculative, as inconsistent reagents prevented conclusive testing of these mechanisms.

Lastly, the clinical relevance and translational potential of BHB remain untested. While preclinical models of obese asthma and allergic asthma suggest beneficial effects of augmenting systemic BHB concentrations, no human clinical trials have been conducted to assess directly whether therapeutic ketosis or exogenous ketone supplementation could improve lung function in asthma patients. Additionally, pharmacological strategies to elevate BHB levels, such as ketone esters or precursors, may differ in efficacy and tolerability compared to dietary approaches like ketogenic diets. Future studies should focus on validating these findings in human subjects, evaluating long-term effects, and

optimizing dosing strategies for potential therapeutic applications. Overall, while the studies described in this dissertation provide strong support for the potential beneficial effects of BHB in asthma, further *in vivo* studies, receptor-targeting experiments, and clinical trials are necessary to confirm its mechanisms and therapeutic potential.

#### **5.2.4. Food for Thought**

Therapeutic ketosis, particularly the ketone body  $\beta$ -hydroxybutyrate (BHB), has demonstrated significant potential in modulating airway hyperresponsiveness, inflammation, and bronchial smooth muscle contraction in asthma. Interventions that may or deliberately increase circulating ketone body concentrations are being applied to respiratory diseases in the clinical setting, including asthma<sup>92,93</sup> and cystic fibrosis<sup>94</sup>, respectively. Whereas the asthma trials are providing medium-chain triglyceride supplementation as a substrate for ketone body formation *in vivo*, the cystic fibrosis trial is providing the ketone ester precursor that more rapidly and efficiently boosts circulating ketone body concentrations and is the same compound we have employed in mouse models of obese asthma and allergic asthma<sup>1,2</sup>. The study of therapeutic ketosis in clinical trials reflects growing scientific interest and recognition of its potential. Providing ketone ester supplementation in asthmatic subjects merit future study. Positive outcomes from these studies would provide robust support for incorporating therapeutic ketosis into treatment protocols, encouraging further exploration of its use as a complementary or alternative asthma therapy. Independent of asthma, therapeutic ketosis is being assessed as a treatment in numerous other various pathological diseases and conditions involving several organ systems, cellular targets, and molecular mechanisms<sup>74,95-119</sup>.

The studies on bronchial smooth muscle described in this dissertation are clearly related to the pathophysiology of allergic asthma but are also relevant to obesity-associated asthma. Given its role in modulating energy metabolism, appetite regulation, and inflammation, ketosis may offer a complementary or alternative strategy for obesity management. Ketone bodies like  $\beta$ -hydroxybutyrate (BHB) have been shown to enhance mitochondrial efficiency, preserve lean muscle mass, and improve metabolic flexibility, which are critical factors in long-term weight management<sup>70,72</sup>. Additionally, ketosis may influence appetite control by interacting with gut hormones such as ghrelin and GLP-1, potentially enhancing satiety and reducing overeating<sup>74,86</sup>. Since chronic low-grade inflammation is a hallmark of obesity, the anti-inflammatory properties of BHB could also mitigate obesity-related complications, including insulin resistance and cardiovascular dysfunction<sup>75,76</sup>. These findings suggest that therapeutic ketosis, whether achieved through dietary interventions or ketone ester supplementation, warrants further investigation as a metabolic tool for obesity treatment. Whereas it would be a challenge for the general population to accept a treatment other than a once-a-week injection of semaglutide, the ever-popular Ozempic, perhaps a once-a-day bolus of ketone ester would have less side effects and still be an attractive therapeutic.

Dietary composition plays a significant role in regulating endogenous ketone production, even in the absence of fasting, when consuming a ketogenic diet, or during ketone ester supplementation. High-fiber foods that promote gut microbial fermentation, such as resistant starches and prebiotic fibers, can enhance the production of short-chain fatty acids (SCFAs) like butyrate, which serves as a precursor for ketogenesis<sup>86</sup>. The ability of diet to shape both microbial composition and ketone availability highlights the potential

of dietary interventions to harness the benefits of ketosis without strict carbohydrate restriction or exogenous supplementation. Future research should investigate the long-term effects of dietary-driven ketosis on metabolic health and whether specific dietary patterns can optimize endogenous ketone production to support therapeutic applications.

This research on  $\beta$ -hydroxybutyrate (BHB) and its effects on bronchial smooth muscle contraction has significant implications for the broader field of histamine research, particularly in relation to vascular smooth muscle function. Histamine plays a critical role in allergic responses, inflammation, and vascular tone, acting through multiple histamine receptors (H1-H4) to regulate smooth muscle contraction, endothelial permeability, and immune cell activation<sup>17</sup>. The findings of this dissertation, demonstrating that BHB attenuates histamine-induced bronchial smooth muscle contraction, suggest a potential role for therapeutic ketosis in mitigating histamine-mediated vascular dysfunction. If similar mechanisms apply to vascular smooth muscle, BHB may help regulate blood vessel constriction and dilation, offering a novel approach to managing conditions characterized by excessive histamine activity, such as mast cell activation syndrome (MCAS), anaphylaxis, and chronic urticaria<sup>32</sup>. In patients with pulmonary arterial hypertension or chronic thromboembolic pulmonary hypertension, conditions in which vascular resistance in the lungs is increased, BHB infusion decreases pulmonary vascular resistance and improves cardiac output<sup>120</sup>. Similar beneficial effects of BHB were observed *ex vivo* in the isolated pulmonary arteries and right ventricle of healthy rats<sup>120</sup>. Beyond its effects on smooth muscle tone, BHB's potential interaction with free fatty acid receptor 3 (FFAR3) and other signaling pathways could have broader implications for vascular health. Histamine is known to increase endothelial permeability, leading to fluid leakage and tissue

edema, a key feature of allergic and inflammatory conditions<sup>33</sup>. If BHB stabilizes endothelial function by modulating histamine signaling or enhancing nitric oxide (NO) bioavailability, it could serve as a therapeutic target for vascular inflammatory diseases, including histamine-related migraines, postural orthostatic tachycardia syndrome (POTS), and chronic inflammatory disorders. Additionally, since histamine release is often accompanied by oxidative stress and inflammatory cytokine production, the antioxidant and anti-inflammatory properties of BHB may further contribute to vascular protection<sup>76</sup>. Future research should explore whether ketone supplementation can mitigate histamine-mediated vascular dysfunction in both acute allergic responses and chronic inflammatory diseases, providing a novel link between metabolic interventions and histamine-related vascular pathology.

Independent of pathological conditions or disease phenotypes,  $\beta$ -hydroxybutyrate (BHB) and its effects on smooth muscle contraction, inflammation, and metabolic regulation has significant implications for athletics and human performance. Exercise places substantial demands on the respiratory system, cardiovascular function, and skeletal muscle metabolism, all of which may benefit from the metabolic and anti-inflammatory properties of ketone bodies<sup>75,121</sup>. One of the primary challenges in endurance and high-intensity sports is maintaining metabolic flexibility—efficiently switching between carbohydrate and fat oxidation to sustain energy output. BHB has been shown to enhance mitochondrial efficiency, reduce oxidative stress, and improve fuel utilization, which may help athletes delay fatigue and optimize performance during prolonged exertion<sup>121</sup>. Additionally, given that BHB attenuates histamine-induced bronchial smooth muscle contraction, therapeutic ketosis may offer benefits for athletes prone to exercise-induced

bronchoconstriction (EIB), also known as exercise-induced asthma, potentially reducing their reliance on bronchodilators and improving respiratory efficiency in endurance sports. Beyond respiratory function, the role of BHB in muscle recovery and inflammation modulation could be particularly relevant for athletes engaging in high-impact or resistance-based training<sup>122</sup>. Intense exercise leads to transient increases in systemic inflammation and oxidative damage, which contribute to delayed-onset muscle soreness (DOMS) and prolonged recovery times<sup>123</sup>. Ketone bodies have been shown to exert anti-inflammatory effects by inhibiting the NLRP3 inflammasome and reducing pro-inflammatory cytokine production<sup>75</sup>. This suggests that therapeutic ketosis could serve as a recovery-enhancing strategy, minimizing muscle damage and accelerating repair processes following intense training sessions. Furthermore, since muscle cramping and fatigue can be exacerbated by metabolic acidosis, the pH-buffering properties of ketones may help regulate acid-base balance, improving muscular endurance and reducing cramp susceptibility in endurance athletes<sup>121</sup>. Future research should explore whether exogenous ketone supplementation can enhance athletic performance, optimize recovery, and improve respiratory function in individuals with exercise-induced airway sensitivity, offering a novel metabolic approach to sports performance and recovery.

It is recommended that a dissertation should go *above and beyond* the scope of its research, but it turns out, therapeutic ketosis may have benefits that are truly *out of this world*. While this dissertation focuses on the effects of therapeutic ketosis on airway inflammation and smooth muscle function, its findings have significant relevance for human health research in extreme environments, including space exploration. The notion of therapeutic ketosis, and its effects on airway inflammation and smooth muscle function,

has significant implications for NASA's human health research, particularly in addressing the physiological challenges of long-duration space travel. One of the major concerns for astronauts is exposure to space radiation, which induces oxidative stress and DNA damage, leading to increased inflammation and cellular dysfunction. Ketone bodies, particularly  $\beta$ -hydroxybutyrate (BHB), have been shown to upregulate antioxidant pathways and reduce inflammatory responses<sup>121</sup>. By leveraging the protective effects of therapeutic ketosis, astronauts may be able to mitigate some of the detrimental effects of radiation exposure, preserving cellular integrity and reducing long-term health risks associated with extended missions. Additionally, since astronauts must adhere to strict nutritional protocols with limited food availability, ketone metabolism—whether induced through intermittent fasting or ketone supplementation—could serve as a metabolic countermeasure to maintain energy balance and metabolic during spaceflight<sup>70,72,121,124</sup>. Another critical application of the findings demonstrated herein is in respiratory health, particularly in the context of airway irritation and inflammation due to space dust exposure. Lunar and Martian dust particles pose significant risks to astronauts' pulmonary systems, with the potential to induce airway hyperresponsiveness and inflammatory reactions similar to those seen in asthma<sup>125-127</sup>. Our findings indicating that BHB reduces bronchial smooth muscle contraction and histamine-induced airway constriction, suggests that therapeutic ketosis could serve as a protective strategy against dust-induced pulmonary inflammation, potentially reducing reliance on traditional bronchodilators or anti-inflammatory medications, which may be beneficial when the nearest pharmacy is 225 million kilometers away<sup>128</sup>. Moreover, stress-induced immune dysfunction is another major challenge in space travel, as prolonged isolation and environmental stressors can impair immune resilience.

Given that ketone bodies have demonstrated neuroprotective and anti-inflammatory effects in other contexts, therapeutic ketosis could be explored as a means to bolster immune function and mental resilience during extended missions. Future research should aim to translate these findings into spaceflight models, investigating the feasibility of ketone supplementation as a multi-faceted countermeasure for radiation damage, metabolic adaptation, and respiratory protection in space environments.

### **5.2.5. Final Remarks**

This dissertation has demonstrated that  $\beta$ -hydroxybutyrate (BHB), a key metabolite of therapeutic ketosis, exerts multifaceted effects on bronchial smooth muscle function, airway remodeling, and inflammatory signaling in asthma. By inhibiting protease activity, modulating cytokine production, and attenuating bronchoconstriction, BHB presents a promising avenue for asthma management. While this work provides strong mechanistic evidence for the therapeutic potential of ketosis, future research must validate these findings in *in vivo* models and human clinical trials. A deeper exploration of receptor-mediated pathways, such as FFAR3 activation, and the potential for BHB to complement existing asthma therapies, will be critical for translating these findings into clinical applications. Ultimately, this research advances our understanding of metabolic interventions in airway disease and lays the foundation for novel therapeutic strategies targeting bronchial smooth muscle dysfunction in asthma.

Beyond its implications for asthma, the therapeutic potential of ketosis extends to a diverse range of physiological and pathological conditions, from metabolic disorders to neurodegenerative diseases. The ability of BHB to regulate inflammation, oxidative stress, and cellular remodeling highlights its versatility as a metabolic intervention. As research

continues to uncover novel roles for ketone bodies in human health, it is clear that their impact reaches far beyond energy metabolism. Whether in the clinic, the athletic field, or even in the extreme environments of space, therapeutic ketosis may provide a powerful tool for optimizing resilience and adaptation in the face of physiological stressors. The findings presented in this dissertation underscore the importance of exploring metabolic therapies as an innovative and interdisciplinary approach to disease management and human performance.

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## **Appendix A: Cytokine Production from Bronchial Smooth Muscle Cells Is Augmented by Exposure to Adipocyte-Conditioned Media**

**Objective:** To gain insight into the effects of adipocyte-derived products on bronchial smooth muscle function.

**Rationale:** Obesity is a risk factor for asthma. Individuals with asthma and obesity often have poor asthma control and do not respond as well to therapies such as inhaled corticosteroids and long-acting bronchodilators as normal-weight asthmatics. Weight loss improves asthma control for obese asthmatics, with a 5%–10% loss in body mass necessary and sufficient to lead to clinically relevant improvements. Preclinical studies have demonstrated the pathogenic contribution of adipocytes from obese mice to the augmented production of pro-inflammatory cytokines from airway epithelial cells and the salutary effects of diet-induced weight loss to decrease these consequences. However, the effects of adipocyte-derived products on bronchial smooth muscle function in human obesity remain incompletely understood.

**Methods:** As described previously<sup>1</sup>, we utilized samples collected from a 12-month longitudinal study of subjects with obesity undergoing weight loss (bariatric) surgery, including subjects without asthma and subjects with allergic and nonallergic obese asthma. Visceral adipose tissue (VAT) samples were collected during bariatric surgery and from recruited normal weight control subjects without asthma undergoing elective abdominal surgery and cultured as described<sup>1</sup>. Human bronchial smooth muscle (HBSM) cells, as described in Chapters 2, 3, and 4, were exposed to adipocyte-conditioned media in the absence or presence of the model inflammatory agonists; 50 µg/mL *Dermatophagoides pteronyssinus* house dust mite extract (HDM; Part No. XPB70D3A25, Lot No. 343205;

Stallergenes Greer, Lenoir, NC), 50 ng/mL of ultrapure *Escherichia coli* O111:B4 lipopolysaccharide (LPS; Invivogen, San Diego, CA, LPS-EB), or 10 ng/mL interleukin (IL)-1 $\beta$  (Stem Cell Technologies, Cat No.78034.1) for 24 hours. A custom 24-plex [CCL2, CCL4, CCL11, CCL17, CCL20, CCL24, CCL26, Chitinase 3-like 1, CXCL1, CXCL2, CXCL8, GCSF, IFN $\beta$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12p70, IL-13, IL-17E, IL-18, IL-33, periostin, TNF $\alpha$ , and thymic stromal lymphopoietin (TSLP)] human magnetic Luminex assay (R&D Systems, Cat. No. LXSAM-24) was first used, following manufacturer's instructions, to determine the cytokines produced by HBSM cells under all conditions.

**Results:** An initial assessment of the cytokines substantially produced by unstimulated or stimulated primary human bronchial smooth muscle (HBSM) cells revealed that CCL2, CCL4, CCL20, CXCL1, CXCL2, CXCL8/IL-8, G-CSF, and IL-6 were produced by this cell type (**Figure A.1**). In the absence of stimulation, Ad-CM alone induced only a modest increase in cytokine secretion by the HBSM cells, with no significant differences found when compared with lean controls. The addition of agonist in combination with Ad-CM increased cytokine levels detected in the cell supernatants overall and revealed some differences among groups. Notably, cells treated with HDM in the presence of Ad-CM from nonallergic obese individuals tended to be significantly lower for all cytokines as compared with the obese non-asthma group but not the normal weight controls<sup>1</sup>.

**Conclusion:** In human bronchial smooth muscle cells, exposure to Ad-CM alone was insufficient to induce differences in Ad-CM groups, but the addition of agonist revealed that cells exposed to Ad-CM from non-allergic obese subjects tended to be lower than obese non-asthma but not normal weight controls (Fig. A)<sup>1</sup>. These data suggest that adipocyte-derived factors that are present in the Ad-CM may not be the main drivers for the

improvements in asthma management observed in subjects with obesity who had undergone bariatric surgery and experienced weight loss.

### **Figure Legends:**

#### **Figure A. Cytokine secretion from bronchial smooth muscle cells is augmented on exposure to Ad-CM.**

Human bronchial smooth muscle cells were exposed to Ad-CM in the presence or absence of house dust mite (HDM), lipopolysaccharide (LPS), or IL-1 $\beta$ . Data showing cytokine levels in supernatants after 24 h. Normal weight controls (C)  $n = 5$  (all female), obese non-asthma (OB)  $n = 9$  (2 males, 7 females), nonallergic obese asthma (N)  $n = 11$  (2 males, 9 females), and allergic obese asthma  $n = 7$  (2 males, 5 females). Male subjects = open circles; female subjects = closed circles. Data were analyzed through one-way ANOVA with Tukey's multiple comparisons test. Statistical significance is presented as  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$  as compared with lean control,  $\#P \leq 0.05$ ,  $\##P \leq 0.01$ ,  $\###P \leq 0.001$  as compared with OB group. Ad-CM, adipocyte-conditioned media.

### **References:**

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**Figure A.1. Cytokine secretion from bronchial smooth muscle cells is augmented on exposure to Ad-CM.**

