Hyperpnoea-induced bronchoconstriction: Prevalence in athletes, novel measure of airway inflammation, & treatment with the prebiotic Bimuno-Galactooligosaccharide

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Abstract

Asthma affects ~5,300,000 people in the UK. It is a significance public health burden costing the NHS an estimated one billion pounds per year. Approximately 50% of asthma patients suffer from hyperpnoea-induced bronchoconstriction (HIB), defined as a transient narrowing of the airway following hyperpnoea. HIB affects ~10% of the general population but is very prevalent in athletes, affecting approximately 35% of athletes and is severely under diagnosed and undertreated. Both asthma and HIB can be treated through pharmacological therapies, but these are neither curative nor preventative and chronic use can cause significant side-effects. The purpose of this thesis was to further understand the prevalence of HIB in athletes, and assess the effect of the dietary prebiotic, Bimuno-Galactooligosaccharide (B-GOS), supplementation on asthma and HIB. To establish the effect of B-GOS we first sort to evaluate the efficacy of a novel method to measure airway inflammation.

Consequently, this thesis investigated: (i) the prevalence of HIB in university field hockey athletes and assessed the effect of sex and diagnostic criteria on prevalence rates. Additionally, the thesis assessed the relationship between symptoms of dyspnoea and HIB *in situ*; (ii) the efficacy of the RTube device to collect exhaled breath condensate (EBC) with measurable cytokine concentrations; (iii) the effect of B-GOS supplementation on HIB severity, asthma control, and markers of airway inflammation in asthma participants with HIB.

The prevalence of HIB in university field hockey players was found to be 19% with a higher prevalence in males (30%) than females (5%). Diagnostic criteria influenced prevalence rates by 19% suggesting that less stringent criteria overestimate HIB prevalence. Symptoms of dyspnoea namely the “unpleasantness or discomfort of breathing” and the intensity of sensory dimensions (IPDS) were higher in HIB^{+} than HIB^{-} athletes, correlated with HIB severity, and

were highly sensitive and specific in predicting HIB (A1: Sensitivity = 100%; Specificity = 63%; IPDS: Sensitivity = 89%; Specificity = 66%). This is the first time respiratory symptoms and HIB have been assessed *in situ*, and one of the few reports to find an associate between symptoms and HIB.

This thesis found that IL-13, TSLP, IL-5, and TNF-α could not be detected in EBC samples collected with the RTube device in participants with HIB and/or asthma, or healthy controls. Device material and protein adsorption was suspected in the poor recovery of biomarkers. Attempts to reduce biomarker adsorption by coating the RTube in 1% bovine serum albumin and 0.01% Tween20, alongside an 8-fold sample concentration did not results in the detection of biomarkers. As such, future work should use alternative methods of measuring airway inflammation to assess the effect of B-GOS supplementation on airway inflammation in participants with asthma and HIB.

It was found that 21 days supplementation with 3.65g/d of B-GOS did not affect HIB severity, asthma control and systemic markers of airway inflammation. These findings contrasted previous work (Williams et al, 2016) and sheds doubt on the efficacy of B-GOS to attenuate HIB. GOS dosage, however, was noted as a potential influencing factor, as such, future work should look to confirm the efficacy of B-GOS using a B-GOS dosage providing ≥5.28g/d of GOS in a larger sample size and taking direct measures of airway inflammation.

Chapter 1 – General Introduction

1.1. Introduction and Rationale

Asthma is a very prevalent chronic respiratory diseases that is estimated to effect 350,000,000 people worldwide (GINA Report, 2020). It is the most prevalent chronic respiratory disease globally with twice the number of cases than chronic obstructive pulmonary disease (COPD) (GBD 2015 Chronic Respiratory Disease Collaborators). Asthma is defined by the presence of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and coughing which vary over time in their presence and intensity (GINA Report, 2020). In tandem with episodic respiratory symptoms asthma patients suffer from reversible but episodic bronchoconstriction that is primarily inflammatory driven leading to expiratory airflow limitation (GINA Report, 2020). Symptoms and expiratory airflow limitation in asthma are induced by triggers such as viruses, allergens, irritants, and exercise (Vernon, Wiklund, Bell, Dall, & Chapman, 2012). The pathophysiology of asthma includes airway hyper-responsiveness, airway inflammation, and airway remodelling. Whilst clearly defined asthma is a heterogeneous disease encompassing a variety of different phenotypes which vary based on their clinical characteristics and inflammatory profiles (Wenzel, 2012). The burden of asthma in the UK is high affecting ~5,300,000 people and costing the NHS an estimated one billion pound per year to treat (Asthma UK accessed 18/09/2020). In addition, it is estimated that 200,000 asthma patients suffer with severe asthma in the UK in which they require the highest doses of medication to treat their asthma. Furthermore, on average one person a day dies from asthma in the UK (Asthma UK accessed 18/09/2020).

Exercise-induced bronchoconstriction (EIB) is another prevalent respiratory condition affecting approximately 10% of the general population (Aguiar, Anzolin, & Zhang, 2018; Molphy, Dickinson, Hu, Chester, & Whyte, 2014). EIB shares similar pathophysiological features with asthma including AHR and airway inflammation and as such it is present in approximately 50% of asthma patients (Weiler et al, 2016; Sano, Sole, & Naspitz, 1998;

Cabral, 1999). The prevalence of EIB is exceptionally high in elite athletes (34%) and university athletes (43%) (Dickinson, McConnell, & Whyte, 2011; Burnett, Burns, Merritt, Wick, & Sharpe, 2016). The high prevalence in athletes is attributed to the high training intensities, frequency and durations sustained by athletes (Kippelen & Anderson, 2013). Bouts of high intensity exercise requiring high ventilation can place the airway epithelium under osmotic and mechanical stress resulting in airway damage. This coupled with high training frequencies leaves little time for airway repair resulting in a cycle of airway damage and inadequate repair over time. This accumulating airway damage overtime may lead to AHR and the development of EIB (Kippelen & Anderson, 2013). In addition, many sports including but not limited to swimming and ice rink sports involve exposure to respiratory irritants which additionally contribute to AHR and the development EIB (Kippelen & Anderson, 2013). In athletes the eucapnic voluntary hyperpnoea (EVH) test is recommended by the International Olympic Committee Medical Commission for confirming EIB in athletes. The EVH test was originally designed and validated by the US army as a surrogate to exercise testing to identify EIB (Eliasson, Phillips, Rajagopal, & Howard, 1992; Hurwitz, Argyros, Roach, Eliasson, & Phillips, 1995). While the EVH test is used to identify EIB in athletes a positive response to the test is more specifically called hyperpnoea-induced bronchoconstriction (HIB). The pathogenesis and pathophysiology of EIB and HIB is the same involving airway dehydration leading to increased airway surface osmolality and the induction of AHR and airway inflammation (Weiler et al, 2016). Additionally, EIB and HIB involves airway cooling during exercise/hyperpnoea and subsequent rewarming inducing vascular engorgement and airway oedema (Weiler et al, 2016). While the pathogenesis and pathophysiology of HIB is the same as EIB it is definitively differentiated by the type of test which is used to induce bronchoconstriction, exclusively hyperpnoea per say for HIB rather than exercise.

The prevalence of HIB varies drastically between sports (8-68%) which may in part be explained by difference in exercise demands (e.g. ventilation requirements, duration) and exposure to air conditions (e.g. respiratory irritants, humidity, temperature) between sports (Parsons et al, 2007; Dickinson et al, 2011; Levai et al, 2016; Weiler et al, 2016). The estimation of prevalence rates in individual sports, however, is tarnished by low cohort numbers ($n \leq 15$) and the variability in diagnostic criteria used to diagnose EIB and HIB (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016). Additionally, data suggests that there may be sex differences in the prevalence of HIB and EIB, however this is not conclusive as the prevalence of EIB and HIB in athletes has been reported to be both higher (42% vs 38%) and lower (18% vs 26%) in males compared to females (Parsons et al, 2007; Wilber et al, 2000). The prevalence of HIB in individual sports assessed using larger sample sizes and the effect of diagnostic criteria and sex on prevalence is required to understanding the true burden of HIB in individual sports and sexes. This is important as studies suggest HIB is commonly undiagnosed in athletes (Dickinson et al, 2011; Ansley, Kippelen, Dickinson, & Hull, 2012). Athletes not receiving treatment for HIB due to a lack of diagnosis or a false-negative diagnosis is problematic as untreated HIB can reduce exercise performance and is a risk factor for exercise-induced asthma mortality (DeJuilio, 2016; Becker, Rogers, Rossini, Mirchandani, & D'Alonzo, 2004; Jackson, Hull, Hopker, & Dickinson, 2018).

The diagnosis of HIB cannot be accurately made using respiratory symptoms alone as HIB is not associated with respiratory symptoms during exercise (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016; Ansley, Kippelen, Dickinson, & Hull, 2012; Jackson et al, 2018). The use of respiratory symptoms in HIB diagnosis leads to both high rates of false-positive and false negative diagnoses (Burnett et al, 2016). This is likely the reason for the high under diagnosis and under treatment of HIB in athletes (Dickinson et al, 2011). Previous

studies assessing the diagnostic accuracy of respiratory symptoms in HIB and EIB have measured symptoms retrospectively with participants at rest and asymptomatic, rather than shortly after an exercise or EVH test when bronchoconstriction is present. This is problematic because the severity of EIB may relate poorly to respiratory symptoms that are assessed retrospectively and reliant on memory (Conner & Barret, 2012). Conversely, momentary conscious respiratory symptoms are more likely to relate to the severity of bronchoconstriction if assessed *in situ*, i.e. shortly after an EVH test (Conner & Barret, 2012). Previous exercise may have also lacked sufficient stimulus to provoke bronchoconstriction (e.g. exercise $\dot{V}_E$ <85% MVV). All these conditions could reduce the validity of establishing an association between respiratory symptoms and HIB. As such there is a need to assess the association between respiratory symptoms and HIB *in situ*, following an EVH test were the presence or absence of HIB can be established.

Both asthma and HIB can be treated using pharmacological therapies with inhaled corticosteroids (ICS) and β_2-agonsists recommended in the initial treatment by the British Thoracic Society (BTS) and Scottish Intercollegiate Guidelines Network guidelines (2014). The chronic use of ICS particularly at high doses however can have undesirable side-effects, including dysphonia, adrenal suppression, growth suppression, bruising, osteoporosis, cataracts, glaucoma, metabolic abnormalities, and psychiatric disturbance (Williamson, Matusiewicz, Brown, Greening, & Crompton, 1995; Barnes, 2010). In the UK approximately 200,000 people require high dose ICS alone or in conjunction with addition therapies for the treatment of severe asthma (Asthma UK, https://www.asthma.org.uk/about/media/facts-and-statistics/ accessed: 05/01/2021). Chronic and frequent use of β_2-agonists in asthma patients has also been show to lead to tolerance and evening worsening bronchoconstriction in response to airway triggers (Swystun, Gordon, Davis, Zhang, & Cockcroft, 2000; Bhagat, Kalra, Swystun, & Cockcroft, 1995).

The World Anti-Doping Association (WADA) prohibited list permits the administration of inhaled salbutamol (1600 μg in 24-h and 800 μg in 12-h), formoterol (≤54 μg in 24-h), salmeterol (≤200 μg in 24-h), vilanterol (≤25 μg in 24-h) and ICS therapy in athletes with a diagnosis of asthma without the need for a therapeutic use exemption (TUE). All other inhaled β_2-agonists and the systemic use of all β_2-agonists (injected or oral) and oral corticosteroids are either prohibited or require a TUE (WADA. Medical Information To Support The Decision of TUECS – ASTHMA. https://www.wada-ama.org/en/resources/therapeutic-use-exemption-tue/medical-information-to-support-the-decisions-of-tuecs-asthma. Access: 03/01/2021). While most asthma medications in commonly prescribed methods and doses are permitted, athletes perceive there is a negative stigma surrounding the use of asthma therapies in competitive sport (Allen, Price, Hull & Backhouse, 2021). This may lead to the avoidance of asthma medication in athletes with asthma and/or HIB.

On the other hand, speculation exists on the potential performance-enhancing effect of asthma therapies. Evidence suggests there is limited ergogenic benefits of inhaled β_2-agonists and ICS on endurance, strength, or sprint performance in healthy athletes while some evidence supports an ergogenic effect of systemic β_2-agonists on athletic performance (Pluim et al, 2011; Kuipers et al, 2008. Multiple asthma therapies which require TUEs however, have need linked to meaningful improvements in athletic performance (Allen, Backhouse, Hull & Price, 2019). These therapies could therefore be misused to gain a competitive advantage in sport. The issues of adverse side-effects, stigma and avoidance, and the potential for misuse of pharmacological asthma therapies in sport highlights the need to identify novel non-pharmacological alternatives to treat asthma and HIB.

The gut microbiota is a potential target for therapeutic intervention in asthma and HIB. The host immune system is influenced by the gut microbiota by direct bacterial interaction and the

production of gut bacterial metabolites (Koh, Vadder, Kovatcheva-Datchary, & Backhed, 2016). As such the gut microbiota can affect immune responses in the gut as well as distal sites including the lung which has led to the emergence of the gut-lung axis (Dang & Marsland, 2019; Budden et al, 2017). The promotion of healthy gut bacteria species including Bifidobacterium and lactobacilli and the production of gut derived metabolites such as short chain fatty acids (SCFAs) have long been linked to host health and shown to moderate and attenuate inflammation at distal sites including the lung (Dang & Marsland, 2019; Budden et al, 2017). Dietary prebiotics are a method to promote the growth of healthy gut bacteria and enhance the production of SCFAs by the gut microbiota (Gibson et al, 2010). Bimuno-galactooligossacharide (B-GOS) is a prebiotic which possess exceptional bifidogenic properties and increases the production of SCFAs compared to other dietary prebiotics (Tzortis, Goulas, Gee, & Gibson, 2005; Depeint, Tzortzis, Vulevic, I'Anson, & Gibson, 2008; Vulevic, Drakoularakou, Yaqoob, Tzortis, & Gibson, 2008). In patients with asthma and HIB 21-days B-GOS supplementation has been shown to attenuate HIB by 40% and attenuate systemic markers of airway inflammation (Williams et al, 2016). B-GOS therefore has major potential as a treatment for asthma and HIB and warrants further investigations. Specifically, the effect of B-GOS on airway inflammation requires investigation as it is a major pathophysiological feature of asthma and HIB. To achieve this, novel methods in measuring airway inflammation are also required. This is because common methods to measure airway inflammation such as induced sputum and the collection of bronchoalveolar lavage fluid are not practical during HIB. While fractional exhaled nitric oxide (FeNO) can provide a non-invasive airway sample its utility is limited to the diagnosis of eosinophilic asthma and in predicting ICS responsiveness in asthma patients (Menzies-Gow, Mansur, & Brightling, 2020). In addition, FeNO is reduce following exercise and spirometry in asthma patients potentially due to changes in airway calibre (Bjermer et al, 2014). This limits the

utility of FeNO in assessing HIB, were FeNO values will be influenced by hyperpnoea, bronchoconstriction, and the frequent spirometry maneuverers required in the assessment of HIB. A sample called exhaled breath condensate (EBC) however, can be collected non-invasively using a device named the RTube. This method, if effective, would allow for the assessment of B-GOS supplementation on airway inflammation in participants with HIB and therefore needs investigating.

In summary, the high prevalence of HIB presents a significant burden for athletes, however, the prevalence of HIB in individual sports and differences between sexes requires further investigation. Furthermore, while previous literature has suggested a poor association between HIB and respiratory symptoms this has not been assessed in situ where an association may still exist. While HIB is effectively and routinely treated using common asthma medications these therapies, particularly in high doses, present a risk for adverse side effects. In relation to athletes there is a real threat of the misuse of some asthma medications to gain a competitive advantage, while on the other hand the negative media portrayal of some athletes using TUEs for asthma medications can contribute to the avoidance of pharmacological therapies in some athletes who require treatment. These issues highlight the need to investigate new non-pharmacological therapies for the treatment of HIB of which prebiotics have shown potential that warrants further investigation. In particular, the effect of prebiotics on airway inflammation in HIB is required, however, new novel ways of measuring airway inflammation in the context of HIB require investigating.

1.2.Thesis aims

In relation to the gaps in the literature highlighted above this thesis aims to investigate the following research questions:

I. What is the prevalence of hyperpnoea-induced bronchoconstriction in university field hockey athletes?

This research aimed to:

- Determine the prevalence of HIB in British University field hockey athletes and determine sex differences in HIB prevalence.
- Assess the effect of different HIB diagnostics criteria on prevalence rates.
- Evaluate the associations between symptoms of dyspnoea and HIB assessed *in situ*.

II. Can cytokines be measured in exhaled breath condensate collected with the RTube device from participants with asthma and hyperpnoea-induced bronchoconstriction?

This research aimed to:

- Determine whether the RTube device can collect exhaled breath condensate from participants with asthma and HIB in which cytokines can be measured.
- If effective this will allow for airway inflammation to be measured in response to B-GOS supplementation in participants with asthma and HIB.

III. What is the effect of 21-days supplementation with the prebiotic, Bimuno-galactooligosaccharide (B-GOS), on HIB severity, asthma control and airway inflammation?

This research aimed to:

- Determine whether B-GOS supplementation attenuates HIB severity and improves asthma controls.
- Assess whether B-GOS supplementation attenuates markers of airway inflammation in EBC.

Chapter 2 – Review of literature

2.1. Pathogenesis and pathophysiology of exercise-induced bronchoconstriction

Exercise-induced bronchoconstriction (EIB) is a transient narrowing of the airways during or following an exercise which is diagnosed by an ≥10% drop in forced expiratory volume in 1 second (FEV_1) from pre to post challenge (Weiler et al, 2016; Parson, 2013). Sustained high intensity exercise requires an athlete maintain a high minute ventilation which can cause airway dehydration and rapid cooling and rewarming during and following exercise. These responses are suggested to initiate airway inflammation, oedema, and smooth muscle contraction in EIB susceptible individuals resulting in reduced airway calibre and enhanced expiratory airflow limitation which reduces FEV_1 (Weiler et al, 2016; Parsons, 2013).

Due to airway dehydration and cooling/rewarming being fundamental in the development of EIB, surrogate tests to assess EIB have been created. Surrogate tests have been created to allow for cheaper tests, greater accessibility, greater sensitivity, and the ability to control sport specific characteristics such as ventilation rates and air conditions. A description of individual test and the positive and negative of each test is detail in Section 2.3.1. Diagnosis of Exercise-induced Bronchoconstriction. One test, the eucapnic voluntary hyperpnoea (EVH) test requires participants to hyperventilate at 85% maximal voluntary ventilation for 6-minute while breathing a dry gas (<5% humidity) and as such is a controlled method to induce airway dehydration, cooling and rewarming. Initial studies identified that individuals who had EIB were susceptible to bronchoconstriction following the EVH test (Eliasson et al, 1992). The response to the EVH test is determined as hyperpnoea-induced bronchoconstriction and is diagnosed in the same manner as EIB, and ≥10% decrease in FEV_1 from pre to post test. This highlighted that exercise was not exclusively required to elicit bronchoconstriction in susceptible individuals as volitional hyperpnoea, particularly of dry air (>5% humidity), could elicit a similar response. As such, the EVH test is a commonly used test to identify EIB in athletes.

While both EIB and HIB (E-HIB) are diagnosed as a ≥10% drop in FEV_1 from pre to post challenge (Parson, 2013; Weiler et al, 2016), the peak drop in FEV_1 can be defined as mild (≥10 - <25%), moderate (≥25 - <50%), and severe (≥50%). The majority of EIB patients, particularly those without asthma, have healthy baseline lung function (e.g. percentage predicted FEV_1 ≥80%) (Same et al, 2003). Following an exercise-challenge lung function can drop dramatically and in severe cases of EIB lung segments are subject to closure or near closure following an exercise-challenge (Same et al, 2003).

Decreases in FEV_1 occur quickly following exercise/hyperpnoea in positive responders, peaking between 3-15 minutes post-test. The drop in FEV_1 is generally short-lived and resolved spontaneously within 30-90 minutes post challenge although the recovery can be accelerated with the use of a β_2-agonist. A refractory period is present in approximately 50% of EIB cases (Belcher, Rees, Clark, & Lee, 1987). This means that the initial induction of EIB induces a period, typically 1-3 hours post challenge, where a subsequent exercise/EVH does not induce the same drop in FEV_1. Interestingly, E-HIB induce refractoriness interchangeably owing to common pathways involved in both (Belcher et al, 1987).

2.1.2. Pathogenesis of EIB

The mechanisms of E-HIB have been attributed to two classical hypotheses, the thermal and osmotic hypotheses. The thermal hypothesis proposes that E-HIB manifests from rapid airway cooling and rewarming following sustained bouts of high ventilation leading to airway vasodilation and oedema (Anderson & Kippelen, 2010). The osmotic hypothesis proposes that evaporation of airway surface liquid (ASL) caused by high ventilation increases airway osmolality which initiates a cascade of inflammation, airway oedema and

bronchoconstriction (Anderson & Kippelen, 2010). Both these pathways reduce airway calibre and can work synergistically to reduce FEV_1 post exercise and hyperpnoea.

2.1.2.1. Thermal hypothesis

The heating and conditioning of large volumes are air to body temperature during exercise or EVH is accompanied by intrapulmonary temperature drops (Deal, McFadden, Ingram, & Jaeger, 1978). Thermal mapping of the airways has shown that ventilation rates of $100L \cdot min^{-1}$ alone decrease proximal and distal airway temperatures 2.8 and 1.6°C, respectively. The breathing of cold air exaggerates these temperature drops to 11.5°C in proximal and 3.9°C in distal airways, respectively (McFadden 1985). In individuals with EIB, the breathing of cold air during exercise therefore accentuates the bronchoconstrictive response (Weiler et al, 2016). This initial cooling process stimulates cholinergic receptors within the airways, which increases airway smooth muscle tone and airway secretions (Barnes, 1987).

Subsequent airway rewarming following the initial cooling process was found to be paramount to the thermal hypothesis. McFadden, Lenner, & Strohl (1986) found that increasing inhaled air temperature following cessation of exercise to facilitate airway rewarming exacerbates bronchoconstriction. The size of the temperature gradient from airway cooling during exercise to rewarming following cessation is linearly relatied to the magnitude of bronchoconstriction (Gilbert & McFadden, 1992). Moreover, airway cooling during exercise in not different between EIB and healthy subjects, but airway temperature rises twice as fast in EIB participants following cessation of exercise (Gilbert, Fouke, & McFadden, 1987). The hyperplastic capillary bed of asthmatics likely exaggerates the rebound hyperaemia leading to oedema and excess airway narrowing, likely owing to the high prevalence of EIB and HIB in asthma populations. In support, attenuation of mucosal

blood supply with norepinephrine reduces the airway rewarming process and attenuates bronchoconstriction (Gilbert & McFadden 1992). The airway cooling-rewarming process is proposed to initiate vascular dilation and permeability leading to vascular engorgement, leakage and oedema at the airway surface contributing to transient airway narrowing and causing expiratory airflow limitation (McFadden, 1990).

The thermal hypothesis likely contributes to the development of EIB/HIB particularly when breathing cold air (Stensurd, Bernsten, & Carlsen, 2007). The observation that bronchoconstriction can be elicited while breathing hot but dry air (Anderson, Schoeffel, Black, & Daviskas, 1985; Eschenbacher, Moore, Lorenzen, Weg, & Gross 1992), and is not exaggerated by cold air when air humidity is maintained (Hahn, Anderson, Morton, Black, & Fitch, 1984) suggests that thermal mechanisms are not the primary cause of E-HIB but are agonistic factors for reduced airway caliber. Other mechanisms are paramount in the development of E-HIB. Eschenbacher et al (1992) found that peak drops in FEV_1 following exercise were similar between cold and dry air (-20.7%), and hot and dry air (-20.4%) breathing. In line with this, Hahn et al (1984) found the fall in FEV_1 following an exercise challenge test did not differ when air temperature was reduced (35 to 10°C) if water content of inspired air is maintained. These observations led to the creation of the osmotic theory suggesting airway dehydration as a prerequisite and predominant mechanism for the initiating EIB/HIB.

2.1.2.2. Osmotic hypothesis

The osmotic hypothesis proposes that evaporation of the ASL to humidify large volumes of air during sustained hyperpnoea leads to increased ion concentration and ASL osmolality (Anderson & Kippelen, 2010). The small volume of ASL (<1mL in the first 12 generation of

airways) makes the airway surface susceptible to dehydration during high ventilation rates. Mathematical modelling devised by Daviskas, Gonda, & Anderson (1991) suggests generation 8 to 10 are recruited in the humidifying process at ventilation rates of $60L \cdot min^{-1}$ with substantial water loss from these small proximal airways. Ventilation rates higher than this are common during high intensity exercise and may engage airway generation as low as 12 in the humidifying process (Kippelen & Anderson, 2013). Generations this low are classified as the small airways with an internal diameter < 2 mm. Within such condition's airway water return to the airways may be inadequate leading to substantial dehydration and increased ASL osmolality. In children with EIB rapid respiratory water loss is evident during exercise with expiratory water vapour remaining lower compared to EIB negative children following exercise (Tabka, Jebria, Vergeret, & Guenard, 1988). This supports airway mucosa dehydration in the creation of a hyperosmotic environment within the airways of EIB-positive individuals.

Within animal models, the breathing of dry air reduces water content of the airways (Van Oostdam et al, 1986) and increases ASL osmolality (Freed, 1999) resulting in enhanced bronchoconstriction. In humans, reduction in ASL depth are estimated by measuring ciliary beat frequency. Decreases in the periciliary fluid layer depth by evaporation decreases the rate of mucociliary clearance (MCC) (Shephard & Rahmoune, 1994). MCC is reduced following isocapnic hyperpnoea in both healthy and asthma participants (Daviskas et al, 1995). Reductions are isolated to the large airways in healthy individuals but additionally include the small airways in individuals with asthma which may be a contributing factor to the high prevalence of E-HIB in asthmatics. ASL dehydration is a clear response to hyperpnoea and high intensity exercise which leads to increase ASL osmolality. Increased airway surface osmolality can be directly achieved by inhalation of mannitol, an osmotic agent. As such there is close agreement in individuals who have EIB and HIB also having

bronchoconstriction induced upon mannitol inhalation, providing evidence that increased airway osmolality is an important factor in EIB/HIB (Barben et al, 2011; Brannan, Koskela, Anderson, & Chew, 1998). Increased airway osmolality initiates the release of inflammatory mediator, cysteinyl leukotrienes (Cyst-Lts) and prostaglandin D_2 (PGD2) which induce airway smooth muscle (ASM) contraction (Hallstrand, Altemeier, Aitken & Henderson, 2013). Hyperosmolality can also activate sensory nerve to induce ASM contraction.

While the osmotic theory is the primary pathway in inducing E-HIB, both the osmotic and thermal pathways happen in sync to induce bronchoconstriction. Additionally, there can be an additive effect whereby inflammatory mediator release due to osmotic changes and reactive hyperaemia due to temperature changes promote greater airway immune cell infiltration and oedema. On top of this airway surfactant function is compromised in the presence of airway inflammation, particularly with lowering temperatures (Enhorning, Hohlfeld, Krug, Lema, & Welliver, 2000). Therefore inflammatory mediator release via osmotic changes and reduced airway temperatures during exercise/hyperpnoea may diminish surfactant function and subsequently increase airway resistance in EIB/HIB.

The thermal and osmotic response to high intensity exercise can be simulated using surrogate challenges such as the EVH test. During an EVH test participants typically sustain ventilation of ~85% MVV for 6-minutes while breathing dry air (Humidity: ~5%). The bronchoconstrictive and inflammatory response to an EVH test mirrors those of exercise-challenges (Anderson & Kippelen, 2012), although the EVH test is typically a more sensitive test due to the use of dry air which exaggerates the airway dehydration process. The response to an EVH test, while inducing bronchoconstriction via the same mechanisms as EIB, is referred to as HIB. Both EIB and HIB present with the similar pathophysiological features of bronchoconstriction, airway inflammation, and airway injury.

2.1.3. Pathophysiology of EIB

Certain airway cells respond in a pro-inflammatory manner to a hyperosmolar environment in vitro. Eosinophils produce more LTC_4 upon osmotic stress, this response is greater in eosinophils from asthmatics (Moloney, Griffin, Burke, Poulter, & O'Sullivan, 2003). Mast cells and basophils release histamine in a hyperosmolar environment while mast cells also produce greater PGD_2 and LTC_4 (Gulliksson, Palmberg, Nilsson, Ahlstedt, & Kumlin, 2006). The release of histamine by mast cells and basophils is partially Ca^{2+}-dependant (Eggleston, 1986). In vivo histamine, tryptase, and leukotrienes are elevated following exercise and EVH challenges in EIB/HIB positive individuals and are well established contributors to EIB and HIB (Barnes, Wilson, & Brown, 1981; Hallstrand et al, 2005). PGD_2 and Cyst-LTs are elevated in the airways during EIB and peak approximately 30-60 minutes post challenge. PGD_2 and Cyst-LTs are potent vasodilators and bronchoconstrictors (Johnston, Freezer, Ritter, O'Toole, & Howarth, 1995). Indeed, Cyst-LTs are approximately 5000 times more potent than histamine in eliciting bronchoconstriction (Dahlen, Hedqvist, & Hammarstrom, 1982). The initial drop in FEV_1 with EIB/HIB is suggested to be histamine mediated and subsequently sustained by leukotrienes. In addition to leukotrienes, eosinophilic cationic protein is elevated in the sputum of EIB positive asthmatics and is associated with the severity of EIB (Yoshikawa et al, 1998), while TNF-α is elevated in blood serum following exercise and EVH challenges in individuals with EIB/HIB (Williams et al, 2016; Mickleborough, Murray, Ionescu, & Lindley, 2003). In vitro, TNF-α enhances ASM sensitivity to other mediators (Anticevich, Hughes, Black, & Armour, 1996).

Mast cells and eosinophils are key sources of eicosanoids (Mendez-Enriquez & Hallgren, 2019). Mast cells are found in close proximity to the airway epithelium in health and are found in greater numbers in asthma patients (Brightling et al, 2002). In asthma, the greatest mast cell densities are found in the distal/membranous airways (Carrol, Mutavdzic, & James,

2002). Intraepithelial mast cell density is 2-fold higher in the Th2- high compared to the Th2-low phenotype of asthma (Dougherty et al, 2010). EIB positive asthmatics also have a higher density of intraepithelial mast cells compared to their EIB negative counterparts (Lai et al, 2014). The mast cells in EIB positive and Th2-high asthmatics express a tryptase and CPA3 high, chymase low phenotype (Lai et al, 2014; Dougherty et al, 2010), this corresponds with increases in blood serum tryptase concentrations following exercise and EVH challenges. The locality of mast cells in the distal airways would expose them to hyperosmolar conditions during exercise/hyperpnoea (Kippelen et al, 2013). The close proximity of mast cells to airway epithelial cells would also support interaction between the two upon osmotic and/or mechanical stress. In addition to elevated mast cells, percentage sputum eosinophils are elevated in EIB positive asthmatics and correlate with the severity of EIB (Yoshikawa et al, 1998). Mast cells and eosinophils respond to osmotic stress by producing high amounts of eicosanoids and are therefore important pathophysiological features of EIB.

A further feature of EIB/HIB is suppression of anti-inflammatory and bronchoprotective mediators. For example, the bronchoprotective mediator PGE_2 is reduced in blood and urine upon an exercise-challenge in EIB individuals, elevating the Cyst-LT's/PGE_2 ratio (Hallstrand et al, 2005). The Cyst-LT's/PGE_2 ratio is further elevated in sputum of EIB positive compared to EIB negative asthmatics (Hallstrand & Henderson, 2009). Inhalation of PGE_2 protects against bronchoconstriction and the major producers of PGE_2 in the airways are epithelial cells. PGE_2 can directly inhibit osmotic stress induced mast cell degranulation and subsequent eicosanoid and cytokine synthesis in vitro (Torres-Atencio, Ainsua-Enrich, De Mora, Picado, & Martin, 2014). PGE_2 acts through prostanoid receptors 2 and 4 to inhibit Ca^{2+} influx and the mitogen-activated protein kinase pathway. The reduction of PGE_2 with EIB therefore limits the ability of the airways to control bronchoconstriction and inflammation. The ratio of anti-inflammatory lipoxin A_4 to the pro-inflammatory leukotriene

B_4 is also decreased with EIB severity (Kazani et al, 2013). This shows an unbalancing between bronchoprovoking and protective mediators in the airway pathology of EIB. The release of protective mediators is thought to contribute to refractoriness found in 50% of EIB patients where bronchoconstriction is less inducible 1-3 hours post challenge (Edmunds, Tooley, & Godfrey, 1978). While the precise mechanism for the refractory period in EIB is not known the release of protective mediators and increased tolerance of ASM to bronchoprovoking mediators are suggested mechanisms (Manning, Watson, & O'Byrne, 1993; Larsson et al, 2011).

Airway epithelial cells also play a role in EIB/HIB by sensing osmotic changes and modulating airway smooth muscle contraction through the release of epithelial derived relaxing factors and excitatory neurotransmission (Fedan, 1999; Wu, 2004). These may initiate smooth muscle contraction in response to an osmotic stimulus and orchestrate the production of eicosanoids from nearby immune cells (Hallstrand, 2012). The epithelial derived alarmin, IL-33, is released from lung tissue in response to increased osmolality (Lai et al, 2014). IL-33 stimulates the production of type-2 cytokines from ILC2 and Th2 cells. Furthermore, $sPLA_2$-X protein is increased in sputum samples and in epithelial cells during EIB (Hallstrand, 2007). $sPLA_2$-X causes the release of lysophospholipids and free fatty acids, including arachidonic acid (AA) and the subsequent synthesis of Cyst-LTs from eosinophils (Hallstrand, Debley, Farin, & Henderson, 2007). This highlights the involvement of epithelial cells in partly orchestrating inflammatory responses from nearby immune cells.

Sensory nerve activation is also implicated in E-HIB. Osmotic stress can activate sensory airway nerves while inflammatory mediator releases such as Cyst-LTs can lower the activation threshold of sensory nerves, contributing to bronchoconstriction (Joos et al, 2004; Freed et al, 2003; Chen et al, 1999). The activation of sensory nerves causes the release of neurokinins, which when blocked, attenuate bronchoconstriction (Joos et al, 2004). The

inhibition of neurokinin-1 and 2 receptors inhibits HIB in canine models but doing so does not attenuate the release of inflammatory mediators Cyst-LTs and PGD_2 (Freed, McCulloch, Meyers, & Suzuki, 2003). The inhibition of Cyst-LTs however inhibits HIB and additionally attenuates neurokinin release (Chen, Chen, & Lai, 1999). This supports a role for osmotic stress and subsequent eicosanoid release to activate sensory nerves and promote bronchoconstriction. This is further supported in humans where there is a relationship between Cyst-LTs and tachykinins neurokinin A (NKA) concentrations in sputum post-exercise challenge in individuals with EIB and asthma (Hallstrand et al, 2007). Hallstrand et al (2007) also found mucin 5AC (MUC5AC) to be the predominant gel-forming mucin in patients with asthma and EIB, and that gene and protein expression of MUC5AC increased following an exercise-challenge. Alongside airway inflammation, the pathology of EIB is therefore accompanied with airway sensory nerve activation and mucus production.

2.1.4. Airway injury in the pathogenesis and pathophysiology of EIB in athletes

The airway epithelium is placed under immense stress during exercise and hyperpnoea which can cause airway damage. Dehydration injury, luminal shear stress during high ventilator flow rates, and the inhalation of irritants can damage the airway epithelium. This process can contribute to the inflammatory processes of EIB/HIB and influence the pathogenesis of E-HIB in elite athletes through damage-repair processes (Kippelen, 2013). One of the key pieces of evidence which supports airway damage in the pathology of E-HIB is the finding of high epithelial cell numbers in the sputum of EIB individuals suggesting of epithelial injury, and increased markers of epithelial damage post exercise and EVH challenges (Chimenti et al, 2010; Randolph, 2009).

Considerable airway epithelial injury following exercise and hyperpnoea have been shown in animal models. Omori et al, (1995) exposed canine peripheral airways to dry air inhalation at flow rates of 2000ml/min and assessed airway injury via light microscopy. Fifty percent of ciliated epithelium was lost with airway damage evident immediately, 1, and 2 hours after hyperpnoea. Peripheral airway damage has also been shown in mice following endurance training (Chimenti et al, 2007). After 45 days mice show progressive airway epithelium damage-repair with a 4-fold lower number of ciliated cells and 56% increase in epithelial thickness compared to control mice (Chimenti et al, 2007). These animal models do implement intense stresses which would be unreflective of recreational exercise, however, elite athletes exercising at high intensities and training frequencies may be exposed to such stresses.

CC16 is a measure of airway epithelial disruption that can be measured in serum and urine. In humans, serum CC16 increases following a half-marathon in amateur runners with no history of asthma (Chimenti et al, 2010), furthermore, bronchial epithelial cell count and apoptosis are increased following half-marathon running. IL-8 concentrations are additionally doubled and correlate with bronchial epithelial cell counts suggesting a possible inflammatory response due to airway injury. IL-8 plays a role in airway neutrophil recruitment and after marathon running sputum neutrophils increase alongside fraction of exhaled nitric oxide (FeNO) (Bonsignore et al, 2001). Interestingly, marathon runners also have elevated sputum neutrophils at rest, a feature also found in XC skiers and swimmers (Randolph, 2009). Neutrophils play a role in inducing airway hyper-responsiveness (Anticevich et al, 1996). Strenuous exercise such as long-distance running therefore acutely induces airway injury, while long term training may also result in enhanced airway neutrophilia. This is of significance as the inflammation induced by airway injury may contribute to EIB in EIB-positive individuals, while the damage repair process and subsequent airway neutrophilia

may contribute to the pathogenesis of EIB in EIB-negative athletes on high training loads. Exercise protocol is important in airway damage with continuous exercise inducing greater airway injury than intermittent exercise. Combes et al (2019) assessed the effect of continuous cycling at 70% work rate max versus a work-matched intermittent exercise on airway epithelial damage. Continuous exercise increased serum CC16 and the CC16/SP-D ratio while mean ventilation during exercise was only 85 l/min^{-1}. Interestingly work-matched intermittent exercise did not increase CC16 suggesting that airway injury did not occur. Mean ventilation was lower with intermittent exercise and the CC16/SP-D ratio correlated with mean ventilation. Therefore, while exercise can induce epithelial damage, exercise modality is influential and as expected ventilation is a contributing factor.

In sports characterised by high ventilation, such as cycling and distance running, airway epithelial damage may be induced by cell shrinkage or mechanical shear force. As modelling by Daviskas et al (1991) suggests, airway generation as low as 10 would be recruited into the air humidifying process at a ventilation of just 60 l/min^{-1}. Ventilation rates >80 l/min^{-1} is common in marathon running which would increase ASL osmolality in peripheral airways and induced epithelial dehydration injury. In addition to dehydration injury airway epithelial cells are exposed to luminal shear stress generated by high airflow rates. High fluid shearing and pressure gradients have been shown to damage epithelial cells in an in vitro model of airway reopening (Bilek, Dee, & Gaver, 2003). These forces are present during mechanical ventilation where lung injury is common. With increased flow rates during exercise shear stress is likely to be increased. Transmural pressure gradients can rise from -8.5cm H_2O with tidal breathing to -20cm H_2O with forced expiration, such as that during exercise. The increased transmural pressure may increase luminal shear stress and contribute to epithelial damage during exercise. Furthermore, shear force is increased with increased wall stiffness (Xia, Tawhai, Hoffman, & Lin, 2010), therefore bronchoconstriction or airway remodelling

(from EIB or asthma) leading to less compliant airways may enhance shear wall stress and epithelial damage (Huh et al, 2007).

Interestingly, urinary CC16 remains unchanged when exercise and EVH are performed with the inhalation of humid air (Bolger et al, 2011; Bolger et al, 2011a). This suggests that epithelial dehydration may be more important than mechanical stress in causing epithelial damage. There can however be an additive effect of both mechanisms in that reductions in ASL volume by dehydration may enhance detachment of epithelial cells caused by high flow rates (Kippelen & Anderson 2013). The ability of the airways to condition large quantities of air is markedly challenged in winter sports such as XC skiing, which are characterised by high ventilation rates in cold environments. Karjalainen et al (2000) found significant airway inflammation and remodelling in elite skiers with and without airway hyper-responsiveness. Bronchial biopsies showed T-lymphocytes, macrophages, and eosinophils counts to be 43-fold, 26-fold, and 2-fold higher, respectively, than in controls with macrophage count correlating with the number of years of skiing experience. Additionally, lymphocytes and macrophage counts were greater in athletes without airway hyper-responsiveness compared to controls, while no difference compared to airway hyper-responsive skiers. Tenascin expression, an extra-cellular matrix protein, was increased in skiers compared to controls. The requirement to condition large quantities of cold and dry air may be involved in the pathogenesis of EIB in XC skiers and likely contributes to the high prevalence of HIB found in winter sports athletes (Dickinson et al, 2011).

Substantial epithelial damage is also present in swimmer where ventilation demands are low and warm humid air conditions are present. Within this context dehydration injury and mechanical shear stress cannot be present to contribute to epithelial damage. The odds ratios for asthma symptoms and current or ever asthma diagnosis is increased with greater number of lifetime hours spent in chlorinated swimming pools (Bernard, Rees, Clark, & Lee, 2009).

Increased serum CC16, SP-A, SP-B, and FeNO concentrations are seen after swimming which is attributed to chlorine/chlorine by-products acting as an airway irritant (Font-Ribera et al, 2010; Bougault, Turmel, & Boulet, 2013; Carbonnelle, Bernard, Doyle, Grutter, & Francaux, 2008). Bonetto et al, (2006) reported that in a group of children accidentally exposed to excessive chlorine concentrations while completing a swimming lesson lung function was impaired and serum CC16 and EBC LTB_4 was increased (Bonetto et al, 2006). In addition, HR-intensity matched swimming results in greater increases in serum CC16 and CC16/SP-D ratios compared with cycling (Bougault et al, 2013). Nitrogen trichloride (NCl_3), an irritant released from indoor chlorinated pools in shown to disrupt the lung epithelium in mice, and it is suspected to cause swimming pool asthma in swimming pool workers who do not enter pools (Thickett, McCoach, Gerber, Sadhra, & Burge, 2002).

The effect of chlorine and chlorine by-products on damaging the airway epithelial likely contributes to the high prevalence of EIB and HIB in swimmers. Castricum, Holzer, Brukner, & Irving (2010) tested elite swimmers for HIB using an EVH test and found over half (55%) had HIB. Interesting, when lung function was assessed following a swimming challenge only one athlete had a positive challenge response. Therefore, while swimming is unlikely to induce EIB directly, the exposure to irritants in swimming pools leads to airway epithelial damage which predisposes swimmers to airway hyper-responsiveness and the development of E-HIB over time. There is evidence however that ≥3 months post retirement from swimming can allow airway hyper-responsiveness to resolve in elite swimmers and this is accompanied with a propensity for sputum eosinophil counts to decrease (Helenius et al, 2002). This supports a damage repair process in elite athlete were frequent high intensity exercise and/or frequent exposure to irritants leads to frequent airway injury that predisposes athletes to developing E-HIB.

Similar to chlorine exposure in swimming other sports expose athletes to high levels of respiratory irritants. There is a high prevalence of EIB in figure skaters (30%) (Provost-Craig, Arbour, Sestili, Chabalko, & Ekinci, 1996). While the high intensity and cold air exposure would inevitably contribute to E-HIB, high levels of fine and ultra-fine particulate matter (PM) are found in ice rinks due to the use of ice-resurfacing machines (Rundell, 2003). Approximately 9% of inhaled fine PM is estimated to be deposited in the lung, 6% reaching the alveolar region, with an 4.5-fold increase estimated with ventilation attained during mild exercise (38 l/min^{-1}) (Venkatarman, Thomas, & Kulkrni, 1999). Deposition of PM in the lung can result in oxidative stress and inflammation (Cutrufello, Smoliga, & Rundell, 2012). Decreased lung function has been reported following the inhalation of fine and ultrafine PM during exercise, and the decline in lung function seen in ice hockey players over time has been linked to the inhalation of PM (Rundell, Slee, Caviston, & Hollenbach, 2008). Other types of pollution such as diesel exhaust fumes, smog and Ozone have been implicated in damaging the airways of athletes. Some runners and cyclists may be exposed to high levels of diesel exhaust fumes and the high ventilation sustained during these exercise modalities may increase fine PM lung deposition.

2.1.4.1. How airway injury may predispose athletes to EIB

Disruption of airway epithelium may predispose athletes to developing E-HIB. The damage repair process of the airway epithelium is accompanied with inflammation, cell proliferation, mucus hypersecretions and plasma exudates (Kippelen & Anderson, 2013). Airway inflammation that accompanies airway injury may contribute to the development of AHR. In long-distance runner's airway injury is accompanied by elevated sputum neutrophils post-exercise (Bonsignore et al, 2001). In elite athlete's sputum neutrophils are elevated at rest

which may be linked to frequent injury repair processes from frequent bouts of high intensity exercise. Neutrophils contribute to increased airway hyper-responsiveness and may change the contractile properties of airway smooth muscle (Anticevich et al, 1996). This could increase airway hyper-responsiveness to airway osmotic/thermal stimuli. Airway injury also contributes to dampened bronchoprotective mechanisms. Airway epithelial cells are the main producers of PGE_2, a bronchoprotective mediator. The loss of airway epithelial cells by epithelial damage will reduce the secretion of PGE_2, inhibiting the airway's ability to relax airway smooth muscle and prevent bronchoconstriction (Folkerts & Nijkamp, 1998). A reduction in the secretion of PGE_2 is observed in individuals with EIB (Hallstrand et al, 2005) and therefore a reduction in PGE_2 might be a contributing factor to the development of EIB/HIB.

The disruption of the airway epithelial barrier may consequently allow allergens, irritants, and pathogens to penetrate the epithelial barrier promoting further airway inflammation. Furthermore, epithelial injury exposes sensory nerve endings to environmental stimuli and inflammatory mediators (Anderson & Kippelen, 2008). Since the bronchial epithelium helps to inhibit neurokinin type-2 mediated contractions (Naline et al, 1988), loss of epithelial cells would lessen this protective function. Epithelial barrier disruption likely enhances the direct exposure of immune cells in the basement membrane to hyperosmolar environments. Mast cell, basophils and eosinophils produce bronchoconstrictive and inflammatory mediators upon direct exposure to a hyperosmolar environment (Gulliksson et al, 2006; Moloney et al, 2003). Additionally, airway epithelial cells help to regulate ASL volume and possess the capacity to absorb and secrete liquid (Boucher, 2003). Airway epithelial damage may inhibit the regulation of ASL and potentially make the airway more susceptible to airway dehydration and hyper-osmolality.

Furthermore, airway remodelling as seen in XC skiers (Karjalainen et al, 2000) will reduce airway calibre over time and increase luminal shear wall stress. This may lead to a feedback loop were initial airway injury leads to airway remodelling and reduces airway calibre. Without appropriate recovery lumen shear stresses are increased and the epithelium becomes more prone to injury in subsequent exercise bouts. Similar feedback loops are likely present with frequent exposure to irritant such as in swimmers. The only way to inhibit these cycles would be to allow airway remodelling to resolve prior to subsequent exposure. This may explain the high prevalence of EIB in competitive swimmer and the subsequent loss/attenuation of EIB following retirement (Helenius et al, 2002).

To summarise, airway injury is a clear contributor to inflammation and bronchoconstriction in EIB and HIB. Airway injury seems to be a key feature in the pathogenesis of EIB in elite athletes particularly those exposed to high ventilation rates, unconditioned air, and respiratory irritants and pollutants during exercise. These stimuli induce airway injury leading to airway remodelling and changes in airway inflammatory profiles that enhance airway hyper-responsiveness. The intense and frequent training nature of elite athletes promotes a damage-repair cycle to the airway epithelial. These mechanisms contribute to the development of EIB and HIB in elite athletes.

2.1.5. EIB phenotypes

Like asthma, EIB encompasses multiple phenotypes, however, unlike asthma these EIB phenotypes are poorly described. The most recognised EIB phenotypes includes EIB which presents in individuals without a diagnosis of asthma and EIB that presents alongside a diagnosis of asthma. In athletes such as XC skiers, runners and swimmer that have EIB without asthma airway neutrophilia has been documented as a phenotypical feature.

Furthermore, XC skiers who suffer with respiratory symptoms and have AHR to methacholine do not respond to budesonide, which is in keeping with the less responsive nature of neutrophilic airway inflammation to ICS (Sue-Chu et al, 2000; Green et al, 2002). ASM contraction, mucus secretion and vasodilation in the airways involve parasympathetic innervation. Parasympathetic activity is increased in athletes and elevated after training and may contribute to development of EIB without asthma in athletes (Stang, Strensurd, Mowinckel, & Carlsen, 2016; Goldsmith, Bigger, Steinman, & Fleiss, 1992).

Airway eosinophilia is a distinct feature of EIB with asthma and is associated with the severity of EIB (Hallstrand et al, 2005; Yoshikawa et al, 1998). Sputum eosinophil percentage predicts the response to ICS in EIB with asthma, with greater improvements found with higher sputum eosinophil percentages (Duong et al, 2008). EIB with asthma also presents with high intraepithelial mast cells and these mast cell present with a distinct tryptase and CPA3 high and chymase low phenotype (Hallstrand et al, 2011; Lai et al, 2014). Following an exercise challenge increased epithelial transcription of tryptase and CPA3, and epithelial repair genes is evident in individuals with EIB, and asthma compared to asthma patients without EIB (Hallstrand et al, 2012) suggesting the involvement of mast cell activation and epithelial repair in the pathology of EIB. Asthma with EIB also presents with greater Cyst-LT concentrations and Cyst-LT/PGE_2 ratio compared to asthma without EIB (Hallstrand et al, 2005). While epithelial injury is implicated in the pathogenesis of EIB without asthma in athletes, epithelial injury is also present at rest and is increased after exercise and hyperpnoea in EIB with asthma (Hallstrand et al, 2005). Some of the underlying features of asthma make the airway more responsive to EIB. Genome-wide association studies in asthma have identified genes related to airway surface and immune interaction and elevated type-2 inflammation are highly expressed in asthma (Moffatt et al, 2010; Demenais et al, 2018). In addition, reduced ASL volume and airway epithelial integrity, and increased

airway vasculature and vascular permeability are pathological features of asthma. These asthma features would exaggerate osmotic and thermal responses to exercise/hyperpnoea and subsequently exaggerate the inflammatory response to such stimuli. The phenotyping of EIB can be further confused by training status with athletes without asthma or EIB having significantly greater sputum basophils compared to non-athletes (Sastre et al, 2013). Therefore, EIB phenotypes may differ between athletes and non-athletes.

In summary, EIB with and without asthma are key phenotypes of EIB with distinct features. Airway neutrophilia seems to be a feature of EIB without asthma in athletes and may relate to airway epithelial injury. EIB with asthma presents with features of airway eosinophilia and mast cell activation. EIB pathology in this phenotype may be exaggerated by the underlying features of asthma. Unfortunately, there is a lack of direct comparisons between patients with EIB and asthma, and EIB without asthma. Such direct comparisons would further help to establish differences and similarities in these phenotypes.

2.2. Pathophysiology of asthma

Asthma is a common disease effecting approximately 350 million people worldwide and ~8% of the UK population (GINA Report, 2020). Asthma presents with respiratory symptoms that limit daily activity and some asthma patients suffer from exacerbation/attacks in which intense symptoms and expiratory airflow limitation occur. Some asthma attacks can require urgent hospitalisation and can unfortunately be fatal, as such on average three people die a day from asthma in the UK. Understanding the pathophysiology of asthma and its different phenotypes has been fundamental in the development of new asthma treatments and in improving efficacy of existing treatments.

2.2.1. Defining asthma: the asthma paradigm

Asthma is defined by the presence of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and coughing which is accompanied by variable and reversible expiratory airflow limitation and airway inflammation (GINA Report, 2020). Respiratory symptoms are a primary feature of asthma and is usually the first presentation of asthma. Asthma symptoms vary over time in their presence and intensity and this pattern of respiratory symptoms is fundamental in distinguishing asthma diagnosis from other common respiratory diseases (GINA Report, 2020). In tandem with episodic respiratory symptoms asthma patients suffer from reversible but episodic expiratory airflow limitation. Expiratory airflow limitation is caused through airway narrowing that is underpinned by the pathophysiological features of asthma. Uncontrolled the variability in expiratory airflow limitation can increase the risk of an asthma attack (Figure 2.1).

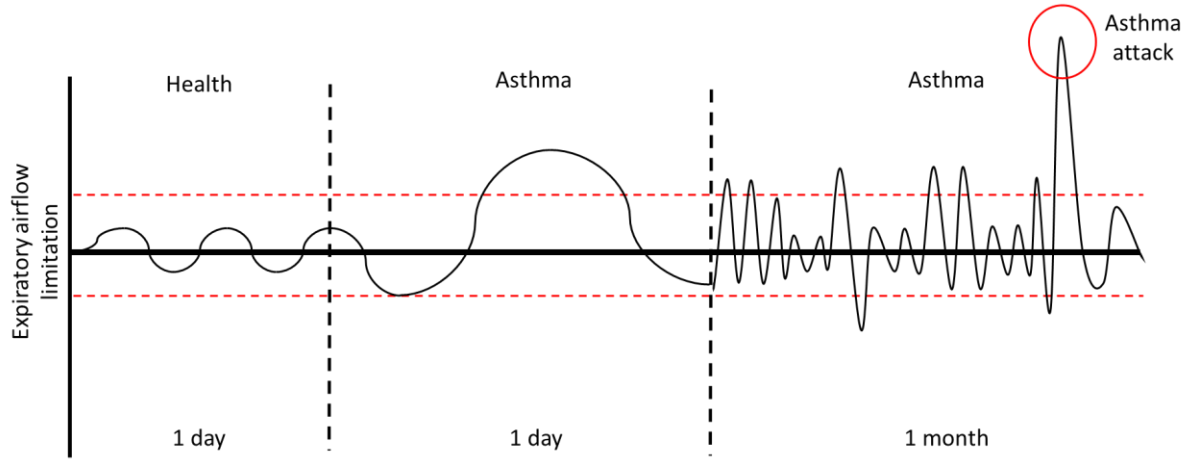

Figure 2.1: Typical variability in expiratory airflow limitation over one day in a healthy individual, one day in an asthma patient, and one month in an asthma patient. Red line indicate the normal homeostatic change in lung function. Asthma involves more frequent and intense expiratory airflow limitation which on occasions can be severe enough to initiate an asthma attack requiring immediate hospitalisation.

These pathophysiological features include airway hyper-responsiveness (AHR), airway oedema, and airway remodelling (Figure 2.8). AHR is an increased airway sensitivity to stimuli which involves a host of airway narrowing factors including airway smooth muscle (ASM) contraction, airway inflammation and mucus production (Cockcroft & Davis, 2006). Direct airway challenge with substances such a methacholine can be used to assess AHR (Cockcroft, 2010). Common stimuli for AHR in asthma patients include viruses, allergens, irritants, and exercise which are referred to as triggers (Vernon et al, 2012). Airway oedema is the swelling of the airways leading to exudation of fluid which plugs the airway lumen creating airflow resistance (Persson, 1986). Airway inflammation and mucus production are key characteristics of asthma that contribute to airway oedema. The pathophysiological features of asthma share bidirectional relationships, for example, certain inflammatory signals can induce ASM contraction and vice versa (Dahlen et al, 1982; Chung, 2000). Controlling these pathophysiological features is key to relieving airflow limitation and symptoms in

asthma. If left uncontrolled a further process called airway remodelling ensues which is defined by structural changes to the airways.

Common features of airway remodelling in asthma include sub-epithelial fibrosis, ASM hypertrophy, goblet cell hyperplasia, angiogenesis, and epithelial disruption (Tang, Wilson, Stewart, & Royce, 2006). ASM hypertrophy causes greater airway narrowing during bronchoconstriction (Moreno, Hogg, & Pare, 1986). Goblet cell hyperplasia increases the production of airway mucus, narrowing lumen diameter in a way which is less responsive to bronchodilators (Wilson, Li, & Pain, 1993). Sub-epithelial fibrosis reduces the elasticity of the airways and correlates with increased AHR and reduced FEV_1 (Chetta et al, 1997; Hishino, Nakamura, Sim, Shimojo, & Isogai, 1998). These structural changes make the airway less pliable and consequently causes fixed airflow obstruction and increased AHR (Fiscus, Van Herpen, Steeber, Tedder, & Tang, 2001). As such the decline in lung function with age occurs at significantly faster rate in adults with asthma than those without asthma (James et al, 2005). Lung function decline in adults >30 years is approximately 25-50mL per year with decreases up to 80mL found in some asthma patients. Progressive airway remodelling in asthma can lead to asthma-COPD overlap syndrome where fixed airway obstruction develops and patients become progressively less response to asthma medication (Postma & Rabe, 2015). These airway changes serve to exaggerate pathophysiological responses to triggers in the future. As such it is imperative to understand and control the pathophysiological features of asthma to prevent airway remodelling, airflow limitation, and ultimately relieve patients of symptoms.

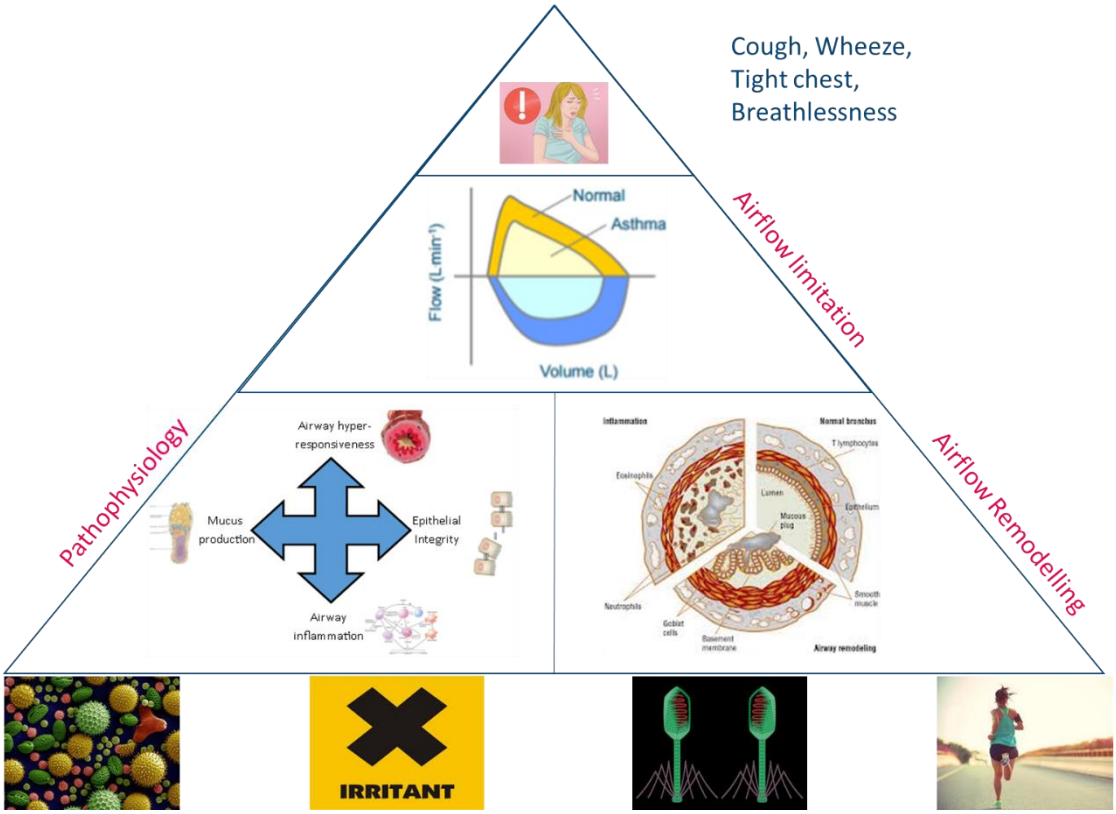

Figure 2.2: Pathophysiological features of asthma which contribute to the development of expiratory airflow limitation and asthma symptoms. Asthma triggers initiate pathophysiological features of asthma such as airway inflammation, hyper-responsiveness, mucus secretion and epithelial disruption. These features interact and induce each other subsequently reducing airway calibre and result in expiratory airflow limitation and the development of asthma symptoms. The pathophysiological features of asthma share a bidirectional relationship with airway remodelling. These features contribute to airway remodelling while airway remodelling can subsequently exaggerate the pathophysiological features of asthma. This can further limit expiratory airflow and increase asthma symptoms if left untreated.

2.2.3. Asthma phenotypes

Asthma is a heterogeneous disease and encompasses multiple subgroups that are known as phenotypes. A phenotype is defined as the ‘observable properties of an organism that are produced by the interactions of the genotype and the environment’. In asthma the observable

traits which have been used to classify phenotypes have been distinguished predominantly by clinical characteristics (e.g. severity; lung function, etc.), inflammatory profiles (e.g. sputum/blood eosinophil/neutrophil counts), and triggers (e.g. allergen, irritant, etc.). Differences in clinical outcomes of asthma such as the exacerbation risk and lung function decline, as well as the response to asthma medications are evident between asthma phenotypes. Therefore, while asthma is defined as a single disease it is better though as a syndrome encompassing multiple different types of asthma. Understanding and identifying asthma phenotypes is important to identify patients at greater risk of poor clinical outcomes and to tailor asthma treatment.

Asthma phenotypes were traditionally classified using clinical characteristics such as triggers, age of onset, and severity via biased approaches. These methods did not however inform on the underlying pathobiology or response to treatments. Molecular phenotyping received interest as a way to link phenotypes to clinical features/outcomes and responses to treatment. This method subgroups patients based on molecular patterns such as sputum white blood cell counts. Wenzel et al (1999) produce a landmark paper which detailed two distinct severe asthma phenotypes based upon airway inflammatory profiles. They identified groups of high and low eosinophils with approximately a 60:40 split. The eosinophil high group was associated with increase lymphocytes, mast cells and macrophages, greater subbasement membrane thickening, and intubations compared to the eosinophil low group, while the eosinophil low group had a lower FEV_1. This showed the link between distinct molecular phenotypes and physiological and clinical features. A study by Woodruff et al (2009) supported these findings by reporting two distinct asthma phenotypes based upon the present of type-2 airway inflammation. Using microarray and polymerase chain reaction analysis on airway epithelial brushings they sub grouped asthmatics as type-2-high and type-2-low. Type-2-high were defined by increased expression of IL-13 upregulated genes. This

phenotype had higher blood and BALF eosinophils, higher IgE and more positive skin prick tests, lower lung function and greater reversibility. Fifty percentage of the cohort fitted into either phenotype. Most profoundly was the finding that the type-2-high phenotype was more responsive to corticosteroids. These studies helped form the two most recognised phenotypes of asthma, Th2-high and Th2-low asthma. However, as these phenotypes are based upon the expression of type-2 derived cytokines which are produced by cells other than just Th2 cells (e.g. type-2 innate lymphoid cells) these phenotypes will be referred to as type-2-high and type-2-low from here in.

Subsequent transcriptomics approaches used in the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED), Severe Asthma Research Program (SARP), and Airways Disease Endotyping for Personalized Therapeutics (ADEPT) cohorts have identified asthma phenotypes in much greater detail. Within bronchial biopsies and epithelial brushing from the U-BIOPRED Kou et al (2017a) identified four phenotypes via transcriptomics. Phenotype-1 was type-2-high and with patients having high submucosal eosinophils, high FeNO, high exacerbation rates and oral corticosteroid (OCS) use. Group-3 was also type-2 high and but these patients had low CS response, high sputum eosinophils, and high BMI. Groups-2 and 4 were less well categories but were likely to have eosinophilic airway inflammation. Work by Kou et al (2017) also revealed three distinct phenotypes using sputum transcriptomics from the U-BIOPRED cohort. TAC1, presented a type-2-high phenotype with increased gene expression for IL-33, CCR3 and TSLP receptors, Th2 cells, and group 2 innate lymphoid cells (ILC2). This phenotype presented features such as high sputum eosinophils and FeNO, OCS dependency, frequent exacerbations, and severe airflow obstruction. TAC2 presented with a type-2-low phenotype expressing IFN-γ, TNF-α, and inflammasome-associated genes, and features such as sputum neutrophilia, high serum c-reactive protein, and high eczema prevalence. TAC3, also presented with a type-2-low

phenotype, high gene expression of metabolic pathway and ubiquitination and mitochondrial function. The phenotype had normal to moderate sputum eosinophils and a healthy FEV_1.

Asthma can therefore be split into two distinct molecular phenotypes of type-2-high asthma which presents with sputum eosinophilia, and type-2-low asthma which presents with neutrophilic or paucigranulocytic airway inflammation, and mixed granulocytic which presents with a mixture of sputum eosinophilia and neutrophilia (Lambrecht & Hammad, 2015). The underlying pathology within these phenotypes is beginning to be resolved leading way to asthma endotypes and tailors' treatments.

2.2.3.1. Type-2-high asthma

Type-2-high asthma is defined by the high protein concentrations or gene expression of type-2 cytokines (IL-4, IL-5, IL-9, IL-13). Since type-2 cytokines result in eosinophil recruitment, activation and survival in the lung type-2-asthma is synonymous with sputum eosinophilia. As such type-2-high and eosinophilic asthma are commonly used interchangeably and represent similar if not the same phenotype. Patients with type-2-high asthma typically have higher rates of exacerbations and hospital admissions. Fortunately type-2-high patients are generally responsive to corticosteroids however a subgroup of type-2-high patients are unresponsive to corticosteroids. Types-2-high patients and in particular those with uncontrolled eosinophilic asthma have the highest cost of care for any asthma group (Kerkhof et al, 2018). The poor clinical outcomes and high cost of care has meant this phenotype of asthma has received great attention. This attention has helped to define its pathobiology and find therapeutic targets for treatment. In doing so endotypes of type-2-high asthma involving Th2 cells and ILC2 pathways have been proposed (Lambrecht & Hammad, 2015).

Type-2 cytokines (IL-4, IL-5, IL-9, IL-13) are key contributors to the hallmarks of type-2-high asthma such as airway eosinophilia, mucus production, allergen specific IgE, and AHR (Wegmann et al, 2009). IL-4 has been shown to be fundamental in allergic asthma in mice models, where its deficiency drastically blunts allergic airway inflammation (Brussell et al, 1995). It contributes to Th2 cell differentiation and proliferation of by upregulating GATA3 expression. In addition, IL-4 is involved in IgM to IgE class-switching required for allergic sensitization within the airways (Lloyd & Hessel, 2010). IL-5 is a Th2 cell derived cytokine vital in eosinophil differentiation, recruitment, survival, and activation (McBrien & Menzeies-Gow, 2017; Palframan, 1988). Benralizumab, mepolizumab, and reslizumab, MAbs which target IL-5 or the IL-5 receptors are associated with decreased asthma exacerbations in patients with eosinophilic asthma (Ramonell et al, 2020). IL-9 is also shown to exacerbate mice models of asthma with anti-IL-9 antibodies eliciting a protective effect in mice. IL-9 can also stimulate the production of CCL11 (eotaxin-1) by airway smooth muscle cells in turn contributing to the recruitment of eosinophils to the airways. IL-13 is a potent initiator of AHR and goblet cell metaplasia (Wills-Karp et al, 1998; Grunig et al, 1998). Th2 cells are a major producer of these cytokines and alongside these cytokines are elevated in BALF of asthma patients. The number of Th2 cells in the airways of asthma patients is therefore associated with eosinophilia as expected (Woodruff et al, 2009). T cells are shown to be important for airway type-2 cytokines and eosinophil accumulation following allergen challenge in mice (Garlisi et al, 1995). Th2 cells are therefore a key cell that increase airway type-2 cytokines and eosinophils which characterise type-2-high asthma (Figure 2.3).

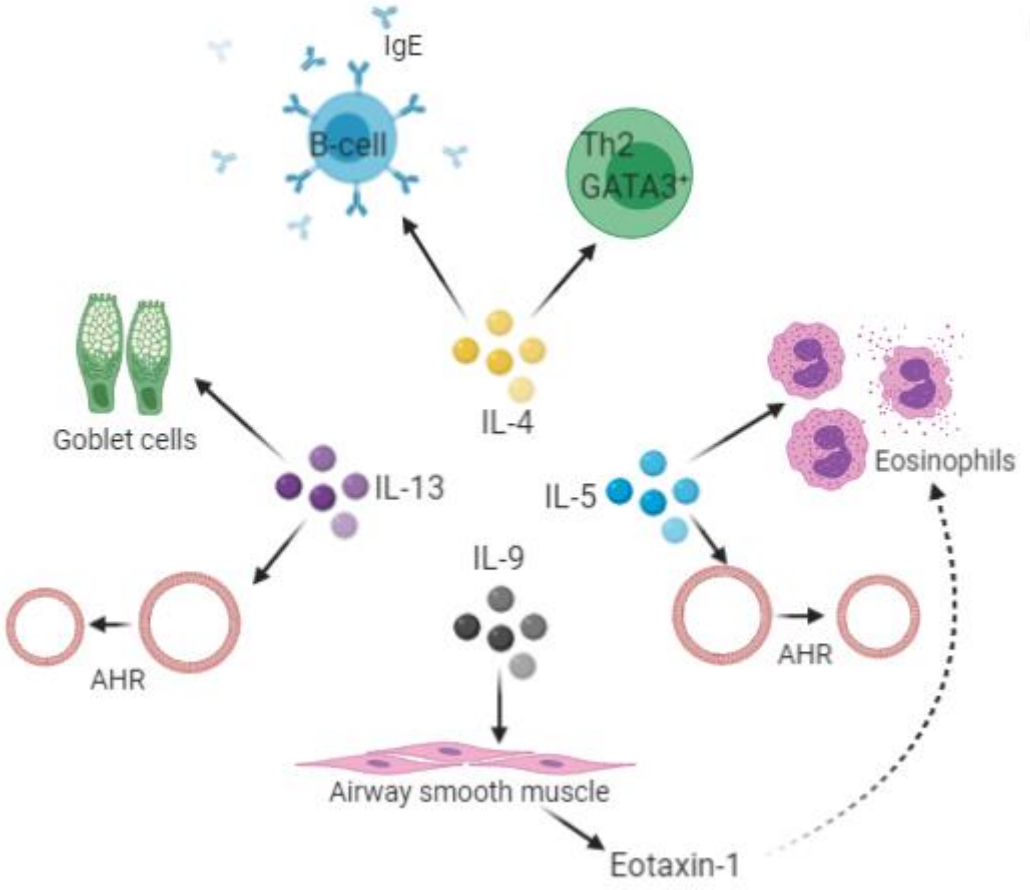

Figure 2.3: Effects of type-2-cytokines on pathophysiological features of asthma.

Airway epithelial cells and DCs orchestrate Th2 cell polarisation, recruitment to the airways, and maturation upon sensitization to an allergen and subsequent challenge (Lambrecht & Hammad, 2015). Epithelial cells and DCs are the first immune defence mechanisms exposed to allergens. House-dust mite (HDM) allergen induces asthma in a toll-like receptor 4 (TLR4) dependant manner. TLR4 expressed on structural airway cells stimulates the release of the epithelial derived cytokines, IL-33, TSLP, IL-25, and granulocyte-macrophage colony–stimulating factor (GM-CSF) which drive DCs activity and effector T helper cell responses (Hammad et al, 2009). Upstream of these responses is the release of IL-1α from bronchial epithelial cells upon HDM allergen exposure. IL-1α induces the release of DC chemo-attractants, IL-33 and GM-CSF (Willart, 2012). Proteolytic activity of the HDM allergen Der p 1 can stimulate the recruitment of DCs by epithelial cells upon exposure (Pichavant et al,

2005). A.*fumigatus* also possesses protease activity which causes fibrinogen cleavage, elevating fibrinogen cleavage products which then activate TLR4 signalling on airway epithelial cells (Millien et a, 2013). Other allergens such as the cat dander protein Fel d 1, are similarly involved in TLR signalling, while *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* have alternatively been shown to activate DCs via lectin receptors (Herre et al, 2013).

Epithelial derived cytokines, TSLP, IL-33, IL-25, and GM-CSF, that are secreted upon allergen exposure are involved in stimulating DCs, facilitating their migration to lymph nodes, and subsequent priming of Th2 cells (Soumelis et al, 2002; Zhan, Xu, & Lew, 2012). IL-33 and TSLP can activate the conventional DC subset, $CD11b^+$ DCs and upregulate OX40 ligand expression. $CD11b^+$ DCs are required to induce allergic sensitization and OX40L is involved in the skewing of Th2 cells by DCs (Plantinga et al, 2013; Blazquez & Berin, 2008). $CD11b^+$ are major sources of TNF-α, CCL17, and CCL22 which attract Th2 $CD4^+$ and $CD8^+$ effector T cells into the lung (van Rijt, 2005). The $CD11b^+$ DCs migrate to mesenteric lymph nodes upon allergen challenge and induce Th2 cell differentiation and proliferation (Plantinga et al, 2013).While DCs are primed into a Th2 cell promoting state via interaction with epithelial cells they can also sample allergens directly and activate adaptive immune responses which promote Th2 cell polarisation from naïve T cells. In the lungs, as with other mucosal sites, DCs can extend their dendrites between epithelial cells into the airway lumen and sample allergens. DCs subsequently migrate to lymph nodes and present the antigen to T cells as part of adaptive immunity (Lambrecht & Hammad, 2015). DCs are elevated in the airways of asthma patients and increase following allergen challenge (Bertorelli et al, 2000; Bratke et al, 2007). Additionally, the placement of ovalbumin activated DCs in the airways of naïve mice induces a Th2 response and features of asthma following ovalbumin challenge, while depletion of DCs protects against AHR in these models (van Rijt et al, 2005). DCs

therefore play a pivotal role in allergic asthma by inducing Th2 cells which subsequently secrete type-2 cytokines and induce pathological features of type-2-high asthma such as airway eosinophilia, AHR, mucus production and airway remodelling (Figure 2.4).

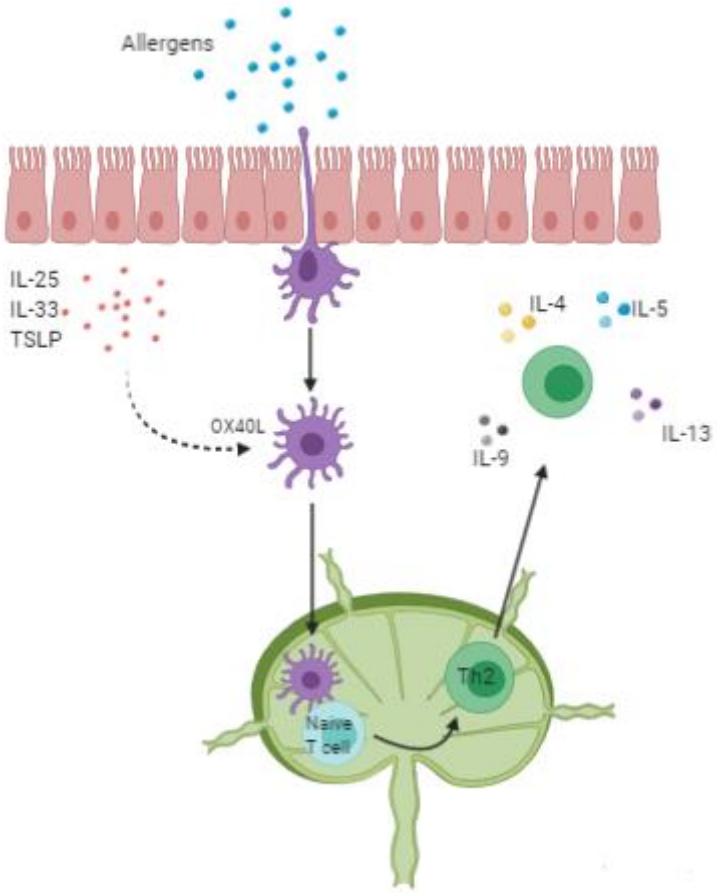

Figure 2.4: Adaptive immune response to inhaled allergens in asthma. Dendritic cells samples allergens and migrate to lymph nodes and present allergen to naïve T cells promoting the production of T helper 2 (Th2) cells. IL-25, IL-33, and TSLP production by airway epithelial cells upregulate OX40L on DCs which promotes their migration to lymph nodes and polarisation of naïve T cells to Th2 cells.

In mice deficient in RAG recombinase which lack mature T and B cells airway eosinophilia to HDM allergens is maintained, suggesting a pathway exists that promotes eosinophilic

asthma without adaptive immune responses (Lambrecht & Hammad, 2015). Another key source of type-2 cytokines is ILC2 which secrete high levels of IL-5 and IL-13 in response to epithelial derived cytokines (IL-25, IL-33, and TSLP). ILC2 therefore resemble Th2 cells in there cytokine responses but ILC2 differ in that they lack antigen-specific receptors. ILC2 are approximately 2-fold higher in the airways of asthma patients compared to controls (Christianson, 2015). In mild to moderate asthma patients with high blood eosinophilia (≥300 cells/μL) ILC2 increase in BALF and decline in blood after an allergen challenge, suggesting the recruitment of ILC2s to the airways during inflammation in asthma.

IL-33 is one of the most potent stimulators of ILC2s. IL-33 provokes large increases in IL-13 by ILC2s which enhances AHR in mice and airway contraction in lung slice models (Barlow et al, 2013). The high production of IL-13 by ILC2s in asthma damages airway epithelial tight-junctions (Sugita et al, 2018). Epithelial damage is a stimulus for IL-33 production which suggest the existence of an IL-33-ILC2-IL-13 positive feedback loop. Such a feedback loop has been documented in a mouse model of asthma by Christianson et al (2015). They found IL-33 increases IL-13 and IL-5 production by ILC2 and IL-13 can subsequently increase IL-33 production from epithelial cells. Additionally, IL-33 is involved in autoinduction, and IL-13 facilitates IL-33 autoinduction by upregulating the IL-33 receptor (Figure 2.5). This would promote continuous high levels of IL-5 and IL-13 contributing to substantial airway eosinophilia and AHR. As a result of such a positive-feedback loop ILC2 contributes to persistent asthma.

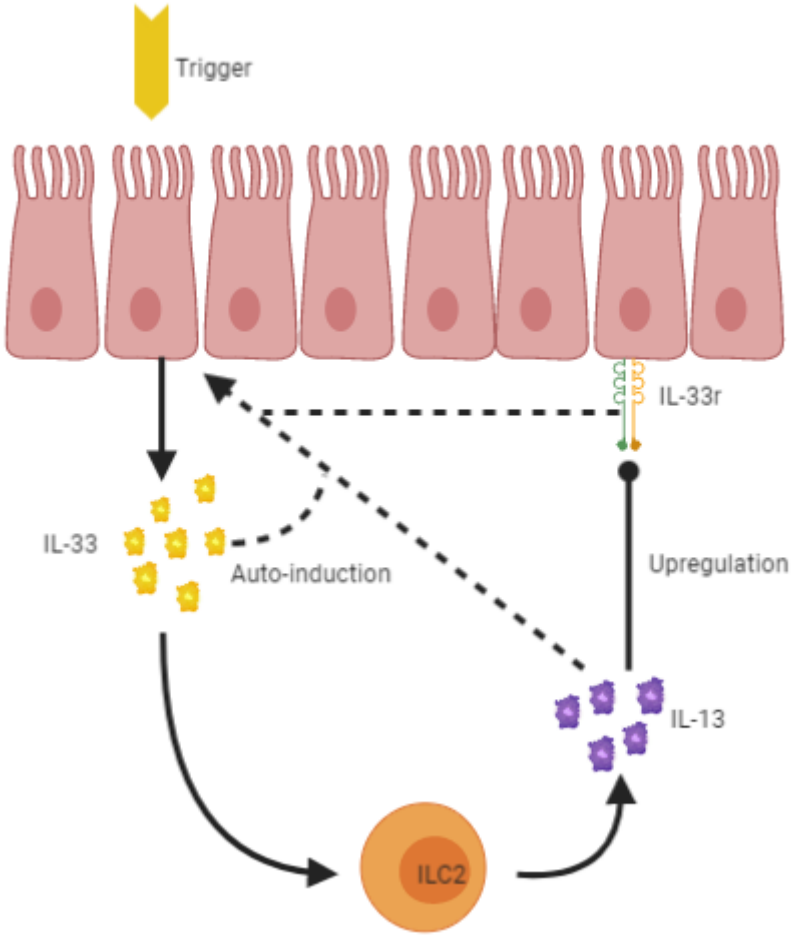

Figure 2.5: The IL-33-ILC2-IL-13 axis. IL-33 production by airway epithelial cells causes the release of IL-13 from group 2 innate lymphoid cells (ILC2s). IL-33 engages in auto-induction while IL-13 upregulates the IL-33 receptor on airway epithelial cells supporting IL-33 auto-induction. This leads to persistent high lung concentrations of IL-13 which can initiate airway hyper-responsiveness and mucus production.

Asthma triggers other than allergens can stimulate the release of epithelial derived alarmins including epithelial damage, osmotic stress, and viruses (Lai et al, 2014; Beale et al, 2014). IL-25 is a pivotal mediator in rhinoviruses (RV) induced asthma exacerbations, one of the most common triggers of asthma exacerbations (Beale et al, 2014). In mice, ILC2s are also implicated in influenza infection (Chang et al, 2011). The release of IL-33 my macrophages during infection activates ILC2 and promotes the secretion of IL-5 and IL-13 which contributes to AHR. This may partly describe the mechanisms involve in viral-induced asthma exacerbations. ILC2 may additionally contribute to asthma exacerbations from stimuli

that disrupt the airway epithelium, such as pollutants and irritants, and osmotic stress during exercise-induce bronchoconstriction.

Besides cytokines, ILC2 activity is arbitrated by lipid mediators and eicosanoids. Lipids and eicosanoids can be produced rapidly by mast cells, eosinophils, epithelial cells, macrophages, and dendritic cells. PGD_2 increases IL-13 secretion from ILC2 while in the presence of IL-33 and IL-25 (Kuruvilla, Lee, & Lee, 2019). ILC2s activity is also increase by LTB_4 and the CysLTs, LTC_4, LTD_4, and LTE_4. CystLTs work synergistically with IL-33 by increasing IL-33-induced activation of ILC2s. CystLTs causes Ca^{+2} influx in ILC2s and nuclear translocation of the transcription factor, nuclear factor of activated T cells (NAFT) which enhances IL-33 induction (von Moltke et al, 2017). Conversely, PGE_2, PGI_2, LxA_4, and maresin-1 suppress ILC2 function. IL-25, IL-33, and TSLP induced IL-5 and IL-13 production by human blood ILC2s is suppressed with the addition of PGE_2 (Maric et al, 2018). PGE_2 suppresses GATA3 in ILC2s, a transcription factor which is fundamental in ILC2 development and function (Zhou et al, 2018). Maresin-1 augments the generation of Treg cells that suppress ILC2s and reduces IL-5 and IL-13 expression (Krishnamoorthy et al, 2015). Given the imbalanced pro to anti-inflammatory lipid profile in E-HIB patients ILC2 may play a role in EIB as well as asthma pathology. Additionally, the release of TSLP and IL-33 upon osmotic stress and epithelial damage, two features of EIB/HIB, would also support potential activation of ILC2 in EIB/HIB.

While ILC2 play a major role in non-allergic type-2 responses in asthma they can work alongside Th2 cells and contribute to adaptive immunity involved in allergic type-2 asthma responses. The high production of IL-5 and IL-13 from ILC2 can support the activation of DC and Th2 cells during allergic asthma and because many allergens cause the release of IL-33, TSLP, and IL-25 by epithelial cells ILC2 are activated and release IL-5 and IL-13 during allergen exposure. Indeed, during ovalbumin and HDM-induced asthma in mice ILC2 are

estimated to make up more than half the cells producing IL-5 and IL-13 in the lung (Wolterink et al, 2012). ILC2 derived IL-13 has been reported to promote $CD40^+$ DC migration to draining lymph nodes in response to lung papain exposure in mice. These DCs promote Th2 differentiation showing the contribution of ILC2 to adaptive immune responses to allergens (Halim et al, 2014). Lung ILC2s enhance the proliferation and activity of $CD4^+$ T cells to promote adaptive immunity. ILC2 therefore additionally contribute to adaptive immune responses to allergens in asthma alongside their involvement innate immune responses to non-allergic asthma triggers.

Type-2-high asthma presents with an airway inflammatory phenotype characterised by high airway eosinophilia. Eosinophil recruitment, activation and survival is enhanced by type-2 cytokines IL-4, IL-5, IL-9, and IL-13. Key producers of these cytokines are Th2 cells and ILC2. Th2 cells alongside DCs and airway epithelial help orchestrate allergic asthma responses leading to airway eosinophilia while ILC2 play a prominent role in non-allergic eosinophilic airway inflammation and asthma responses. Together these cells help orchestrate eosinophilic airway inflammation in response to allergic and non-allergic stimuli in asthma and are fundamental to the pathobiology's underpinning the type-2-high asthma phenotype.

2.2.3.2. Type-2-low asthma

Fifty percent of asthma patients are non-eosinophilic (Douwes, Gibson, Pekkanen, & Pearce, 2002; McGrath et al, 2012) which is defined by sputum percentage eosinophils <2%, as 1.9% is the upper limit of normal range in the healthy population (Belda et al, 2000). Neutrophilic and paucigranulocytic asthma are two phenotypes within non-eosinophilic asthma and are characterised by their airway inflammation profiles. Neutrophilic asthma is diagnosed using a sputum percentage neutrophil cut-off typically ranging from ≥60% to ≥76% while

paucigranulocytic asthma is characterised by normal sputum white blood cell counts and percentages (Chung, 2016; Tliba & Panettieri, 2019). In addition to eosinophilic, neutrophilic and paucigranulytic asthma, mixed granuolytic asthma (MGA) also exists. MGA presents with both sputum eosinophilia and neutrophilia and appear to be an intermediate phenotype (Pignatti et al, 2019). MGA patients are typically older and have a lower prevalence of nasal polyposis than eosinophilic asthma and have higher blood eosinophils and a lower prevalence of gastroesophageal reflux disease than neutrophilic patients (Pignatti et al, 2019).

Eosinophilic and paucigranulocytic are the predominant asthma phenotypes. Schleich et al (2013) found out of 508 asthma patients that 42% had eosinophilic, 40% had paucigranulocytic and only 16% had neutrophilic asthma. Similar finding were found by Ntontsi et al (2017) with 40% eosinophilic and 48% paucigranulocytic. A slightly higher predominance of neutrophilic asthma was found by Simpson et al (2006) with 41% eosinophilic, 31% paucigranulocytic and 20% neutrophilic. Differences in diagnostic cut-off for differential cell counts likely explain the slight discrepancies. Neutrophilic asthma is associated with a late age of onset, has a low response to corticosteroids and lacks the sub-epithelial layer thickening seen in eosinophilic asthma (Shaw et al, 2007). Fortunately, neutrophilic asthma is associated with lower exacerbations and hospital admissions than type-2-high asthma. Understanding of the triggers in neutrophilic asthma is limited but infections, cigarette smoke, pollution, and occupational exposure are suggested inducers (Esteban-Gorgojo, Antolin-Amerigo, Dominguez-Ortega, & Quirce, 2018).

While much is unknown about the pathobiology of neutrophilic compared eosinophilic asthma Th1 and Th17 cells have been implicated. Severe asthma patients with high sputum neutrophils and low eosinophils have higher concentrations of sputum CXCL1, CXCL10, CCL2, IL-6, IL-8, IL-1β alongside a trend for higher G-CSF and IL-22 (Manni et al, 2014; Simpson et al, 2014). These inflammatory cytokines, chemokines and growth factors are

increased in response to the Th17 cytokines IL-17A, IL-17F, and IL-22. Sputum gene expression of IL-8, IL-8Rα, IL-8Rβ, and neutrophil elastase protein is higher in neutrophilic compared to non-neutrophilic asthma (Woods, Baines, Fu, Scott, & Gibson, 2012). Higher levels of systemic inflammation have also been found in neutrophilic asthma with higher concentrations of C-reactive protein and IL-6 (Woods et al, 2012). Blood neutrophils from non-eosinophilic asthma patients produce more IL-8 at rest than those from eosinophilic asthma while there is no difference in cytokine gene expression and sputum neutrophil protein production (Baines, Simpson, Bowden, Scott, & Gibson, 2010). This suggests that non-eosinophilic and potentially neutrophilic asthma may relate to mechanisms of enhanced blood neutrophil chemotaxis and survival.

In addition inflammasomes have been implicated in neutrophilic asthma with increased sputum gene expression of NLRP3, caspase-1, caspase-4, and caspase-5 (Simpson et al, 2014). Inflammasomes are innate immune receptors that activate caspase enzymes to induce inflammation in response to microbes (Schroder & Tschopp, 2010). Interestingly the airway microbiology of neutrophilic asthma is distinct compared to other inflammatory phenotypes (Taylor et al, 2018). Airway microbiology of neutrophilic asthma is less diverse and is characterised by a greater frequency and relative abundance of pathogenic taxa and reduced *Streptococcus*, *Gemella*, and *Porphyromonas* taxa relative abundance. Total abundance of Moraxella catarrrhalis, and Haemophilus and Streptococcus genera in the airways is positively correlated with sputum neutrophil counts and IL-8 concentrations (Green et al, 2014).

Two large scale randomise double blind control trials (AZISAST and AMAZES) have found azithromycin, an antibiotic therapy, to be effective in improving primary endpoints of asthma and in reducing exacerbations in severe non-eosinophilic, and severe uncontrolled asthma patients (Brusselle et al, 2013; Gibson et al, 2017). Microbes may therefore play a role in this

phenotype, but greater insight is needed to reveal underlying endotypes of neutrophilic asthma. Treatments targeting neutrophils in asthma reveal that neutrophils may be a bystander in the mechanisms of neutrophilic asthma. IL-8 is a major ligand involved in the recruitment of neutrophils to the airways and alongside its receptors (IL-8Rα and IL8-Rβ) is elevated on the gene level in sputum of neutrophilic asthma patients. SCH527123 and AZD5069, antagonists of the IL-8 receptors IL-8Rβ, reduce sputum neutrophil percentage but do not provide benefits to clinical outcomes in severe asthmatics with airway neutrophilia (Nair et al, 2012; O.Byrne et al, 2016). The lack of asthma improvement while targeting neutrophils suggests that neutrophils are not causing asthma but responding to asthma in this phenotype.

One of the key features of neutrophilic asthma is steroid resistance, making this phenotype hard to treat. While steroids induce apoptosis of eosinophils, they reduce apoptosis of neutrophils and increase neutrophil release from bone marrow (Saffar, Ashdown, & Gounni, 2011; Cox, 1995). Impaired nuclear recruitment of HDAC2 has been suggested as a potential mechanism for steroid resistance. In mice dexamethasone fails to increase HDAC2 activity and reduce NF-κβ (Ito et al, 2008). HDAC2 activity down-regulates pro-inflammatory gene expression. Reduced autophagy of $CD11c^+$ cells has also been reported to induce neutrophilic airway inflammation that is glucocorticoid resistant (Suzuki et al, 2016). Autophagy in pulmonary $CD11c^+$ cells is impaired during severe airway inflammation. Additionally, deletion of autophagy-related gene 5 (Atg5) in $CD11c^+$ cells of mice causes increased airway IL-17A concentrations, neutrophilic airway inflammation and AHR that is glucocorticoid resistant.

Paucigranulocytic asthma (PGA) lacks evidence of airway inflammation, presenting with sputum eosinophil and neutrophil counts and differential counts within the normal range. Although inflammation is a major contributor to AHR, AHR can occur independent of airway

inflammation (Benayoun, Druilhe, Dombret, Aubier, & Pretolani, 2003). AHR independent of airway inflammation would allow airflow limitation and subsequent symptoms to develop without the presence of airway eosinophilia or neutrophilia as seen in PGA. In mice AHR comparable to allergen induced AHR can be achieved independent of airway inflammation using nerve growth factors (Braun et al, 2001). Various signalling molecules have been linked to inducing AHR without enhance airway inflammation. Using mice knockout models, the absence of G protein signalling 5, caveolin-1, early growth response 1, and estrogen receptor have all individually been shown to enhance AHR without affecting AI following allergen sensitization and challenge (Balenga et al, 2014; Gabehart et al, 2013; Kramer et al, 2009; Carey et al, 2007). In addition, overexpression of genes gasdermin B (GSDMB) and orosomucoid like 3 (ORMDL3) in mice results in increased AHR, airway remodelling, airway smooth muscle hypertrophy, sub-epithelial fibrosis and mucus in the absence of airway inflammation (Das et al, 2016; Miller et al, 2014). This shows potential pathobiological pathways and genetic factors which can result in AHR independent of airway inflammation. It has been proposed that PGA may be a consequence of inflammatory burn out in which prolonged inflammation depletes inflammatory cell pools leading to airway immune cell paucity (Zhang & Wenzel, 2007). The pathobiology of PGA likely reflects markers of airway remodelling such as TGF-β in the absence of airway inflammation.

The different phenotypes of asthma show that asthma is a heterogeneous disease. While asthma is defined by its distinct presentation of symptoms and symptom patterns there are many ways in which these symptoms are caused in difference asthma phenotypes. The molecular features of different asthma phenotypes influence clinical outcomes and responses to treatment. As such asthma should not be thought of as a single disease but multiple syndromes with distinct molecular features that can cause the same presentation of symptoms and symptom patterns.

2.3. Diagnosis of asthma

The National Institute for Health and Care Excellence (NICE) guidelines for asthma diagnosis (2020) recommends starting the diagnostic process for asthma by obtaining a clear clinical history. These include checking for respiratory symptoms such as wheeze, cough, breathlessness and chest tightness, patients experiencing ≥2 of these symptoms supports an asthma diagnosis (GINA guidelines, 2020). Any daily or seasonal variations in these symptoms and a history of potential triggers that worsen symptoms is additionally checked. Symptoms varying over time and in intensity especially symptoms worsening at night and early morning also supports an asthma diagnosis (GINA guidelines, 2020). Variability in symptom presence and intensity over time specifically in response to an asthma trigger is a key feature distinguishing asthma from other respiratory disorder that share similar symptoms such as COPD and bronchiectasis (Athanazio, 2012). Common asthma triggers include viral infections, exercise, allergies, changes in weather, irritants, smoke, and strong smells. Furthermore, a collection of a history or family history of asthma and/or atopy are recommended. This is collected as there is a clear genetic component to asthma while atopy is strongly associate with asthma, particularly the allergic asthma phenotype (Moffatt et al, 2010; Heinzmann & Deichmann, 2001). Clinical history is followed by a physical examination to assess the presence of expiratory polyphonic wheeze and signs of other causes of respiratory symptoms. If asthma is suspected following clinical history and physical examination objective tests are used to confirm an asthma diagnosis (NICE guidelines, 2020).

To support an asthma diagnosis objective testing is performed, these tests assess some of the pathophysiological features of asthma including expiratory airflow limitation, airway inflammation, and airway hyper-responsiveness (AHR). The use of objective testing is important as there is a significant misdiagnosis of asthma when clinical history is solely relied upon for diagnosis (Yang, Simons, Foty, Subbarao & Dell, 2016). Spirometry helps to

support an asthma diagnosis, specifically a FEV_1/FVC ratio <0.7 indicated expiratory airflow limitation, and while alone this is not enough to confirm a diagnosis of asthma it supports a diagnosis of asthma (Quanjer & Weiner, 2014). A spirometry score within the normal range however cannot be used to rule out asthma (Schneider et al, 2009). Reversible expiratory airflow limitation is suggestive of asthma and therefore a reversibility test is commonly performed during asthma diagnosis. Reversibility testing involves a pre-bronchodilator assessment of lung function, the patient then administers a short-acting β_2-agonists (e.g. 4*100mcg of salbutamol) through a spacer and subsequently re-performs spirometry 15 minutes post bronchodilator administration (Pellegrino et al, 2005; Graham et al, 2019). A positive test is defined by a >200mL or >12% improvement in pre to post bronchodilator FEV_1. Reversibility can also be assessed chronically my measuring the change in FEV_1 following 4-weeks of anti-inflammatory treatment. A positive test cut-off is defined the same as an acute reversibility test (NICE, 2020).

Variability in expiratory airflow is a key feature of asthma which can be assessed by measuring the variability in average daily diurnal peak expiratory flow (PEF). PEF naturally varies throughout the day in healthy individuals, but this diurnal variability is much larger in asthma patients (Hetzel & Clark, 1980). Variability over 20% is suggested as the threshold for a positive test by the NICE guidelines (2020), while variability over 10% in adults and 13% in children is suggested by the GINA guidelines (2020). The presence or absence of reversibility alone cannot be used to run in or out asthma. AHR another defining feature of asthma is assessed using bronchoprovocation tests such as a methacholine challenge test. These tests assess the hyper-responsiveness of the airways to inhaled substances. Airway narrowing in response to these substances happens at a lower dose in individuals with asthma or AHR compared to healthy participants (Coates et al, 2017). A fall in FEV_1 ≥20% with a methacholine concentration ≤8 mg/mL is defined as a positive test showing the presence of

AHR and supporting an asthma diagnosis (NICE guidelines, 2020). The presence or absence of AHR alone cannot be used to run in or out asthma. Fractional exhaled nitric oxide (FeNO) is a test used to assess airway inflammation in individuals suspected of having asthma. A score greater >40 ppb in adults and >35 ppb supports a diagnosis of asthma (NICE guidelines, 2020). It is recommended that FeNO is used to support the diagnosis of asthma but cannot be relied upon alone for a diagnosis. The cut-off for a positive test of these objective tests which supports an asthma diagnosis is summarised in table 2.2.

Table 2.2: Positive test thresholds which support a diagnosis of asthma in adults and children. Adapted from the NICE guidelines (2020).

Test	Cut-off supportive of asthma diagnosis (adults)	Cut-off supportive of asthma diagnosis (children)
Fractional exhaled nitric oxide (FeNO)	≥40 ppb	≥ 35 ppb
Spirometry (FEV_1/FVC ratio)	<0.7	<0.7
Average daily diurnal PEF variability	>20% >10%**	>20% >13%**
Change in absolute FEV_1 from pre to post bronchodilator use	>200ml and >12%	>12%*
Change in absolute FEV_1 pre to post 4 weeks of anti-inflammatory treatment	>200ml and >12%	>12%*
Direct bronchial challenge test with methacholine	≥20% fall in FEV_1 with ≤8 mg/mL concentration of methacholine	n/a

PEF = peak expiratory flow rate; FEV_1 = forced expiratory volume in 1 second. *change in percentage predicted value instead of absolute. **positive test threshold based on the GINA guidelines, (2020).

A positive response to objective tests supports an asthma diagnosis, however, a diagnosis of asthma cannot be made on individual tests alone. An asthma diagnosis can still be confirmed follow a negative test response to one test if other tests are positive. Alternatively, a positive test response cannot conclusively support an asthma diagnosis if other tests are negative. As

such the diagnosis of asthma usually involves applying a diagnostic algorithm in which the response to multiple objective tests is used collectively to support or refute an asthma diagnosis. The diagnostic algorithm suggested by the NICE guidelines (2020) to support a diagnosis of asthma in adults (age 17 and over).

2.3.1. Diagnosis of Exercise-induced bronchoconstriction

EIB is defined by a ≥10% reduction in FEV_1 during or following exercise (Weiler et al, 2016). The diagnosis of EIB relies upon the use of objective testing using an exercise-challenge test or surrogate tests. The utility of exercise-challenge tests and surrogate tests are detailed below. Whilst respiratory symptoms are sometimes used for diagnosis, they have been shown to have poor diagnostic accuracy in predicting EIB leading to both false-negative and false-positive diagnoses (Dickinson et al, 2011; Parsons et al, 2007) (for more details please see section 2.1.1.3. Respiratory symptoms and hyperpnoea induced bronchoconstriction).

2.3.1.1. Respiratory symptoms and exercise-induced bronchoconstriction

The associate between exercise related respiratory symptoms and EIB is poor with approximately only 50% of individuals suffering from EIB reporting respiratory symptoms (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016; Ansley et al, 2012; Jackson et al, 2018). Parsons et al (2007) assessed the prevalence of EIB in university athletes alongside the presence of respiratory symptoms during exercise (e.g wheezing, dyspnoea, chest pain). They found a EIB prevalence of 39% overall, 36% in athletes with no respiratory symptoms and 35% in athletes with respiratory symptoms, as such, symptoms were not predictive of EIB. In elite British athletes who demonstrated a positive EVH test response Dickinson et al

(2011) found only 59% to report at least one symptom of EIB. Similar findings are found in university athletes with a positive exercise challenge test response (Burnett et al, 2016). Burnett et al (2016) found most EIB-positive athletes (59%) reported no symptoms of EIB during exercise. Strikingly, 79% of athletes tested positive for EIB did not have prescribed medication (Burnett et al, 2016). Therefore, the lack of association between respiratory symptoms and EIB and HIB means many athletes go untreated (Dickinson et al, 2011; Burnett et al, 2016). Currently there is no diagnostic questionnaire for EIB, and diagnosis must be made by an objective challenge test (e.g. exercise challenge test or EVH test) (Weiler et al, 2014).

Simpson et al., (2015) assessed respiratory symptoms (cough, wheeze, chest tightness, and mucus secretion) in athletes 15 minutes following an EVH test using visual analog scales. EVH-positive athletes scored significantly higher for wheeze compared to EVH-negative athletes, and wheeze was moderately positively correlated with the maximal fall in FEV_1. Additionally, pooled symptoms score was significantly greater in EVH-positive athletes than EVH-negative athletes, but no other symptom individually differed between groups. Treatment with terbutaline in EVH-positive athletes, leading to bronchoprotection, significantly reduced symptoms scores compared to placebo, however, the degree of bronchoprotection was not correlated with symptoms scores.

The dyspnoea-12 questionnaire (D-12) has been assessed using machine learning in its ability to differentiate EIB-positive and negative athlete who report exertional symptoms (Price, Allen, Hull, Backhouse, & Beggs, 2019). While D-12 was unable to differentiate EIB positive and negative athletes' questions Q4 (I have difficulty catching my breath) and Q11 (My breathing makes me agitated) were identified as the most influential in ruling in a diagnosis of EIB, although the authors concluded that the D-12 should not be used to confirm EIB. Instead of using questionnaires Price, Hull, Ansley, Thomas, & Eyles (2016) conducted

semi-structured interviews in endurance athletes to try and identify respiratory symptoms linked to EIB diagnosis. They identified few features which distinguished EIB-positive and negative athletes, however, they found EIB-positive athletes typically present symptoms lower down the respiratory tree in the chest/lung in comparison to EIB-negative athletes suffering with exercise dyspnoea who present with symptoms in the larynx region. Additionally, recovery time for symptoms was usually present between 2-10 minutes post exercise for EIB-positive athletes while 10-60 minutes is common for EIB-negative athletes presenting with dyspnoea (Price, Hull, Ansley, Thomas, & Eyles, 2016). These findings give hope in being able to link exercise induced respiratory symptoms with EIB and/or HIB in the future.

The poor diagnostic accuracy of respiratory symptoms has been attributed to athletes failing to associate respiratory symptoms of exercise with EIB or HIB, instead believing they are a normal sensations of exercise exertion (Dickinson et al, 2011). Alternatively, for EIB and HIB to develop sufferers must reach a high ventilation rate (~85% MVV). Many athletes may not be achieving such high levels of ventilation during their sport to induced EIB and as such do not develop bronchoconstriction and subsequently do not develop exercise induced respiratory symptoms to be associated with positive objective test responses. This is exemplified in swimmers who have an exceptionally high prevalence of HIB but rarely suffer from bronchoconstriction following swimming, a sport with a low ventilation demand (Castricum et al, 2010). Following an EVH test Castricum et al (2010) found prevalence of EIB at 55% in swimmers, however, only 3% of these athletes suffered with bronchoconstriction post a swimming challenge. Furthermore, previous studies assessing the relationship between respiratory symptoms and EIB/HIB assess symptoms retrospectively with participants at rest and asymptomatic, rather than shortly after an exercise or EVH test when bronchoconstriction is present. This is problematic because the severity of EIB may

relate poorly to respiratory symptoms that are assessed retrospectively and reliant on memory (Conner and Barret 2012). Due to the uncertainty in the presence of EIB/HIB in previous studies and the retrospective recall of symptoms a true association between symptoms and bronchoconstriction cannot be established. To truly assess the association between EIB/HIB and respiratory symptoms, respiratory symptoms need to be assessed when bronchoconstriction is present and can be documented such as post an EVH test. Assessing these associations in such a manner will help to identify symptoms/questions which may have diagnostic value in EIB and HIB and therefore warrants investigation.

2.3.1.2. Exercise-challenge test

Due to the poor association between the respiratory symptoms and EIB and the current lack of a validate symptoms questionnaire the diagnosis of EIB relies upon objective testing. The primary test to assess the presence of EIB is the exercise-challenge test. Within an exercise challenge test exercise intensity is rapidly increased within 2-4 minutes to achieve a high exercise intensity (80-90% HR max) and ensure a high level of ventilation (≥60% MVV) is achieved (Parsons et al, 2013). This exercise intensity is then sustained for a further 4-6 minutes with FEV_1 assessed pre and periodically post-test. The test is primarily conducted in a laboratory setting on a treadmill or cycle ergometer but can be conducted as a field test to replicate sport-specific exercise in athletes (Parson et al, 2013). The lower limit reference range for the fall in FEV_1 following an exercise challenge test in healthy controls without a history of asthma, atopy, or recent upper respiratory tract infection is 7%. As such ≥10% fall in FEV_1 is suggested as evidence of AHR and a clinically significant fall in FEV_1 based upon the mean fall in FEV_1 plus two standard deviations (Rundell, Anderson, Spiering, & Judelson, 2000; Eliasson et al, 1992). Under well controlled conditions exercise-challenge

tests show average reproducibility with Anderson et al., (2010) reporting a 76% agreement between two tests conducted within 4 days of each other. Ventilation rates and air conditions are key determinants during the airway response to exercise with higher sustained ventilation and drier and colder air conditions produce greater responses to an exercise-challenge test (Noviski, Bar-Yishay, Gur & Godfrey, 1987). This can limit the utility of the exercise-challenge tests in the diagnosis of EIB, as unless these factors are well controlled the efficacy of the test can be reduced. Furthermore, data suggests that laboratory exercise challenges tests fail to provide the conditions required to assess EIB in cold weather athletes leading to a high percentage of false negatives (>78%) (Rundell et al, 2000).

2.3.1.3. Surrogate tests to identify EIB

The EVH test is a recommended alternative test to identify EIB in athletes. The EVH test is a 6-minute breathing test in which participants breath at a target ventilation at approximately 85% of their predicted maximal voluntary ventilation while breathing a dry gas mixture (<2% humidity; 21% O_2, 5% CO_2, balance N_2, 21°C). Pulmonary function is assessed pre and post-test (typically: 3, 6, 10, 15, 20, and 30 minutes). The EVH test was originally designed and validated by the US army as a surrogate to exercise testing to identify exercise-induced bronchoconstriction (EIB) (Eliasson et al, 1992; Hurwitz et al, 1995). It was subsequently adopted for identifying EIB in elite athletes and became the recommended test by the International Olympic Committee Medical Commission for confirming EIB in athletes. (Anderson et al, 2001). While used to assess EIB in athletes a positive test response to the EVH test is specifically defined as HIB. Due to the very harsh stimulus of the EVH test involving high ventilation and dry air breathing the American Thoracic Society (ATS) guidelines define a positive test response as a ≥10% fall in FEV_1 from pre to post-test at two

consecutive time points (Parsons et al, 2013; Weiler et al, 2016). The use of two consecutive time points improves the specificity of predicting EIB in athletes using the EVH test. The severity of EIB following an exercise or EVH test can be further defined as mild (≥10% - <30%), moderate (≥30% - <50%), and severe (≥50%) based on the peak fall in FEV_1 from pre to post-test (Weiler et al, 2016). In an individual taking inhaled steroids a decline in FEV_1 ≥30% is considered as severe (Parsons et al, 2013).

Rundell, Anderson, Spiering & Judelson, (2004) compared the EVH test and a field exercise test to identify AHR in elite cold weather athletes. Out of 38 athletes, 19 had AHR defined as by a positive test to exercise or EVH. The exercise test identified 11 positive athletes while the EVH test identified 17 positive athletes. Additionally, the EVH test identified 9 of the 11 subjects positive to exercise. The EVH test therefore shows greater sensitivity in identifying AHR in cold weather athletes. In figure skaters similar finding are reported with the EVH test able to identify 41% of athletes with AHR compared to only 31% identified using a on-ice exercise challenge test with rink-side spirometry (Mannix, Manfredi & Farber, 1999). Similarly, within a cohort of elite swimmers the EVH test was able to identify AHR in 55% of swimmers compared to only 3% using a field swimming test with spirometry assessed pool side (Castricum et al, 2010). Compared to a laboratory exercise-challenge test the EVH test also shows greater sensitivity in identifying EIB (Eliasson, Phillips, Rajagopal & Howard, 1992). The EVH test therefore has a greater sensitivity in identifying AHR in athletes compared to exercise, most likely due to the high ventilation sustained and the breathing of dry air during the test. In addition, the EVH test elicits good day to day reproducibility in the bronchoconstrictive response over 21 days (coefficient variation: 12%; reproducibility: 328 mL) and 70 days (coefficient variation: 10%; reproducibility: 196 mL) in physically active males with asthma and HIB (Williams, Johnson, Hunter, & Sharpe, 2015). Compared to exercise challenge tests the EVH test is more sensitive to identifying AHR and has superior

reproducibility. The high sensitivity and good reproducibility of the EVH test coupled with it ease to implement make it an ideal test to identify EIB in athletes and to be used to assess the effectiveness of interventions to treat EIB/HIB.

The mannitol challenge is another surrogate test used to identify EIB. Mannitol is an osmotic agent. During the mannitol inhalation test increased doses of dry powder mannitol (5, 10, 20, 40, 2 × 40, 4 × 40, 4 × 40, 4 × 40 mg) are delivered to the participants airways with spirometry assess pre-test and after each accumulating dose (Hallstrand et al, 2018). The test is stopped if FEV_1 decreases ≥15%, a 10% decrease in FEV1 between two consecutive doses occurs or the cumulative dose reaches a total of 635 mg. A positive test is defined by a 15% decrease in FEV_1 from baseline in response to the cumulative total dose, or a 10% decrease in FEV_1 between two consecutive mannitol doses. A negative test is defined when a cumulative dose of 635mg of mannitol has been administered and the FEV_1 has not fallen by ≥ 15% from baseline (Hallstrand et al, 2018).

Mannitol is an osmotic agent which causes an increase in airway surface osmolality, which subsequently induces mast cell activation and the release of leukotrienes (Brannan, Gulliksson, Anderson, Chew, & Kumlin, 2003). These mechanisms are involved in the pathogenesis and pathophysiology of EIB (Kippelen & Anderson, 2013; Barnes et al, 1981; Mickleborough et al, 2003; Hallstrand et al, 2005). As a mannitol test induces similar mechanistic pathways leading to bronchoconstriction as exercise and EVH tests there is good diagnostic agreement between tests. In 50 elite summers sport athletes a positive mannitol challenge test had a sensitivity of 96% and a specificity of 92% in identifying a positive EVH test response (Holzer, Anderson, Chan, & Douglass, 2003). In individuals with asthma there is good agreement in a positive test response between mannitol and exercise and EVH tests (Aronsson, Tufvesson, & Bjermer, 2011; Brannan, Koskela, Anderson & Chew, 1998). Brannan, Koskela, Anderson & Chew., (1998) conducted EVH, exercise and a mannitol tests

in 36 asthma patients. They found that all participants positive to EVH and all but one participant positive to exercise were positive to a mannitol challenge. In addition, the test has been shown to be highly reproducible in children with moderate to severe asthma (Barben, Roberts, Chew, Carlin & Robertson, 2003). Compared to exercise and EVH the mannitol challenge is arguably simpler and inexpensive. These benefits coupled with good agreements with exercise and EVH test responses and good reproducibility make it a useful test in the diagnosis of EIB.

The methacholine challenge test is a direct bronchoprovocation challenge used to identify AHR. Methacholine is an inhaled drug that directly interacts with muscarinic receptors on ASM, resulting in ASM contraction and airway narrowing (Coates et al, 2017). Following baseline spirometry, a methacholine challenge involves challenging the airways with increasing doses of methacholine. A positive test is defined by an ≥20% fall in FEV_1 from baseline after a cumulative dose ≤ 2 mg of inhaled methacholine. A negative test is defined when a cumulative dose of 2 mg of inhaled methacholine has been administered and the FEV_1 has not fallen by ≥ 20% from baseline (Coates et al, 2017). In 25 elite summer athletes with a positive EVH test response Holzer, Anderson, & Douglass (2002) found only 9 athletes to have a positive methacholine challenge response. Pedersen, Winther, Backer, Anderson & Larsen., (2008) conducted EVH, laboratory exercise, field exercise and methacholine tests in 16 elite swimmers. Five swimmers were positive to EVH, four with field exercise and four with laboratory exercise tests, however, no athlete had a positive response to the methacholine test. When assessed in non-athletes the methacholine test responses are poorly correlated with EVH test responses ($r = 0.11$) (Holley et al, 2012). The sensitivity of the methacholine challenge test is therefore poor at identifying EIB compared to exercise, EVH and mannitol challenges. While the methacholine challenge test is routinely

used to identify AHR in asthma patients, the exercise-challenge, EVH and mannitol challenge tests are far superior in identifying EIB.

2.4. Prevalence of exercise-induced bronchoconstriction and hyperpnoea-induced bronchoconstriction

The prevalence of exercise-induced bronchoconstriction (EIB) and hyperpnoea-induced bronchoconstriction (HIB) is estimated between 5-20% in the general population (Aggarwal, Mulgirigama, & Berend, 2018; Johansson et al, 2014; Aguiar et al, 2018; Molphy et al, 2014). Unfortunately, there are very few studies conducted in adult non-asthmatic populations for an accurate estimation of prevalence in the adult general population. A single study in 136 recreationally active adults conducted by Molphy et al (2014) reported a prevalence of HIB, as assessed by the eucapnic voluntary hyperpnoea (EVH) test, at 13.2%. Similarly, in 146 adolescents undertaking a treadmill exercise challenge test EIB prevalence was reported at 19% by Aguiar et al (2018). A meta-analysis incorporating 33 studies including general populations of children and adolescents estimated the global prevalence of EIB at approximately 10%, highlighting EIB as significant global public health problem (Aguiar et al, 2018). In children however there is substantial variability in the prevalence of EIB between studies conducted in different countries. In Kenya for example to prevalence of EIB has been estimated at 18%, while in Quebec Canada a prevalence of 5.9% has been reported, although differences in exercise protocols and air condition partly contribute (Ng'ang'a et al, 1998; Hemmelgarn & Ernst, 1997).

These variabilities may be partly explained by differences in exercise challenge methodologies that are implemented such as exercise intensity, duration, modality as well as the method of assessing bronchoconstriction (FEV_1 vs PEF). There does however exist

differences in the prevalence between demographic groups assess under the same exercise challenge conditions. EIB prevalence is much higher in urban communities compared to rural ones (Calvert & Burney, 2005; Ng'ang'a et al, 1998; Sudhir & Prasad, 2003; Yobo, Custovic, Taggart, Asafo-Agyei, & Woodcock, 1997). In Kenya, a difference of 9.2% in EIB prevalence is present between urban (22.9%) and rural (13.2%) communities (Ng'ang'a et al, 1998). Lower prevalence rates are generally found in less westernized communities and in rich urban communities compared to urban poor communities (Yobo et al, 1997; Van Niekerk, Weinberg, Shore, Heese, & Van Schalkwyk, 1979). While the effect of ethnicity is unclear a study conducted in English inner city and Scottish school children (9 years old) found the prevalence of EIB to be >2-times greater in Afro-Caribbean (9.1%) and >3-times greater in Asian (12.3%) compared to white inner-city children (4.5%) (Jones, 1996). HIB prevalence has also been reported to be higher in recreationally active adult males (16%) compared to females (7%) (Molphy et al, 2014).

Compared to the general population EIB prevalence is much higher in asthma patients, estimated at ~50%. In 114 adolescents Correia, Costa, Sarinho, Rizzo, & Sarinho (2017) found the prevalence of EIB to be 47% in asthma participants and 7% in controls. Furthermore, higher prevalence's have been found with increased asthma severity (Sano et al, 1998; Cabral et al, 1999). Sano et al (1998) found the prevalence of EIB to be 45% in a cohort of children with asthma. In mild, moderate, and severe asthma patients the prevalence was 19%, 64% and 85%. In children with intermittent, mild, moderate, and severe asthma the prevalence of EIB has been reported at 27%, 40%, 70% and 58% by Cabral et al (1999). The increased EIB prevalence in asthma patients may be unsurprising as EIB and asthma share similar pathophysiological features such as airway hyper-responsiveness (AHR) and airway inflammation (Weiler et al, 2016) In addition, EIB is suggested to be an indicator of inadequate control in asthma patients (Weiler et al, 2016). A significant portion of the general

population therefore suffer with EIB and HIB with an exceptionally high prevalence found in asthma patients.

2.4.1. Prevalence of exercise-induced bronchoconstriction in athletes

In elite athletes the prevalence of HIB has estimated at 34%, while in university athletes the prevalence of HIB and EIB has be estimated at 39% and 43% (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016). Estimations of prevalence varies drastically between sports, Dickinson et a. (2011) assess 228 elite British athletes from eight sports (rugby, cricket, badminton, rowing, hockey, athletics, football, biathlon, short track speed skating) for the present of HIB using the EVH test (Table 2.3.). HIB prevalence ranged from 18-62% across these eight sports. Strikingly, in 107 university athletes from 13 sports Parsons et al (2007) found the prevalence of HIB to range from 0-67% across sports. Parson et al (2007) found a higher prevalence of HIB in sports which involve high ventilation demands (41%) compared to sports only requiring low ventilation (36%). In contrast the prevalence of HIB has been reported at 2.7%, much lower than reported in other studies (Parsons et al, 2012). The nature and environmental factors of certain sports/exercises such as exercising in cold and dry air condition, exercise at high ventilation, and exposure to respiratory irritants or allergen has been suggested to predispose athletes from certain sports to EIB and HIB (Weiler et al, 2016).

Table 2.3: The prevalence of hyperpnoea-induced bronchoconstriction and exercise-induced bronchoconstriction in athletes as assessed in large scale prevalence studies.

Study	Sports/population (*n*)	Test	Prevalence (%)
Dickinson et al. (2011)	**Elite athletes:**	EVH test	
	Rugby (108)		32
	Badminton (8)		38
	Rowing (42)		31
	Hockey (21)		38
	Athletics (14)		21
	Football (11)		18
	Biathlon (13)		62
	ST speed skating (11)		55
	Total (228)		34
Parsons et al. (2007)	**University athletes:**	EVH test	
	Football (10)		50
	Gymnastic (1)		0
	Cheerleading (3)		0
	Fencing (13)		38
	Volleyball (8)		38
	Basketball (6)		67
	Cross country (3)		0
	Ice hockey (13)		31
	Lacrosse (4)		25
	Soccer (3)		33
	Swimming (12)		58
	Wrestling (7)		43
	Track (14)		36
	Total (107)		39
Parsons et al. (2012)	**University athletes:**	EVH test	
	Ice hockey (49)		2
	Soccer (36)		3
	Lacrosse (59)		3
	Total (144)		2.7
Burnett et al. (2016)	American college athletes (80)	Treadmill exercise challenge test	43

EVH = eucapnic voluntary hyperpnoea test.

Air quality and conditions is associated with a greater prevalence of EIB and HIB in athletes (Helenius & Haahtela, 2000). The prevalence of HIB in competitive figure skaters was reported to be 41% when assessed in 29 athletes using the EVH test (Mannix et al, 1999). Interestingly, following a field test in which figure skaters had spirometry assessed pre and 5-minutes post intense skating 31% of athletes had an ≥10% decline in FEV_1 (Mannix et al,

1999). Similarly, Provost-Craig et al (1999) used rink side spirometry to assess the prevalence of EIB in 100 skater and reported a prevalence of 30%. The high prevalence of EIB and HIB in figure skaters has been attributed to production of fine and ultrafine particulate matter (PM) by ice-cleaning equipment (Rundell, 2012; Rundell et al, 2008). Fine and ultrafine PM penetrates deep into the airways and induce oxidative stress, airway inflammation and airway damage (Venkataman et al, 1999; Cutrufello et al, 2012). Rapid lung function declines in ice hockey players has been attributed to the inhalation of PM (Rundell et al, 2008). These effects likely increase the risk of developing AHR, EIB and HIB in ice rink athletes. Diesel exhaust fumes are a major producer of PM and therefore athletes such as cyclists and distance runners frequently exercising close to busy roads may be at an increased risk. Alongside the inhalation of PM, ice rink athletes inhale cold and dry air during exercise which are two characteristics involved in the pathogenesis of EIB and HIB (Weiler et al, 2016). This likely explains the high prevalence of EIB following field testing in figure skaters (~30%), cross-country (XC) skiers (50%), and short-track speed skaters (43%) (Mannix et al, 1999; Provost-Craig et al, 1999; Wilber et al, 2000).

Swimmers are also exposed to respiratory irritants in the form of Trichloramine, a gas by-product in the air of chlorinated indoor swimming pool, which disrupt the airway epithelium and induced airway inflammation (Font-Ribera et al, 2010; Bougault et al, 2013; Carbonnelle et al, 2008). Elite swimmers have been shown to have an exceptionally high prevalence of EIB (55-69%) (Castricum et al, 2010; Levai et al, 2016). This further supports that chronic exposure to respiratory irritants in athletes is potentially involved in the development AHR, EIB and HIB in athletes. Interestingly, in many swimmers EIB resolves after three months or more post retirement suggesting that the removal of respiratory irritants for an extended period of time may allow AHR to resolve (Helenius et al, 2002). In contrast to winter athletes such as figure skaters and XC skiers who exercise at high ventilation and in cold dry air

environments, swimmers exercise in warm and humid air conditions. This provides an interesting paradigm in which the prevalence of EIB/HIB may be similar between sports, however, athletes from certain sport may be more likely to suffer from bronchoconstriction after partaking in their sports due to ventilation requirements and air conditions.

Variations in the prevalence of EIB and HIB are partly explained by the nature of different sports (e.g. ventilation requirements, duration) and the exposure to different air conditions (e.g. respiratory irritants, humidity, temperature), however, the estimation of prevalence rates in individual sports is cofounded by low cohort numbers and the variability in diagnostic criteria used to diagnose EIB and HIB. While the prevalence of HIB and EIB has been assessed in large cohort of athletes, prevalence in individual sports is commonly estimated on small sample sizes ($n \leq 15$) (Parsons et al, 2007; Dickinson et al, 2011). These sample sizes likely hinder the estimation of prevalence as they do not represent the population. Small sample sizes may not yield a sufficient number of 'positive' and 'negatives' cases to accurately assess prevalence (Arya, Antonisamy & Kumar, 2012). Sample size may be especially significant in team sports where multiple positions exist, and the training and match play demands may vary substantially between positions. In addition, small sample sizes may encourage recruitment bias as athletes who suffer with respiratory symptoms may be more receptive to partake in a study and coaches may encourage these athletes to partake. While there is no association between respiratory symptoms following exercise and the presence of EIB/HIB, respiratory symptoms can be indicative of other respiratory disorders such as asthma, vocal cord dysfunction and exercise-induced laryngeal obstruction. The prevalence of EIB and HIB in people with asthma for example is much higher than non-asthmatics which could distort the prevalence of EIB/HIB in a cohort.

Furthermore, the prevalence of EIB and HIB in athletes has been estimated using different diagnostic criteria. For example, Dickinson et al (2011) adopted the ATS criteria in their

estimation of EIB prevalence, alternatively, Parsons et al. (2007) and Burnett et al (2016) estimated prevalence using a less stringent criteria in which a FI ≥10% at any one time point post exercise/EVH is used to diagnose EIB/HIB. The use of less stringent criterion has been reported to influence prevalence rate by up to 18% (Koch, Sinden, & Koehle, 2018). The diagnosis criteria likely cofound the estimations of EIB and HIB within individual sports. This limits understanding of the true prevalence and burden of EIB and HIB within individual sports. It is important to know this as EIB is commonly undiagnosed in athletes resulting in some athletes not receiving treatment which in turn could increase the risk of exercise-induced mortality (DeJuilio, 2016; Becker et al, 2004). Furthermore, the identification of EIB and its subsequent treatment in elite soccer players is shown to improve VO_{2peak} by 3.4 $mL.kg^{-1}.min^{-1}$ leading to a potential improvement in exercise capacity (Jackson et al, 2018). Understanding the burden in individual sports will help to ensure tailored screening is provided to those most at risk.

Data suggests that there may be sex differences in the prevalence of HIB and EIB. An assessment of HIB in university athletes from 22 different sports found HIB prevalence to be lower in females compared to males (38% vs 42%; Parsons et al, 2007). Conflictingly, in winter sports athletes the prevalence of EIB was found to be higher in females compared to males (26% vs 18%) (Wilber et al, 2000). An explanation for this discrepancy is unclear but might be related to sport-specific differences such as environmental conditions (Parsons & Mastronarde, 2005) and/or the type of challenge (exercise vs. EVH). Difference in prevalence is expected however, as the severity of EIB is shown to be significantly greater during the mid-luteal phase (-17.4%) of the menstrual cycle in comparison with the mid-follicular phase (-12.8%) (Standford et al, 2006). Greater severity was significantly correlated with salivary testosterone levels. Differences in sex hormones may therefore influence the prevalence of HIB and EIB between sexes and may also influence prevalence rates in females between

studies dependant on the phase of the menstrual cycle in which tests were conducted. Comparisons of prevalence between sexes is warranted given the low number of studies making these comparisons.

The prevalence of HIB and EIB is high in athletes, particularly winter sports athletes, athletes which are exposed to respiratory irritants, and athlete partaking in sports with a high ventilation demand. The prevalence of bronchoconstriction following exercise is more frequently reported in athletes exercising at high ventilation rates whilst exercising in dry and cold air conditions. Data suggests that sex differences exist in the prevalence of EIB/HIB but the direction of this differences is unknown (Molphy et al, 2014; Parsons et al, 2007; Wilber et al, 2000). The prevalence of EIB/HIB in individual sports in confounded by low sample sizes and the use of difference diagnostic criteria. This limits the understanding of the true burden of EIB/HIB within individual sports, a factor important to know as under treatment is linked to exercise-induced asthma deaths and reduced exercise performance (Juilio, 2016; Becker et al, 2004; Jackson et al, 2018).

2.4.2. Prevalence of asthma

Asthma is a common respiratory disease with a large global burden, worldwide asthma is estimated to effect 358 million people, this number is projected to reach 400 million by the year 2025 (GINA Report, 2020; Masoli, Fabian, Holt, & Beasley, 2004). The prevalence of asthma varies greatly between countries with estimates between 1% and 21% when established by the means of a doctor's diagnosis of asthma (To et al, 2012). Between 2002 and 2003 the World Health Survey was developed and implemented by the World Health Organization to estimate the prevalence of doctor diagnosed asthma, clinically treated asthma, and wheeze (To et al, 2012). A total of 178,215 individuals completed the

questionnaire, the results determined the prevalence rates of doctor diagnosed asthma, clinically treated asthma, and wheeze at 4.3%, 4.5%, and 8.6%. Exceptionally high rates of doctors diagnosed asthma was found in Australia (21%), Sweden (20%), the United Kingdom (UK) (18%), and the Netherlands (15%). Alternatively, the lowest prevalence rates were found in Vietnam (1%), Bosnia (1.3%), Kazakhstan (1.4%), and Estonia (2%). A trend for a greater prevalence of asthma in resource rich countries was found. A similar trend was reported by the European Community Respiratory Health Survey conducted between 1988 and 1994 in which asthma symptoms and asthma attacks were found to vary greatly between countries being most frequent in British Isles, New Zealand, Australia and the United States compared to northern, central, and southern Europe (Chinn, Burney, Jarvis, & Luczynska, 1997). Current reports by the GBD 2015 Chronic Respiratory Disease Collaborators suggest that asthma prevalence has increased globally by 12.6% from 1990 to 2015, whereas asthma related deaths has reduced by 26.7%. Asthma was identified as the most prevalent chronic respiratory disease worldwide in 2015 with twice the number of cases than chronic obstructive pulmonary disease (COPD) (GBD 2015 Chronic Respiratory Disease Collaborators).

The prevalence of asthma in children worldwide has been assessed by the International Study of Asthma and Allergies in Childhood (ISAAC). The most recent ISAAC study, phase three, surveyed ~1,200,000 children from 233 centres and 98 countries (Mallol et al, 2012). The prevalence of asthma in 6–7-year-olds was estimated at 11.5%, in 13–14-year-olds asthma prevalence was estimated at 14.1%. The gross national income per capita was positively correlated with asthma symptoms suggesting a similar trend as seen in adults with a greater asthma prevalence in more affluent countries. In 6–7-year-olds the prevalence of asthma was greater in males than females (12.7% vs 10.3%), concomitantly, in 13–14-year-olds asthma

prevalence was lower in males than females (13.6% vs 14.6%). Indeed, asthma is frequently shown to be more prominent in males than females during childhood between the ages 0-10 years (Almqvist, Worm, & Leynaert, 2008; Marco, Locatelli, Sunyer, & Burney, 2000). Prevalence begins to balance between males and females as they approach puberty (10-15 years) (Marco et al, 2000). After puberty, the risk of asthma flips and becomes higher in females (Almqvist et al, 2008; Marco et al, 2000). Although not conclusively known the higher prevalence of asthma in females post puberty is partly attributed to alterations in sex hormones with estrogenic and androgenic signalling identified as mediators of type-2 airway inflammation, while testosterone attenuates both type-2 and non-type 2 inflammation (Yung, Fuseini, & Newcomb, 2018). Additionally, the generally smaller lung size of females may predispose females to asthma symptoms for a given degree of airway narrowing.

The UK was reported to have the 3rd highest prevalence of asthma in the World Healthy Survey (To et al, 2012). The prevalence of asthma in the UK is estimated at 8%, effecting approximately 5,300,000 people of which 200,000 are estimated to suffer from severe asthma requiring the highest doses of asthma medication to attain asthma control or remaining uncontrolled even on the highest doses of medication (BTS/Sign asthma guidelines 2019; Asthma UK accessed 18/09/2020). Additionally, 48% of asthma suffers in the UK have been identified as having not well controlled asthma (Demoly, Gueron, Annunziata, Adamek, & Walters, 2010). In addition, asthma is estimated to costs the NHS one billion pounds per year (Asthma UK accessed 18/09/2020). When assessed against 11 other countries (Australia, Canada, Germany, UK, Hong Kong, Italy, Japan, The Netherlands, Spain, Sweden, The USA) the UK ranks 2nd for asthma hospital admissions (Gupta, Anderson, Strachan, Maier, & Watson, 2006), as such, on average three people a day dies from asthma in the UK (Asthma UK accessed 18/09/2020).

Over the past 50 years UK asthma trends have shown a drastic upwards projection (Anderson, Gupta, Strachan, & Limb, 2007) during a timeframe including the introduction of inhaled corticosteroids and an increase in their use. Current trends however suggest the prevalence of adult asthma has plateaued (Anderson et al, 2007). In children, the same observation is present. The prevalence of childhood asthma and wheezing was reported to increase by approximately 2-3 fold between the 1950s and 1990s (Anderson et al, 2007). Between 1991 and 2002 the prevalence of wheeze in London children increased from 12.9% to 29.8% (Butland, Strachan, Crawley-Boevey, & Anderson, 2006). Similarly, in Merseyside between 1991 and 1998 self-reported doctor diagnosed asthma increased from 17.7% to 29.8% (Rizwan et al, 2004), with this trend also consistently seen in South Wales with reported asthma increasing between 1973 (5.5%) and 2003 (27.3%) (Burr et al, 2006). Promising research of recent has found childhood wheeze and asthma is on the decline by as much as 8.2% since the start of this century (Patil et al, 2015; Malik, Tagiyeva, Aucott, McNeill, & Turner, 2011). The reason for the increasing trends over time are likely related to increased societal awareness of asthma and improved diagnostic algorithms, however, obesity has substantially increased during the same timeframes and is a risk factor for asthma (Mohanan, McWilliams & Dulin, 2014). It must be noted however, that these trends are likely influenced by some confounders such as demographic changes in the population of study over time, in addition, many studies only have two time points assessing prevalence over time which reduces the reliability of assessing a trend.

In summary the UK highly ranks word-wide for asthma prevalence whilst also ranking highly for the use of asthma medication. Asthma trends have been on the rise since the 1950s although recent trends (21st century) suggest that prevalence has at least plateaued if not on the decline. The current asthma prevalence is high which is reflected by the large costs (£1 billion per year; Asthma UK) and frequent loss of life from the disease (3 people a day die

from asthma in the UK; Asthma UK). Asthma imposes a major burden worldwide and in the UK.

2.5. Treatment of asthma and exercise-induced bronchoconstriction

Currently there is no known cure for asthma, exercise-induced bronchoconstriction (EIB), or hyperpnoea-induced bronchoconstriction (HIB) however there are a range of pharmacological therapies available to help control asthma symptoms. In the UK Asthma is treated under the stepwise approach outline by the British Thoracic Society (BTS) and Scottish Intercollegiate Guidelines Network guidelines (2014). In athletes suffering with EIB the same guidelines are used with treatment using β_2-agonsists and inhaled corticosteroids (ICS) recommended by the BTS. The step-wise approach is a 5-step progressive protocol used to find the ideal asthma medications and dosages required to gain asthma control. Patients start the programme on the most appropriate step based on their initial asthma severity. Patients with mild intermittent asthma start on step one, involving treatment with an inhaled short-acting β_2-agonist (SABA) as and when required. If asthma is not well controlled on this step and symptoms persist patients are moved onto the next step. This process is continued until a step is reached in which the prescribed medication results in the patient's asthma being well controlled.

2.5.1. Inhaled β_2-agonists

B_2-agonsits are a quick acting form of asthma medication used to combat airway narrowing and provide a rapid relief of symptoms. B_2-agonsits act on β_2-adrenoceptors located on ASM cells resulting in ASM relaxation which helps to resolve bronchoconstriction and airway narrowing (Billington, Penn, & Hall, 2017). Three groups of β-agonists exist: SABAs, Long-

Acting β Agonists (LABAs) and Ultra-Long Acting β Agonists (ultra-LABAs). The bronchoprotective effect of β_2-agonists occurs within minutes and last between 4-6 hours for SABAs, ~12 hours for LABAs, and ~24 hours for ultra-LABAs. While LABAs sound more effective than SABAs they are only introduced for the treatment of asthma as an add-on therapy in patients already taking regular ICS but with inadequate control, this is due to the safety issues surrounding LABAs outlined in more detail in section (2.5.3. Issues with current medication).

In EIB sufferers it is recommended they take SABAs 30 minutes prior to exercise. In a Cochrane systematic review including 53 trials consisting of 1139 participants short-term administration of β_2-agonists reduces the peak fall in FEV_1 by 17.7% (95% CI: 15.84% to 19.51%) compared to placebo (Bonini et al, 2013). In EIB, β_2-agonists therefore provide valuable protection. If patients are using there SABA more than three time per week then the addition of a maintenance therapy in the form ICS is recommended (British Thoracic Society Scottish Intercollegiate Guidelines Network, 2014).

2.5.2. Inhaled corticosteroid

ICS are the most common form of maintenance therapy used to treat asthma. They can effectively suppress airway inflammation in asthma. ICS work by switching of activated inflammatory genes leading to a reduction in the number and activation state of inflammatory cells (Barnes, 2010). The activation of glucocorticoid receptors (GR) by glucocorticoids initiates nuclear translocate of GR which subsequently inhibits histone acetyltransferase activity and recruitment of histone deaceytlase-2 which suppresses transcription of inflammatory genes usually activated through the nuclear factorκB (NF-κB) pathway. ICS reduce inflammatory cell numbers including eosinophils, T-lymphocytes, mast cells, and

dendritic cells in asthma patients and additionally attenuate asthma responses in structure cells such as cytokine release from epithelial cells, mucus secretion by goblet cells, and upregulation of β_2-adrenoceptors on ASM (Barnes, 2010).

2.5.3. Issues with current treatments

Chronic and frequent use of β_2-agonists in asthma patients has been shown to lead to tolerance or worsening bronchoconstriction (Swystun et al, 2000). Swystun et al (2000) assessed the effect of salbutamol daily (100 μg, 2 puffs 4 times daily) or matched placebo for 10 days on allergen challenge responses in mild-moderate asthma patients. Salbutamol treatment significantly increased the early-phase fall in FEV_1 compared to placebo (20% vs 15%). This was accompanied by increased serum tryptase levels 1-hour post challenge and increased sputum percentage eosinophils 7-hours post challenge. The loss of a bronchoprotective effect has additionally been reported with the β_2-agonists salmeterol and formoterol (Bhagat et al., 1995; Cheung et al, 1992; Aziz, Hall, McFarlane, & Lipworth, 1998). The safety of LABAs have been questioned, a meta-analysis conducted in 2006 assessed the association between LABA use and the risk of severe, life-threatening, or fatal asthma exacerbations (Salpeter, Buckley, Ormiston, & Salpeter, 2006). In 19 trials including 33826 asthma patients LABAs significantly increased the odds ratio for exacerbations requiring hospitalisation (OR = 2.6), life-threatening exacerbations (OR = 1.8) and asthma-related deaths (OR = 3.5) compared to placebo. Exacerbations requiring hospitalisation was significantly increased for both salmeterol (OR = 1.7) and formoterol (OR = 3.2).

The chronic use of corticosteroids particularly at the highest prescribed doses can have undesirable side-effects (Barnes, 2010). One of the most common airway side effects is dysphonia which may occur in 50% of patients using metered dose inhalers (Barnes, 2010;

Williamson et al, 1995). Systemic side effects include adrenal suppression, growth suppression, bruising, osteoporosis, cataracts, glaucoma, metabolic abnormalities, and psychiatric disturbance (Barnes, 2010). Additionally, there has been concerns over increased risk of pneumonia and lower respiratory tract infections with ICS. A dose response relationship between ICS dose and the risk of pneumonia and lower respiratory tract infection has been reported in asthma patients (McKeever, Harrison, Hubbard, & Shaw, 2013). Patients receiving the highest doses of medication (≥ 1000μg) had a 2.04 increased risk compared to asthma patients who did not have a prescription for ICS in the previous 90 days. Furthermore, reduced anti-viral responses are associated with high-dose ICS in asthma patients while budesonide has been shown to reduce anti-viral responses in vitro (Simpson et al, 2016; Davies et al, 2011). Furthermore, severe asthma patients show little response to ICS, while medication adherence is also a major issue with ICS (Barnes, 2010).

The current WADA prohibited list permits the administration of inhaled salbutamol (1600 μg in 24-h and 800 μg in 12-h), formoterol (≤54 μg in 24-h), salmeterol (≤200 μg in 24-h) and vilanterol (≤25 μg in 24-h) without a therapeutic use exemption (TUE). Athletes with EIB are recommended to take β_2-agonists 15 minutes prior to exercise (Parsons et al, 2013). Athletes with asthma are recommended to additionally initiate ICS therapy to reduce the reliance on β_2-agonists and reduce the risk of developing tolerance. This is important in elite athletes who commonly perform multiple bouts of exercise per day. ICS therapy in athletes with a diagnosis of asthma in permitted by WADA without the need for a therapeutic use exemption. All other inhaled β_2-agonists and the systemic use of all β_2-agonists (injected or oral) and oral corticosteroids are either prohibited or require a TUE (WADA. Medical Information To Support The Decision of TUECS – ASTHMA. https://www.wada-ama.org/en/resources/therapeutic-use-exemption-tue/medical-information-to-support-the-decisions-of-tuecs-asthma. Access: 03/01/2021).

A permitted urine substance threshold for salbutamol is set at 1.0 µg/mL and for formoterol at 40 ng/mL, although no substance thresholds currently exist for salmeterol and vilanterol (WADA. WADA technical document. https://www.wada-ama.org/sites/default/files/resources/files/td2018dl_v1_en.pdf. Accessed: 14/03/21). The decision limit for salbutamol and formoterol are set at 1.2 µg/mL and 50 ng/mL, therefore if an athletes urinary concentration exceeds these values WADA will report an 'adverse analytical finding' (AAF). In this instance an athlete can request an individual pharmacokinetic study to assess whether an AAF has the potential to occur irrespective of adherence to permitted medication doses. Heubergerm Dijkman & Cohen., (2018) reported large variability in urine concentration of salbutamol which can lead to incorrect anti-doping violations whereas some violation may not return AAFs if samples are taken long enough after drug administration. Interestingly between 2013 and 2017 20% of AAF violation for salbutamol have resulted in acquittal (WADA. WADA clarifies facts regarding UCI decision on Christopher Froome. https://www.wada-ama.org/en/media/news/2018-07/wada-clarifies-facts-regarding-uci-decision-on-christopher-froome. Accessed: 14/03/21). There have been some high-profile cases involving elite athletes that have been acquitted of salbutamol anti-doping violations. Unfortunately, these cases are commonly portrayed negatively in the media creating a stigma around the use of asthma medication in athletes. Allen, Price, Hull & Backhouse., (2021) found that athletes perceive there is a negative stigma surrounding the use of asthma therapies in competitive sport. This may lead to the avoidance of asthma medication in athletes with asthma and/or EIB that require treatment.

On the other hand, speculation exists on the potential performance-enhancing effect of asthma therapies. Evidence suggests there is limited ergogenic benefits of inhaled β_2-agonists and ICS on endurance, strength, or sprint performance in healthy athletes while some evidence supports an ergogenic effect of systemic β_2-agonists on athletic performance (Pluim

et al, 2011; Kuipers et al, 2008. Multiple asthma therapies which require TUEs however, have need linked to meaningful improvements in athletic performance (Allen, Backhouse, Hull & Price, 2019). These therapies could therefore be misused to gain a competitive advantage in sport. The issues of adverse side-effects, stigma and avoidance, and the potential for misuse of pharmacological asthma therapies in sport highlights the need to identify novel non-pharmacological alternatives to treat asthma and EIB. In addition, the potential risk of athletes returning an AAF after salbutamol administration within permitted doses highlights the important of finding other therapies which can reduce the dependence on β_2-agonists.

In summary, while pharmacological medications for asthma combats some of the key pathophysiological feature of asthma the side-effects, poor adherence, ineffectiveness in some patients, and development of tolerance are major issues. The limitations of current asthma and EIB treatments highlights the need to find novel alternative therapies which can attenuate some of the key features of these conditions including airway inflammation, airway hyper-responsiveness, and airway remodelling. New therapies may allow for a step-down approach to current pharmacological therapies allowing for a reduction in dosages which may alleviate some of the negative side-effects. New therapies may additionally alleviate the dependence of asthma medication in athletes with asthma and/or EIB and reduce the risk of AAFs and anti-doping violations occurring.

2.6. Dietary fibre and prebiotics in asthma

Pharmacological therapies have been the mainstay for asthma treatment and provide great relief for many patients suffering with asthma. As mentioned in previous sections however, these treatments are not curative nor effective in all asthma phenotypes. As such, there is a need for new therapies to help improve asthma control to help these patients and reduce the burden of asthma. These therapies must be able to either work alongside current therapies to provide additional control, improve the efficacy of current treatments, or be able to replace current therapies due to their superior effects. While the later remains elusive, diet seems to have a large influence on asthma development and control and therefore may satisfy the ability to improve asthma control alongside other therapies and improve the efficacy of current pharmacological treatments.

2.6.1. Influence of diet and dietary fibre on asthma

The risk of developing asthma in childhood is influenced by diet before we are even born via maternal diet. Maternal diets higher in fruit and vegetables, vitamin E and D, alongside a greater adherence to the Mediterranean diet is associated with a reduced incident of wheeze in infants (Nurmatov, Nwaru, Devereux, & Sheikh, 2012). In airway allergen sensitized and challenged mice, maternal resistant starch intake protects offspring from the development of allergic airway disease by epigenetic regulation (Thorburn et al, 2015). Later on in infancy, breast feeding reduces the risk of wheeze and asthma even in infants with a greater genetic susceptibility to asthma (Klopp et al, 2017; Lodge et al, 2015). The nutrients which reduce the risk of wheeze and asthma in maternal diets are paralleled in childhood diets. Individually, fruit and fish intake in children between the ages of 8-12 years old are associated with a ≥14% reduction in the risk of developing wheeze. Higher fruit, vegetable,

and fish intake in combination is associated with a reduced prevalence of asthma, while diets more closely resembling the Mediterranean diet (e.g. a diet high in vegetables, fruits, nuts, seeds, wholegrains, legumes, fish, olive oil) are associated with a reduced prevalence of wheeze and asthma (Nagel et al, 2010). Diet during pregnancy through to childhood and adolescents is therefore influential in the risk of developing asthma (Figure 2.6).

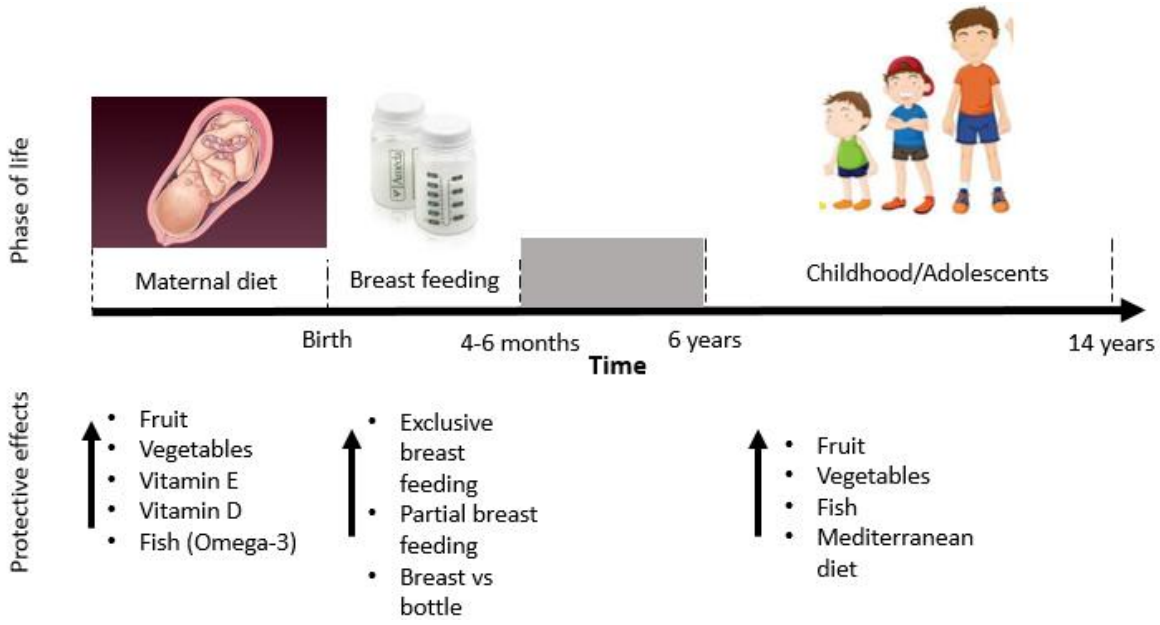

Figure 2.6: Dietary nutrients and practises which are associated with a reduced risk of developing wheeze or asthma from maternal diet through to adolescents. (Nurmatov et al, 2012; Klopp et al, 2017; Lodge et al, 2015; Nagel et al, 2010).

Diet is also influential on asthma symptoms and outcomes in people with already established asthma. Healthier diets have been associated with fewer asthma symptoms (Andrianasolo et al, 2018; Li et al, 2017), even independent of BMI (Lie et al, 2017). Westernised diets in particular have been linked to more frequent asthma attacks (Varraso et al, 2009), while diets expressing a higher inflammatory index have been associated with lower lung function and higher blood interleukin-6 (IL-6) concentrations (Woods, Shivappa, Berthon, Gibson, & Herbert, 2015). Strikingly, for every unit increase in dietary inflammatory index FEV_1 decreased 3.5 times (Woods et al, 2015). Various dietary nutrients such as dietary sodium,

omega-3 fatty acids, dietary antioxidants, and caffeine are shown to influence airway inflammation and hyper-responsiveness in asthma (Javid, Cushley, & Bone, 1988; Stoodley et al, 2020; Williams, Hunter, Johnson, & Sharpe, 2013; Weiler et al 2016).

Dietary fibre seems beneficial for asthma and is associated with reduced asthma symptoms and improved outcomes. Lower dietary fibre intake has been associated with reduced lung function and sputum eosinophilia (Berthon, Macdonald-Wicks, Gibson, & Wood, 2013; Hanson et al, 2015). Concomitantly total, soluble and insoluble fibre intake is inversely associated with asthma control scores and symptom scores (Adrianasolo et al, 2019). These associations have developed interest in the therapeutic potential of dietary fibre for asthma (Williams, Scott, & Wood, 2019). In particular a proportion of dietary fibres classified as dietary prebiotics have shown promise in tackling pathophysiological features of asthma such as airway inflammation, airway hyper-responsiveness (AHR), and lung function which lead to improved asthma control (Van De Pol, Lutter, Smids, Weersink, & Van Der Zee, 2011; Williams et al, 2016; Halnes et al, 2017; McLoughlin et al, 2019).

The CODEX Alimentarius Commission (2009) defines dietary fibre as groups of carbohydrate polymers with ten or more monomeric units which cannot be completely broken down or absorbed in the small intestine. Under these guidelines fibres belong to the following categories: (1) edible carbohydrate polymers naturally occurring in foods as consumed; (2) edible carbohydrate polymers which have been obtained from food raw materials by physical, enzymatic, or chemical means and which have a beneficial physiological effects demonstrated by generally accepted scientific evidence, and: (3) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence. Dietary fibre can be further classified into insoluble and soluble fibre. Insoluble fibres are generally poorly fermented by the gut microbiota, but

soluble forms are generally highly fermentable in the large intestine. Thus, many forms of dietary fibre possess prebiotic qualities.

Prebiotics are defined as 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' (Gibson et al, 2017). While prebiotics have historically been viewed as non-digestible food ingredients acting via the gut microbiota (Gibson & Roberfroid, 1995), the definition has changed over time to align with new findings which recognise that substrates other than dietary fibre (e.g. polythenols) possess prebiotic qualities and other sites have microbiotas (e.g. lung, vaginal, oral, skin) that are linked to host health. Dietary prebiotics are however, additionally defined as 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring a benefit upon host health' (Gibson et al, 2010). Most recognised dietary prebiotics are classified as dietary fibre, although it must be noted that not all fibres are classed as prebiotics (Slavin, 2013).

2.6.2. Prebiotics and synbiotics in human asthma and HIB

Dietary prebiotics have shown promise in the treatment of asthma and HIB. The first study involving prebiotic supplementation (7.2g/d GOS; 0.8g/d FOS) in asthma was by Van De Pol et al (2011) in combination with a probiotic (*Bifidobacterium Breve:* 10^{10} CFU). Four weeks supplementation increasing evening PEF by ~10 L/min and morning PEF by ~30 L/min across the 4-week compared to placebo. Although statistically significant this improvement is not a clinically significant improvement (≥ 60 L/min; Quanjer, Lebowitz, Gregg, Miller & Pedersen, 1997). House dust mite challenge (HDM)-induced increases in serum IL-5 were attenuated. Concomitantly, ex-vivo production of type-2 cytokines (IL-4, 5, 13) from peripheral blood mononuclear cells (PBMCs) upon HDM exposure were attenuated

Conversely, there was no change in airway hyper-responsiveness (AHR) to methacholine and HDM, or HDM-induced increases in sputum eosinophils. This study provided the first evidence to support that supplemention with prebiotics and probiotics reduces asthma severity. It does however remain unknown the individual or combined contribution of GOS, FOS, and *B.Breve* to the positive effects seen by Van De Pol.

Williams et al (2016) conducted a double-blind, randomised, placebo controlled cross-over study in ten adults with mild to moderate asthma and hyperpnoea-induced bronchoconstriction (HIB) to evaluate the effect of 21 days supplementation with 11g/d of Bimuno-galactooligosaccharide (B-GOS) (48% GOS content). B-GOS significantly attenuated HIB by 40% comparison to placebo. B-GOS supplementation also abolished the increases in TNF-α observed following an EVH test. In addition, resting serum concentrations of CCL17 and CRP were significantly reduced with B-GOS compared to placebo. This study showed B-GOS to be effective in attenuating HIB and systemic marking of airway inflammation in individuals with asthma and HIB. To date, this is the only study to test the effect of prebiotics on HIB, utilizing individuals that have the HIB with asthma phenotype. However, Williams et al (2016) did not measure airway inflammation so the effect of B-GOS on airway inflammation is unknown. Additionally, no faecal samples collected so the effect of B-GOS on gut bacteria composition and activity was not known.

Similar findings have been found with inulin ingestion by McLoughlin et al (2019). Inulin supplementation (12g/d for 1-week) in asthma patients significantly reduced sputum percentage eosinophils (Δ -1%) and reduced asthma control scores (Δ -0.3) at 1 week compared to baseline. The mechanisms underpinning these changes were supported by reduced HDAC9 gene expression in sputum cells. HDAC9 down-regulates Treg cell function (Beier et al, 2012) and therefore HDAC9 inhibition may contribute to increased Treg function in the airways. Faecal *Anaerostipes* bacteria, a SCFA producing genus (Riviere, Selak,

Lantin, Leroy, & De Vuyst, 2016), was also increased. SCFAs have HDAC inhibitory abilities (Smith et al, 2013; Park et al, 2015), suggesting a possible pathway in which inulin acts on airway inflammation. This supports inulin as another prebiotic with therapeutic potential in asthma.

Interestingly, however, these outcome measures were unaltered when inulin was combined with a multi-strain probiotic (> 25 billion CFU), even though inulin-alone and the synbiotic altered bacterial taxonomic numbers significantly compared to placebo. This highlights that synbiotics while may not always yield better outcomes in host health. This suggests that there is complex interplay between the gut microbiota and lung health and supports the idea that specific interactions along the gut-lung axis are required for improved lung health rather than simply altering gut microbial composition.

While chronic supplementation with prebiotic induces favourable changes in asthma, similar effects are seen with acute supplementation. Halnes et al (2017) assessed the effect of 3.5g inulin in combination with a probiotic yoghurt (*Lactobacillus acidophilis* LA5, *Lactobacillus rhamnosus* GG, and *Bifidobacterium lactis* Bb12) on lung function and airway inflammation pre and 4-hours post ingestion. In the synbiotic group absolute FEV_1 (Δ 0.1 L), percentage predicted FEV_1 (Δ 4%), and percentage predicted FEV_1/FVC ratio (Δ 3.8%) increased from baseline, however, a clinically important change in FEV_1 is typically deemed as >12% or >0.2 L (Pellegrino et al, 2005). Additionally, sputum lymphocyte, neutrophil, macrophage, total sputum cell counts, IL-8 concentrations and fraction of exhaled nitric oxide were reduced from baseline. These effects were accompanied by increased gene expression of G-couple protein receptor-41 (GPR41) and GPR43 in sputum cells.

GPR41 and GPR43 signalling inhibits the NF-κB pathway (Woods et al, 2019) and are stimulated by the short-chain fatty acids, acetate, butyrate, and proprionate (Koh et al, 2016)

which can be produced through bacterial fermentation of prebiotics in the gut. GPR43 deficient mice exhibit more extensive and unresolved airway inflammation (Malowski et al, 2009) highlighting the importance of this pathway in tackling airway inflammation. The production of SCFAs by fermentation of inulin may provide a protective effect against airway inflammation via GPRs, although SCFA concentrations in faecal and blood samples were not measures by Halnes et al (2017). It is interesting that these beneficial changes occurred after a single dose of inulin and probiotic, which is unlikely to cause significant changes in gut bacterial composition. This suggests that gut bacterial activity and metabolite production is important, which is supported by the increased gene expression of receptors (GPR41 and GPR43) found by Halnes et al, (2017).

Chronic and acute supplementation with prebiotics show therapeutic potential in the treatment of asthma by improving outcome measures such as AHR, lung function, and airway inflammation. The indefinite mechanisms is not known from these human studies. The data does however suggest involvement of GPR41 and GPR43 signalling and HDAC inhibition which can dampen airway inflammation. Both these pathways can be stimulated by SCFAs which can be produced from gut bacterial fermentation of prebiotics. These mechanisms have been alluded to in much greater detail in mice models of asthma. Future work needs to establish the effect of prebiotic on airway inflammation in asthma to a greater degree, try and reveal the mechanisms involved, and find the most effective prebiotics and the best supplementation practises.

2.6.3. Prebiotic ingestion in mice models of asthma

Considerable work has been conducted in the effects of prebiotics in mouse models of asthma by Verheijden and colleagues, primarily using GOSs (Verheijden et al, 2015a; Verheijden et

al, 2015b; Verheijden et al, 2015c; Verheijden et al, 2016; Verheijden et al, 2018). These studies have highlighted the ability of GOS to modulate airway inflammation by reducing type-2 inflammation and enhancing anti-inflammatory mechanisms via T regulatory (Treg) cells. Supplementing BALB/c mice's diets with 1% GOS prior to being sensitized and challenged with HDM abrogates AHR and reduces airway inflammation. Markers of airway inflammation suppressed by GOS include lung chemokine ligand-5 (CCL5), IL-13, and IL-33 concentrations, BALF leukocyte counts, and serum mucosal mast cell protease-1 concentrations (Verheijden et al, 2015a; Verheijden et al, 2015b; Verheijden et al, 2015c; Verheijden et al, 2018).

In addition, GOS not only attenuates the airway epithelial alarmin IL-33 but reduces mRNA expression of the IL-33 receptor, ST2 (Verheijden et al, 2015b). This is of importance as IL-33 alongside other airway epithelial derived cytokines (e.g. thymic stromal poietin, IL-25) are key initiators of airway inflammation which respond to a broad range of asthma triggers (e.g. allergens, viruses, osmotic). These cytokines activate strong type-2 responses from Th2 cells and type-2 innate lymphoid cells (ILC2). The effects of GOS on IL-33 is also present in the small intestine of mice suggesting GOS may be effective in a broad range of IL-33 mediated conditions. GOS not only induces its own protective effect but enhances the efficacy of budesonide, an ICS that is commonly prescribed to treat asthma (Verheijden et al, 2018). In lung homogenates supernatant from HDM challenged mice, budesonide and GOS combined but not individually reduces CCL17, CCL22, and IL-33 concentrations. GOS therefore enhances the efficacy of budesonide to attenuate type-2 inflammation. It would be interesting for future work to try and replicate these findings in humans, and to also assess whether GOS can help improve the response to ICS in the eosinophilic/type-2 ICS resistant phenotype of asthma.

The pathways in which GOS attenuates airway inflammation seem to be Treg cell mediated. Verheijden et al (2016) highlighted the important role Treg cells play in the protective effect of GOS in mice models of asthma. BALB/c mice were exposed to phosphate buffer saline (PBS) (i.e. control), or sensitized and challenged with HDM. From two weeks prior mice were fed a 1% GOS, or a control diet. GOS attenuated HDM-induced airway eosinophilia and IL-33 and CCL5 concentrations. However, the depletion of Treg cells, using an anti-CD25 antibody, abolished GOS's protective effects. This suggests that the ability of GOS to reduce airway eosinophilia and IL-33 and CCL5 is mediated in part by Treg cell function.

Resistant starch feeding also has a similar effect in suppressing HDM-induced features in mice asthma models, while increasing faecal and serum acetate concentrations (Thorburn et al, 2015). This suppression of allergic asthma in mice is paralleled by acetate feeding, with both resistant starch and acetates suppressive effects being GPR43-independent, suggesting alternative mechanism are involved. Thorburn et al (2015) revealed that acetate feeding leads to greater Treg cell numbers and suppressive function. Greater acetylation of the FOXp3 promotor region was found with FOXp3 expression indeed increased. Acetate feeding also suppressed lung cell HDAC activity. HDAC-9 is involved in FOXp3 mediated suppression in Treg cells (Beier et al, 2012) and reduced gene expression of HDAC-9 is reduced by inulin supplementation in human asthma (McLoughlin et al, 2019). This supports the role of Treg cells in the protective effect of prebiotics on asthma and suggests that acetate production may mediate these effects via inhibiting HDAC activity (likely HDAC-9) leading to greater Treg cell numbers and activity. Such a mechanism would have a profound effect in suppressing and resolving airway inflammation as shown by prebiotic feeding in both mouse and human asthma studies.

Resistant starch also increases serum propionate (Thorburn et al, 2015; Trompette et al, 2014) and propionate feeding elicits a protective effect against allergic asthma in mice similar to

resistant starch and acetate but in a GPR41 dependant manner (Trompette et al, 2014). Propionate feeding also alters bone marrow haematopoiesis of dendritic cells (DCs) leading to DCs in the airways with a reduced ability to promote Th2 effector activity. Propionate may therefore alleviate asthma by suppressing airway Th2 cell function. Furthermore, butyrate modulates GATA3 expression in pulmonary ILC2s, which subsequently attenuates airway neutrophil recruitment. Introduction of butyrate producing bacteria to germ free mice suppresses ILC2-driven AHR (Lewis et al, 2019).

Prebiotics are able to suppress pathological features of asthma such as AHR and airway inflammation. These effects are mediated by reduced pro-inflammatory and enhance anti-inflammatory capacity of immune cells. The data suggests that the mechanisms involved in immune modulation are mediated partly by GPR41 and 43 signalling, and/or HDAC inhibition. These mechanisms result in enhanced Treg cell suppressive capacity and numbers, alongside reduced Th2 cell function by altered haematopoiesis of DCs and macrophage progenitors. How prebiotics can cause these changes via specific bacterial immune cross talk or bacterial derived metabolites is discussed in the following sections.

2.6.4. Immune modulation by the gut microbiota and microbial derived metabolites

The beneficial effects of prebiotics in asthma seems to be dependent on immune modulation. Dietary prebiotics influence the composition and activity of the gut microbiota. Certain bacteria alongside the metabolites they produce can induce immune modulation and lead to enhanced immune regulatory responses which may be beneficial in inflammatory disorders such as asthma and HIB. It is important to understand the context and mechanisms by which certain bacteria and their metabolites induced these immune regulatory effects as well as identify prebiotics which can induce these beneficial changes in the gut microbial

composition and metabolite production. In doing so a more tailored approach to selecting prebiotics for their intended use can be applied, e.g. asthma treatment.

2.6.4.1. Direct bacterial-GALT crosstalk

The gut itself is ideally designed for immune interactions. Firstly, the gut has an extensive mucosa-associated lymphoid tissues known as the, gut-associate lymphoid tissue (GALT) (Zgair, Wong, & Gershkovich, 2016). The GALT consists of an epithelial layer coated in a mucus layer on the luminal side while the basal side is equipped with the lamina propria, housing immune structures such a preyer's patches, mesenteric lymph nodes, and lymphoid follicles. With these structures reside large and dense populations of lymphocytes, macrophages, and DCs. Secondly, the large intestine encapsulates 10^{14} of bacteria, a microbiota two orders higher than any other microbial site in/on the human body (Sender, Fuchs, & Milo, 2016). Thirdly the large intestine of an average man is estimated to have a mucosal surface area of $2m^2$ (Helander & Fandriks, 2014). The magnitude of immune tissue, microbes, and surface area of the gut is well designed for gut microbiota and immune interactions.

This is exemplified in germ-free (GF) mice, which are born, raised and housed in sterile conditions and therefore do not possess developed gut microbiotas. GF mice are less able to control inflammation and are more prone to allergic sensitisation. This dysregulation can affect the airways, enhancing lymphocytes and eosinophil infiltration (Herbst et al, 2011), and lead to lower Treg cell numbers and suppressive function (Ostman, Rask, Wold, Hultkrantz, & Telemo, 2006). Introduction of diverse microbiotas and even specific bacterial strains via probiotics or faecal transplants from heathy donors can ameliorate these effects (Ostman,2006; Kozakova, 2016). Commensal bacteria are also important for GALT

structural development. Colonic mucus synthesis and function are impaired, alongside reduce expression of intestinal epithelial cell (IEC) tight-junction proteins in GF mice (Birchenough, Schroder, Backhed, & Hansson, 2017; Johansson et al, 2015). While GF models highlight the importance of microbiotas in helping form functional immune responses in the host and the development of the GALT, they cannot only be attributed to the gut microbiota as GF mice lack development in other microbial sites (e.g. lung, skin, mouth).

The gut microbiota, however, is associated with the risk of developing allergic diseases including asthma in humans (Johnson & Ownby, 2017). For example, *Lactobacilli* and *Eubacteria* were more frequently observed in Estonian children's (low allergy prevalence) stool, while *Clostridium* difficile was more frequent in Swedish children's (high allergy prevalence) stool (Sepp et al, 1997). Lower levels of *Bifidobacteria* are consistently found in the stools of one year old children who subsequently develop allergy at 2 years of age (Bjorksten et al, 2001). More diverse gut microbiotas in the first weeks of life are also associated with reduced incidence of asthma at seven years old (Abrahamsson et al, 2014). Infants at risk of asthma present with gut microbial dysbiosis in the first 100 days of life with reduced relative abundance of bacterial generas, *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* (Arrieta et al, 2015). This gut microbial dysbiosis is accompanied by reduced faecal acetate, a microbial derived metabolite with immune tolerogenic tendencies (see section 2.6.4.). Furthermore, taking macrolide antibiotics in early life, which causes adverse changes to the gut microbiota that can be detected >2years post macrolide consumption, are also associated with increased risk of asthma (Korpela & de Vos, 2016).

These observations underpin the hygiene hypothesis which suggests that early life microbial exposure, primarily commensal, helps develop the immune system and protects against allergic and autoimmune diseases (Rook, 2000; Folkerts, Walzl, & Openshaw, 2000). Indeed, early life factors such as breast feeding and natural birth which influence gut microbial

development are linked to lower incidence of asthma and allergy in childhood (Lodge et al, 2015; Thavagnanam, Fleming, Bromley, Shields, & Cardwell, 2008). Probiotics, live bacteria which can be introduced to the gut, may be effective in stimulating beneficial gut microbial development. Meta-analysis of probiotic administration in infants shows a reduced risk (relative risk = 0.8) of atopic sensitization and total IgE alongside a reduced risk of eczema (Cuello-Garcia et al, 2015; Elazab et al, 2013). Supplementation with *Bifidobacterium* and *Lactobacillus* species reduces the risk of developing atopic disease in high-risk infants (Kalliomaki et al, 2001; Gutkowski et al, 2011).

While meta-analysis data does not currently support probiotic use for the prevention of asthma in children (Wei, Jiang, Liu, Sun, & Zhu, 2019), probiotic studies do however use a variety of individual strains, combination, and dosages. It has been shown that immunomodulatory effects are strain specific (Medina, Izquierdo, Ennahar, & Sanz, 2007; Hougee et al, 2010). Therefore, while the conclusion of meta-analysis data suggest no protective effect of probiotics for asthma in infants more research is needed to gain a greater understanding of the potential efficacy of individual strains. This is exemplified in mice models of asthma were strains such as *Lactobacillus rhamnosus GG, Lactobacillus reuteri, and Bifidobacterium longum NCIMB* promote anti-inflammatory and asthma resolving qualities were *Lactobacillus rhamnosus GR-1, Lactobacillus Salivarius, and Bifidobacterium Longum W11* either do not possess these qualities or have lesser efficacy (Spacova, Ceuppens, Seys, Petrova, & Lebeer, 2018, Forsythe, Inman, & Bienenstock, 2007; Medina et al, 2007). This highlights the sensitive interplay between gut microbes and the host immune system and shows that care must be taken to select the appropriate strain(s) for the intended use. These studies show the importance of the gut microbiota in the risk of allergic diseases and support the protective role that beneficial interventions such as pro and prebiotics may have on reducing the risk of developing atopy and asthma in early life.

The gut microbiota composition in adults with asthma does not markedly differ from that of healthy controls (Hua, Goedert, Pu, Yu, & Shi, 2016; Hevia et al, 2016) and, therefore, the relationship between the adult gut microbiome and adult asthma risk remains uncertain (Hua et al, 2016). Hevia et al (2016) did however report that allergic asthmatics had a slightly greater abundance of *Faecalibacterium* and *Bifidobacterium* at the genus level than healthy controls. Moreover, a higher relative abundance of *Bifidobacterium adolescentis* within Bifidobacterium species was found for asthmatics, while *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium bifidum* were the prevailing species in controls. Asthmatics that have suffered from asthma for a longer duration tend to have lower levels of *Bifidobacterium* (Hevia et al, 2016). This genus is positively associated with health and may be a therapeutic target in this sub asthmatic population, however it is not known whether longer asthma durations are predisposed by lower *Bifidobacterium* levels or vice versa, such is the caveat of these established relationships. Buendia et al (2018) also found differences in gut bacterial composition between asthma patients living in the tropics based on phenotypes of airway obstruction. They found asthma phenotypes of no airway obstruction, reversible airway obstruction and fixed airway obstruction could be distinguished by *Streptococcaceae: Streptococcus* and *Enterobacteriaceae:Escherichia-Shigella* while *Veillonellaceae:Megasphaera* further distinguished reversible airway obstruction and fixed airway obstruction. While the gut microbiota in the adult asthma population as a whole is not too dissimilar from health it seems that slight differences exist between phenotypes of asthma. It would be interesting for future work to compare the gut microbiota between severe asthma vs mild/moderate and controls, as well as, eosinophilic versus neutrophilic phenotypes.

How gut microbial altering interventions reduce asthma risk is likely influenced by direct microbial and GALT interactions which shape the host immune system. Bacteria can directly

interact with the gut immune system by penetrating the mucus layer to make contact with epithelial cells (Ivanov et al, 2009), or through direct sampling by specialised immune cells. For example, DCs shuttle bacteria across the intestinal epithelium by extending their dendrites into the intestinal lumen (Rescigno, Rotta, Valzasina, & Ricciardi-Castagnoli, 2001) and M cells can ingest bacteria (Ohno, 2016). DCs play a pivotal role of immune modulation in the gut. Surface expression of toll-like receptors (TLRs) and intracellular nuclear oligomerization domain receptors (NOD) on gut immune cells identify bacteria and bacterial components (e.g. lipopolysaccharide, flagellin) and subsequently drive immune responses. *Bifidobacterium* and *Lactobacillus* have generally been associated with health and probiotic administration of a variety of strains of these generas induced anti-inflammatory cell maturation.

B.adolescentis, B.animalis, L.reuteri, and *L.casei* can modulate DC function in vitro leading to DCs which promote Treg and foxp3+lymphocytes leading to higher productions of the anti-inflammatory cytokine, IL-10 (Baba, Samson, Bourdet-Sicard, Rubio, & Sarfati, 2008; Smits et al, 2005). The effect of *L.reuteri* and *L.casei* was mediated by intercellular adhesion molecule 3-grabbing non-integrin. *L.reuteri* upregulates HLA-DR, CD40, CD80, CD83, and CD86 expression on human myeloid DCs. These changes also result in greater DC production of IL-12 and IL-18 leading to Th1 polarisation over Th2 cells (Mohamadzadeh et al, 2005), this same effect is found with *L.gasseri,* and *L.johnsonii*. Similarly *B.breve C50* upregulates HLA-DR, CD83, and CD86 on DCs resulting in greater IL-10 production in a TLR2-dependant manner (Hoarau, Lagaraine, Martin, Velge-Roussel, & Lebranchu, 2006). *B.bifidum* strains elicit anti-inflammatory effects by upregulating Treg cell function, and increased Th17 cell numbers helping to enhance gut innate immunity. Hart et al (2004) looked at a prebiotic cocktail containing three *Bifidobacterium* and four *lactobacillus* strains in vitro cultures with blood and intestinal DCs. There findings were similar to previous

reports with enhanced DC IL-10 production, this anti-inflammatory response was so potent it diminished LPS induced inflammation while maintaining IL-10 levels. Furthermore, individual strain assessments showed *Bifidobacterium* strains to upregulate IL-10 production from DCs by a greater amount than *lactobacillus* strains.

It seems that while bacteria have these direct effects on gut immune cells similar findings can be found with bacterial extracts. Similar to the effect of live B.bifidum on inducing Treg cell function via DCs, B.bifidum LMG13195 membrane vesicles elicit as similar response (Lopez et al, 2012). DCs cultured with these vesicles prompted naïve T cells induction into foxp3+Treg cells which produce high concentration of IL-10. This highlights that bacteria can act as a vehicle to transport immune modulating antigens. It additionally supports the direct interaction between microbes (provided as probiotics) and intestinal immune cells as it could be argued that increases in populations such as *Bifidobacterium* and *lactobacillus* would also lead to increased intestinal SCFAs which have separate immunomodulatory effects. Thus, whilst dead bacteria/extracts would have little effect on gut SCFA synthesis/concentrations, they may still induce immune modulation, thereby supporting direct gut bacteria-immune crosstalk.

Bifidobacterium and *lactobacillus* supplementation reduces airway inflammation and AHR in mice models of asthma. *Lactobascillus rhamnosus* attenuates AHR in ovalbumin challenged mice, reducing type-2 cytokines and eosinophils in the airways (Wu, Chen, Lee, Ko, & Lue, 2016; Jang et al, 2012; Yu et al, 2010). These changes are associated with increased TGF-β secretion from CD4+/CD3+ T cells in mesenteric lymph nodes and increased bronchial and splenic foxp3+Treg cells (Jang et al, 2012). TGF-β is important in the induction of foxp3 expressing Treg cells. Similar attenuations of asthma with accompanied increases in splenic foxp3+Tregs is found with *Lactobacillus reuteri* (Karimi, Forsythe, & Bienenstock, 2008). *Bifidobacterium lactis*, and *infantis* likewise reduce airway eosinophils and type-2 cytokine

production (Feleszko, Jaworska, & Hamelmann, 2006). The positive effects of Bifidobacterium supplementation transpire into improved quality of life in human children with pollen-induced asthma (Del Giudice et al, 2017). The direct interaction between gut microbes and GALT immune cells is one potential hypothesis in how prebiotics might be effective in asthma and HIB. Prebiotics such as GOS and inulin, which stimulate the growth of *Bifidobacterium* and *lactobacillus* strains, could enhance Treg cell production in the gut which may subsequently translocate to the airways and facilitate the control of inflammatory processes in asthma.

Indeed, studies using bacterial extracts support this gut-lung translocation hypothesis (Narvarro et al, 2011; Strickland et al, 2011). The bacterial extract known as Broncho-vaxom can reduce AHR, BALF eosinophils, and lung IL-4, IL-5 and IL-13 concentration in ovalbumin challenged mice (Narvarro et al, 2011). This effect was MyD88 dependant suggesting the requirement of TLRs and the gut. Interestingly bronch-vaxom increased trachea Treg numbers that had a phenotypical profile (High CCR9 expression) of intestinal origin. Mesenteric lymph nodes and preyers patch DCs imprint CCR9 phenotypes, as such the effect of broncho-vaxom on Tregs was CD103+DC dependant. Increased tracheal foxp3+Treg cells are also found after OM-174 ingestion in mice, an Escherichia coli extract (Strickland et al, 2011). This study highlights an interesting mechanism in that immune cells at the site of the gut can translocate to the airways when the airways are challenged. Therefore, anti-inflammatory cell production at the gut may help control airway inflammation. Prebiotics which stimulate the growth of bacterial strains which induce Treg cell development and reduce Th2 cell polarization may lead to such anti-inflammatory cells superseding the airways following translocation from the gut. This is one potential mechanism in how pro and prebiotics may attenuate asthma.

2.6.4.2. Immune modulation by gut bacterial derived metabolites

The breakdown of certain dietary prebiotics such as GOS, inulin, and FOS by gut microbes results in the production of metabolites, some of which possess immune modulating capacity. The most well-known and established of these are the short-chain fatty acids (SCFAs) acetate, propionate and butyrate. These are likely to be fundamental in the protective effect established by prebiotics in asthma. SCFAs have been shown to induce Treg cells in the gut, and as already discussed these cells may subsequently translocate to the airways. Additionally, SCFAs are absorbed into circulation allowing for potentially systemic and far reaching effects on peripheral tissue by immune modulation within blood and of bone marrow derived progenitor cells. SCFAs may also be present in the airways and influence local airway mucosal resident immune cells.

Fermentation of dietary fibre primarily occurs in the large intestine (Macfarlane and Macfarlane, 2012). The major products of this process are the SCFA metabolites - acetate, propionate, and butyrate - which are present along the large intestine at average concentrations of 54, 21, 20 mmol/kg of content, respectively (Cummings, Pomare, Brancg, Naylor, & Macfarlane, 1987). SCFAs concentrations peak in the caecum and ascending colon but reduce substantially in the descending colon and sigmoid/colon (Cummings et al, 1987). Very low levels are found in the ileum and jejunum. This is in keeping with the bacterial abundance of the large intestine compared to the ileum and jejunum where the bacterial content is three and seven orders of magnitude higher (Sender et al, 2016). Acetate is formed primarily from pyruvate via the Wood-Ljungdahl pathway or via acetyl-CoA (Koh et al, 2016). Most enteric bacteria are producers of acetate. Propionate is formed from either succinate by the succinate pathway, lactate via the acrylate pathway or, fucose and rhamose sugars in the propanediol pathway. Bacteroides spp., Phascolarctobacterium succinatutens, Dialister spp., Veillonella spp, Megasphaera elsdenii, Coprococcus catus, Salmonella spp.,

Roseburia inulinivorans, Ruminococcus obeum are key producers of propionate via these pathways. Butyrate is produced by the phosphotransbutyrylase/butyrate kinase or the butyryl-CoA:acetate-CoA transferase routes. Coprococcus comes, Coprococcus eutactu, Anaerostipesspp.,Coprococcus catus, Eubacteriumrectale, Eubacterium hallii, Faecalibacteriumprausnitzii, Roseburia spp are key producers of butyrate via these routes (Koh et al, 2016).

In mice, colonic SCFA concentrations are positively correlated with Treg cell numbers. Butyrate can induce differentiation of colonic Treg cells in vitro and in vivo and enhances H_3 acetylation in the promoter region of the FOXp3 locus (Furusawa et al, 2013). Interestingly acetate feeding in mice increases FOXp3 expression in Treg cells and enhances acetylation of the H_4 promoter region of FOXp3 (Thorburn et al, 2015). Butyrate can also control colonic inflammation in a GPR109a-dependant manner (Singh et al, 2014). Butyrate increases the anti-inflammatory properties of macrophages and DCs leading to greater Treg induction and IL-10 production, via GPR109a. GPR109a signalling by butyrate also reduces NF-κB activity (Thangaraju et al, 2009). The protective effect of propionate on attenuating Th2 cell activity in airways of mice with asthma is GPR41-depedants (Trompette et al, 2014). GPR43 also plays a role in increasing the size and function of the colonic Treg pool (Smith et al, 2013), and SCFAs stimulate GPR43 in the order of affinity propionate>butyrate>>acetate (Le Poul et al, 2003). Propionate also has epigenetic regulation of Tregs. Specifically, propionate reduces, in a GPR43-dependant manner, HDAC6 and HDAC9 expression in colonic Treg cell (Smith et al, 2013), which are known to downregulate Treg cell function (Beiler et al, 2012). GPR41 and 43 may not however be needed for HDAC inhibition in T cells. This is because SCFAs can be readily absorbed into cells without requiring GPRs. Furthermore, GPR41 and 43 are expressed at low levels on T cells, and SCFAs do not activate the downstream GPR41

and 43 signal extracellular signal-regulated kinases (Park et al, 2015). Regardless, HDAC signalling seems to be another mechanism by which SCFAs induce colonic Treg cell numbers and function alongside GPR-signalling. As mentioned previously, colonic Tregs can potentially migrate to the airways (Narvarro et al, 2011; Strickland et al, 2011). Increased SCFAs production from dietary prebiotic fermentation may therefore be another mechanism by which prebiotics reduce asthma severity.

The effect of GPR41 and GPR43 signalling on attenuating inflammation involves inhibiting the formation and nuclear translocation of nuclear factors-kappa B (NF-κB) (Williams et al, 2019). GPR41 activation initiates G protein αi (Gαi) subunit coupling which in turn inhibits the enzyme adenylyl cyclase (Brown et al, 2003; Le Poul et al, 2003). Adenylyl cyclase catalyses adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Accumulation of cAMP activates protein kinase A (PKA) leading to the phosphorylation of NF-κB subunits NF- κB1 (p50) and RelA (p65) (Christian, 2016) enhancing NF- κB transcription activity.

Alternatively, GPR43 activation increases phospholipase C activity via the Gαq subunit. Phospholipase C is responsible for the generation of inositol triphosphate (IP_3) and diacyglycerol (DAG), which lead to increased intracellular calcium and activation of protein kinase C. Protein kinase C can modulate arachidonic acid (AA) synthesis which produces inflammatory prostaglandins and leukotrienes when metabolised by lipoxygenase and cyclooxygenase enzymes. GPR43 activation reduces phosphorylation of NF-κB via β-arrestin 2 (βArr2) (Lee, 2013). βArr2 upregulates the inhibitor of NF-κB nuclear translocation, IκBα, which sequesters NF-κB to the cell cytosol (Gao et al, 2004; Lee et al, 2013). Activation of GPR41 and 43 therefore dampens inflammation by inhibiting the NF- κB pathway, a pivotal mediator of inflammation by inhibiting its formation and subsequent cytosol-nucleus translocation (Woods et al, 2019).

Alongside local immune modulation, SCFAs can exist systemically and induce immune modulation in sites remote from the gut. SCFAs can be absorbed into the systemic circulation and two reports have found modest concentrations in blood. Cumming et al (1987) reported peripheral vein blood plasma acetate, proprionate, and butyrate concentrations of 70, 5, and 4 μmol/L, respectively, in deceased individuals. Confirming these concentrations and ratios Wolever, Josse, Leiter, & Chiasson (1997) reported fasting blood serum concentrations of acetate, propionate, and butyrate to be 148, 5, and 2 μmol/L in healthy adults. Serum concentrations of SCFAs increase between 4-6hrs post inulin ingestion, owing to the fermentation of inulin by the gut microbiota and subsequent absorption (van der Beek et al, 2018; Tarinin & Wolever, 2010).

In mice, butyrate and propionate increase Treg cell numbers by boosting extrathymic differentiation (Arpaia et al, 2013). In human's direct acetate feeding increases natural killer cell activity (Ishizaka, Kikuchi, & Tsujii, 1993). Park et al (2015) found SCFAs could induce T effector cells as well as T regulatory cells depending on the cytokine milieu/immunological context. They found that SCFAs could generate Th17, Th1, and IL-10+T cells through HDAC inhibition. HDAC inhibition allowed for acetylation of p70 S6 kinase and phosphorylation rS6, upregulating the mammalian target of rapamycin (mTOR) pathway. This pathway was GPR41 and 43 independent highlighting a pathway irrespective of GPRs. Park et al (2015) also found that T cells do not express GPR41 and 43 at functional levels, which suggests that GPR41 and GPR43-independent pathways such as HDAC signalling may be involved in SCFA modulation of T cells.

SCFAs can enter cells by via monocarboxylate transporters (Slc5a8) and inhibit HDACs. This passive diffusion allows SCFAs to enter cells and inhibit HDACs independent of GPRs. Butyrate and propionate are well documented HDAC inhibitors (Schilderink, Verseijden, & de Jonge, 2013; Li, Yoa, & Jiang, 2014). While acetate is less well established Thorburn et al

(2015) showed acetate to increase acetylation of the FOXp3 promotor region of Treg cells and therefore suggests its involvement in epigenetic regulation. Evidence therefore supports both GPRs dependant and independent pathways in the modulation of immune function via SFCAs (Smith et al, 2013; Park et al, 2015). The bodily compartment and local immunological environment may dictate which pathways are dominant, although more work is required the shed light on the subject.

One such factor may be the concentrations of SCFAs in different bodily compartments. Total SCFAs are present in high levels in the large intestine with concentration of 131 mmol/kg of content in the cecum, and 123 mmol/kg in the ascending colon (Cumming et al, 1987). As expected, high levels are also found in faeces, with concentration reported at 479 µmol/kg wet faeces (Huda-Faujan et al, 2010). Much lower concentrations are then found outside the large intestine with concentration at 375 µmol/L in the portal vein, 148 µmol/L in the liver, 140 µmol/L in the hepatic veins, and only 79 µmol/L in peripheral veins (Cummings et al, 1987) (Table 2.3). Alongside lower concentrations outside the large intestine there is a shift in the ratios between acetate, propionate and butyrate. In the colon, molar ratios of acetate:propionate:butyrate are 57:22:21. . In the portal vein butyrates comprise only 8%. This may be due to the large contribution of butyrate as an energy source for colonocytes, which results in small amounts reaching the portal vein. Propionate also drops from 21% in the portal vein to 12% in the hepatic vein as it is absorbed by the liver. In peripheral veins only acetate is found in substantial amounts (acetate = 70 µmol/L; propionate = 5 µmol/L; butyrate = 4 µmol/L) (Cummings et al, 1987) (Table 2.3).

Table 2.3: Concentrations and ratios of acetate, propionate, and butyrate within the ascending colon, portal vein, liver, hepatic veins, and peripheral veins of humans. (Cummings et al, 1987; Wolever et al, 1987; Koliana et al, 2019).

Bodily compartment	Acetate (µmol/L)	Propionate (µmol/L)	Butyrate (µmol/L)	Total (µmol/L)
Ascending colon	63*	27*	25*	115*
Portal vein	258	88	29	375
Liver	_	_	_	148
Hepatic veins	115	21	12	140
Peripheral veins	70 - 148	5	2 - 4	77 - 157
Airways (BAL)	~75	~8	~1	~84

*units are mmol/kg contents instead of µmol/L. Individual acetate, propionate, and butyrate concentrations within the liver are not reported. BAL = bronchoalveolar lavage fluid.

The concentrations of SCFAs influence their ability to provoke responses from GPRs. Studies have attempted to establish the half maximal effective concentration (EC50) of GPR41, GPR43, and GPR109a. EC50 is the concentration of a ligand required to evoke a response halfway between a baseline and maximal response. For GPR43, acetate and propionate are the most potent ligands of the SCFAs (Brown et al, 2003). The EC50 for acetate has been reported between 52 – 537 µmol/L, and between 31 – 290 µmol/L for propionate (Brown et al, 2003; Le Poul et al, 2003). The concentrations of propionate in peripheral blood would be too low to evoke GPR43. Concentrations in the portal vein may be high enough, and concentration in the large intestine will most definitely be high enough to evoke a response. Similarly, acetate is likely to only evoke a small/unmeaningful response to GPR43 in peripheral veins but concentrations within the portal and particularly the large intestine would be within range.

For GPR41, propionate and acetate are the most potent ligands (Le Poul et al, 2003). The EC50 for acetate is high, between 1020 – 1299 μmol/L, while propionate is between 127 – 274 μmol/L (Brown et al, 2003; Le Poul et al, 2003). This suggests that only concentrations in the large intestine are high enough to evoke strong responses for GPR41. For GPR109a, butyrate is the most potent ligand and has an EC50 ~1.6 mmol/L (Koh et al, 2016), again confining the stimulation of GPR109a by butyrate to only the large intestine where concentrations are sufficiently high. This suggests that immune modulation by SCFAs via GPR41, GPR43 and GPR109a primarily occurs in the large intestine or the portal vein, especially for butyrate and propionate which are primarily metabolised in the intestine and liver (Bloemen et al, 2009).

Interestingly the luminal concentration of SCFAs is high enough for continuous saturation of these GPR41, GPR43, and GPR109a (Koh et al, 2016). For example, lumen butyrate concentration is over 10-times greater than the EC-50 for GPR109a (20 vs 1.6 mmol/L) (Thangaraju et al, 2009). This suggests that any further increase in SCFAs would have no effect on immune modulation and further increases in intestinal SCFAs via prebiotic supplementation would not induce immune modulation via these receptors in the gut. The mucus layer of the colon however likely ensures a SCFA gradient. This likely results in GALT being exposed too much lower concentrations then present in the lumen and as such the microenvironment that gut immune cells are exposed to is likely within the functional range of the GPRs, in which case increase SCFA concentrations would induce immune modulation. Another observation is the large drop in SCFAs concentrations between the portal and hepatic veins were the majority of propionate and butyrate are taken up by the liver (Bloemen et al, 2009). The liver plays a role in adaptive and innate immune modulation and is a major site of extrathymic T cell development (Parkers & Picut, 2005). The liver may therefore be a potential site in immune modulation by SCFAs. A hypothesis based on the gut-

liver-lung axis has been proposed in COPD where beneficial effects of dietary fibre on lung function are thought to be influenced by liver innate immune modulation which subsequently attenuated inflammatory airway responses (Young, Hopkins, Marsland, 2016). This is an intriguing hypothesis which warrants further investigation. It would be interesting to understand the effects of SCFAs and gut bacteria/bacterial ligands on immune modulation in the liver, and if so, whether these modulated immune cells translocate to peripheral tissues.

Another hypothesis explaining the role SCFAs play in immune modulation and the attenuation of asthma is that they are transported via the circulation to the lung where they induce immune modulation of airway resident immune cells. Given that SCFAs, particularly acetate, are measurable in peripheral blood and increase following ingestion of fermentable carbohydrates, it is feasible that they are present in the airways. Acetate, propionate, and butyrate have been detected in human bronchial alveolar lavage (BAL) (Acetate: ~75 μmol/L; Propionate: ~8 μmol/L; Butyrate: ~1 μmol/L) and sputum (Acetate: ~300 μmol/L; Propionate: ~60 μmol/L; Butyrate: ~3 μmol/L). Higher concentrations of acetate are found in BAL of asthma participants compared to healthy controls (Koliana et al, 2019). The higher concentration in sputum versus BAL samples is likely due to oral contamination and therefore BAL samples are more likely to reflect airway concentrations. Airway SCFAs are however more likely lung microbiome than gut microbiome derived, which refutes gut SCFA translocation. Lung bacteria can synthesise SCFAs from airway mucus, which likely accounts for the higher concentration of airway SCFAs in people with asthma compared to controls since greater mucus is a characteristic of asthma pathology. Airway SCFAs have not been measured following resistant starch supplementation in mice, even when elevated blood SCFAs is evident. Additionally, propionate and acetate feeding also fails to produce measurable concentrations of airway SCFAs (Trompette et al, 2014; Thorburn et al, 2015). These findings suggest that gut synthesised SCFAs are unlikely to reach the airways but

SCFAs can be synthesised by the lung microbiome by breaking down airway mucus. This evidence refutes the hypothesis that gut derived SCFAs from prebiotic fermentation can translocate to the lung and modulate airway resident immune cells. None the less, it is still important to understand the effect of SCFAs on airway immune cells given that the lung microbiome can be a source.

While SCFAs tend to elicit anti-inflammatory effects on gut and blood immune cells they promote pro-inflammatory responses in airway cells (Rutting et al, 2019; Koliana et al, 2019). Acetate and propionate enhance the production of IL-6, CXCL8 and CCL26 from airway epithelial cells at concentrations of 1 and 10mM. This is further shown by Rutting et al (2019) who found human lung fibroblasts and airway smooth-muscle cells release greater IL-6 and CXCL8 when exposed to large concentrations of SCFAs (10-25 mM). GPR41 and GPR43 can induce inflammation by activating extracellular signal-regulated kinase ½ and p38 mitogen-activated protein kinase signalling pathways, which have been reported to promote inflammation in intestinal epithelial cells within a certain context (Kim, Kang, Park, Yangisawa, & Kim, 2013). It may be that this pathway is preferentially activated in airway immune cells by SCFAs. Additionally, it must be noted that these in vitro studies use supra-physiological concentrations of SCFAs which may lead to toxicity. Regardless, the differing responses induced by gut, blood and airway immune cells by SCFAs shows the contextual effect of SCFAs and the fine tuning of immune responses they play through multiple pathways. In terms of promoting an anti-inflammatory environment in the airway by SCFAs the gut seems to be a better therapeutic target than the lung.

Due to the low concentrations of SCFAs in peripheral blood it is unlikely that GPRs play a large role in immune modulation distal to the gut and liver. This suggests that reports of increased airway Treg cell numbers and function following GOS and acetate feeding in mice is likely due to gut-lung translocation of immune cells and/or HDAC inhibition (Verheijden

et al, 2016; Thorburn et al, 2015). SCFAs may also alter bone marrow haematopoiesis, which promotes a shift in inflammatory cell phenotypes to less inflammatory or anti-inflammatory states that then supersede the airways. These mechanisms associated with the gut-lung axis were highlighted in the eloquent study by Trompette et al (2014) which showed that propionate and resistant starch feeding attenuated asthma in mice. These feeding practises altered macrophage and DC precursors in bone marrow and increased the number of monocyte/macrophage and DC precursors (MDP), and common DC precursors (CDP). These altered cells subsequently populated the lungs and matured into cells less driven in Th2 cell activation, as such, airway inflammation was heavily blunted.

Within the bone marrow CDP can give rise to pre-classical DCs (Pre-cDC) and plasmacytoid DCs (PDC) which can circulate within blood to peripheral tissues (Geissmann et al, 2010). Pre-cDCs can further give rise to classical dendritic cells and CD103+DCs in lamina propria of mucosal sites. CD103+DCs are crucial in the differentiation of intestinal Treg cells. Dietary fibre and SCFAs alongside retinoic acid are involved in the induction of CD103+DCs (Goverse et al, 2017). Promotion of such cells from bone marrow would encourage immune tolerogenic responses. MDPs on the other hand give rise to either Ly-6C+ or Ly6C- monocyte subsets within bone marrow. Both types enter the blood circulation to migrate to peripheral tissue. Ly6C+ monocytes are involved in tissue inflammation giving rise to inflammatory macrophages and DCs within tissue (Fogg, 2006). Ly6C- monocytes however can differentiate into alternative macrophages in the lung with anti-inflammatory and tissue repair capabilities (Landsman, Varol, & Jung, 2007).

Mice fed a high fibre diet exhibit altered bone marrow hematopoesis with enhanced Ly6c- monocytes (Trompette et al, 2018). This leads to a lung population of macrophages that produce less CXCL1, a chemokine involved in the recruitment of neutrophils, which reduce lung neutrophil infiltration (Sawant et al, 2016). These mechanisms extend to attenuating

influenza infection immune pathology. The high fibre diet also increased SCFAs butyrate, propionate, and acetate, with the greatest effect on butyrate. As such, similar responses in terms of bone marrow haematopoiesis and attenuation of influenza pathology were seen with butyrate and propionate feeding. It seems that bone marrow haematopoiesis is another potential mechanism in which prebiotic/dietary fibre may attenuate asthma by the production of SCFAs. Prebiotics/dietary fibre can induce DC and macrophage progenitors via SCFAs to create an airway cell milieu with a greater anti-inflammatory capacity.

In summary the production of the SCFAs: acetate, propionate, and butyrate from dietary prebiotic supplementation results in potent immunomodulatory effects which likely underpin some of the beneficial effects seen in asthma with prebiotic supplementation. SCFAs can induce immune modulation in the gut by inducing DCs to a more tolerogenic state leading to a large and more functional colonic Treg pool while reducing Th2 polarization. GPR41, GPR43, and GPR109a largely contribute to these effects in the gut. Treg cells may engage in gut-lung translocation when airway inflammation is present. Additionally, SCFAs are absorbed into blood circulation, albiet in low concentrations. Therefore, although immune modulation may still occur in the circulation, it would likely favour HDAC inhibition rather than GPR signalling. SCFAs produced in the gut are unlikely to reach the airways in substantial concentrations and therefore are unlikely to induce immune modulation of resident lung immune cells. This may occur, however, due to SCFAs produced by the lung microbiome, although this seems to induce unfavourable pro-inflammatory responses in asthma. Finally, SCFAs can induce bone marrow haematopoiesis of DC and macrophage precursors superseding the airways with anti-inflammatory DC and macrophage phenotypes. Together the immune modulation of immune cells at the gut, blood and bone marrow may translocation to the airways, creating a cell milieu possessing enhanced anti-inflammatory capabilities.

2.6.5. Direct prebiotic-GALT cross talk

Although much less well understood, the structural composition of prebiotics can affect gut immune cell modulation. It is becoming increasingly recognised that certain saccharides have direct effects on immune cells. These effects are independent of immune modulation induced by gut bacterial changes or metabolite production. Glucans, mannans, pectin polysaccharides, arabinogalactans, fucoidans, galactans, hyaluronans, fructans, xylans, and GOS have all been reported to induce direct immune modulation (Ferreira et al, 2015; Searle et al, 2012). This effect by B-GOS is of particular interest as this prebiotic attenuates markers of airway inflammation and HIB in people with asthma and HIB (Williams et al, 2016). B-GOS directly stimulated TNF-α and IL-6 production from macrophages in vitro. The low molecular weight fractions (e.g. GOS) of B-GOS and more specifically its tri (DP3) and ≥tetra-saccharide (DP ≥ 4) fractions seem to be important in this response (Searle et al, 2012). β-Glucan has a similar effect in macrophages (Lee et al, 2001) while human milk oligosaccharides have been shown to influence T cell activation and cytokine production (Eiwegger et al, 2004). DC maturation is unaffected by HMOs 6'-sialyllactose and 2'-fucosyllactose as well as the prebiotic GOS (Perdojk, van Neerven, van den Brink, Savelkoul, & Brugman, 2018). The chemical structure, molecular weight, conformation, functional groups, and branching seem to be features which contributes to the immune-stimulatory effects of saccharides (Ferreira et al, 2015). Whether this can have a positive influence in asthma is unknown. In fact the pro-inflammatory nature of these effects may be suspected to be detrimental, although this is not known. A theoretical idea is that certain saccharides may induce mild immune responses in the gut similar to those seen in the airways in asthma and as such trains the immune system to deal with these responses by enhancing tolergenic responses to these specific immune responses. In support, allergen ingestion in mice can attenuate airway responses to a challenge with the same allergen (Liu et al, 2013). Future research is required to investigate

the effect of direct immune modulation by saccharides to understand whether there is therapeutic potential. Additionally, a greater understand of the features of saccharides which contribute to direct immune modulation would be useful as this could allow saccharides to be engineered for specific immune modulating effects.

2.6.6. Bacterial translocation

Prebiotics can also inhibit gut bacterial translocation either indirectly through stimulating growth of beneficial bacteria or directly by interacting against pathogens. Probiotic bacteria are able to adhere to intestinal mucus in the lumen acting as a defence against pathogenic bacteria. Bifidobacterium and Lactobascillus species have been shown to do this as well as inhibit further pathogen adherence. Furthermore, these species can displace pathogens already adhered to gut mucus (Collado, Meriluoto, & Salminen, 2007). Most gut bacterial strains can translocate to mesenteric lymph nodes (MLNs) and the composition of gut bacteria parallels the population of translocated bacteria in MLNs (Steffen & Berg, 1983). Therefore, the promotion of probiotic species which inhibit pathogenic bacteria adhering to gut mucus and subsequent translocation will help to prevent pathogenic bacteria build up on IECs and in MLNs reducing their chance of inducing pro-inflammatory immune modulation and infection.

As well as inhibiting pathogenic bacterial adhesion, beneficial commensal bacteria can promote colonisation resistance (Buffie & Pamer, 2013). This is encouraged through improved host immune defence systems and by direct competitive exclusion either by competition for bacterial niches or by antimicrobial factors. As such prebiotics such as B-GOS and inulin reduce the incidence of travellers' diarrhoea (Drakoularakou, Tzortis, Rastall, & Gibson, 2010; Cummings, Macfarlane, & Englyst, 2001). Furthermore, SCFAs improve

intestinal barrier function protecting it against damage and promoting repair (Chen et al, 2017). When the intestinal barrier is compromised gut bacterial translocation is enhanced. The promotion of beneficial bacteria and SCFAs can help to prevent the translocation of pathogenic and/or pro-inflammatory immune modulating bacteria. Prebiotic which can do both therefore indirectly protect against pathogenic bacterial colonisation and translocation. Prebiotics can further directly inhibit bacterial adhesion to IECs. Certain oligosaccharides exert an agonistic effect by allowing pathogen binding and subsequent displacement from the GI tract (Monteagudo-Mera, Gibson, Charalampopoulos, & Chatzifragkou, 2019). HMO have this agonistic property (Licht, Ebersbach, & Frokiaer, 2012) while GOS, inulin, oligofructose, pectin-oligosaccharides and xylooligosaccharides have also been shown to inhibit pathogen adherence (Searle et al, 2009; Tzortis et al, 2005; Buddington, Kelly-Quagliana, Buddington, & Kimura, 2002; Ebersbach, Andersen, Bergstrom, Hutkins, & Litcht, 2012).

While there has been no links between these effects and the improvements in asthma with prebiotics so far this is an area which may come to emergence in the future as more research is conducted. Indeed, promoting anti-inflammatory immune modulation in the gut and MLNs by prebiotic seems to benefit asthma. Therefore, inhibiting bacteria which can induced pro-inflammatory immune modulation in the gut and MLNs is likely to also be beneficial. However, more work is required to establish whether this mechanism explains the positive influence of prebiotics in asthma.

2.6.7. Summary of prebiotic supplementation in asthma

The prebiotics B-GOS and inulin have been shown to be effective on asthma in humans, with B-GOS also drastically improving HIB (Williams et al, 2016; McLoughlin et al, 2019). These

prebiotics attenuate AHR and airway inflammation with the mechanisms alluding to GPR signalling and HDAC inhibition of which GPR41, GPR43, and HADC9 are suspected contributors. Mice models of asthma support the beneficially effect of prebiotics, especially GOS and resistant starch (Verheijden et al, 2016; Thorburn et al, 2015; Trompette et al, 2014). In these models increased Treg cell numbers and function are key features in attenuating AHR and airway inflammation. Moreover, altered bone marrow haematopoiesis of macrophages and DCs promote a more anti-inflammatory environment in the airways. GRP41 and GPR43 are implicated in these mechanisms, while other GPR-independent mechanisms such as HDAC inhibition are also involved.

The immune modulating effects of prebiotics are likely due to promotion of beneficial gut bacteria and the production SCFAs. Beneficial gut bacteria such as Bifidobacterium and *Lactobacillus* species can induce intestinal Treg cell development which have potential to supersede the airways. SCFAs produced by gut bacterial fermentation of prebiotics also increases the intestinal Treg pool and function. SCFA induce immune modulation through GRP41, GPR43, GPR109a, and HDAC inhibition. SCFAs are also absorbed into systemic circulation and can induce immune modulation in peripheral tissue. Alteration of bone marrow derived DC and macrophages in less inflammatory phenotypes is one example. Immune modulation by SCFA in the peripheral veins is likely to be GPR-independent due to the low concentrations in respect the EC50 of GPRs to SCFAs. Gut derived SCFAs are unlikely to be present in the airways and airway cells seem to respond in a pro-inflammatory manner to SCFAs. Furthermore, although less well linked to asthma, prebiotics exhibit direct immune-stimulatory characteristics and can inhibit pathogenic and pro-inflammatory promoting bacteria both directly and indirectly.

This highlights that the promotion of bacterial genera such as Bifidobacterium and Lactobacillus, alongside SCFAs production is fundamental to the beneficial immune

modulation which underpins the beneficial effects seen in mice models of asthma and human asthma after prebiotic supplementation. This therefore suggests that a tailored approach to selecting prebiotics which promote such bacteria and increases the production of SCFA are most likely to have therapeutic potential in human asthma. Future work may also want to engineer such prebiotics with these qualities in mind. The next section explores already existing candidate prebiotics with these qualities which may have the most therapeutic potential in asthma.

2.7. Bimuno-Galactooligosaccharide

Galactooligosaccharides (GOS) are a prebiotic derived from lactose compounds by enzymatic treatment with β-galactosidase (Torres, Goncalves, Teixeira, & Rodrigues, 2010). GOS was originally viewed as an undesirable side product during the hydrolysis of lactose, however, due to its structural similarities with human milk oligosaccharides, prebiotic qualities, and health promoting effects it is widely used in formula milk for infants, fortified into food products, and sold as commercial prebiotic supplements (Mozaffar, Nakanishi, & Matsuno, 1985; Fischer & Kleinschmidt, 2018). The stability of GOS is very high being stable in high temperatures and acidic conditions partly due to the presence of β-type linkages within its structure (Sangwan, Tomar, Singh, Singh, & Ali, 2011). In neutral pH, GOS remains unchanged at 160°C for up to 10 minutes, similarly remaining unchanged for 10 minutes at 120°C at a pH of 3 (Sangwan et al, 2011). This allows GOS to be fortified into many food products in which other prebiotics and certainly probiotics would degrade during such harsh manufacturing processes. The stability at low pH and high temperatures also gives GOS a long shelf life making them ideal for use in commercial products.

The synthesis of GOS relies on the hydrolysis of lactose through transgalactosylation by the enzyme, β-galactosidase (Torres et al, 2010). The source of lactose for GOS synthesis is usually whey-derived lactose although cow milk can be used. B-galactosidase is a hydrolase which attacks the o-glucosyl group of lactose with the transgalactosylic nature of β-galactosidase involving multiple subsequent reactions with disaccharides, forming GOS as an intermediate product (Wallenfels and Malhorta, 1960). This generally results in the formation of trisaccharides, 4' and 6' galactosyl-lactose (GOS-3), tetrasaccharide 6'-digalactosil-lactosa (GOS-4), longer oligosaccharides, transgalactosylated disaccharides, and non-reducing oligosaccharides (Fischer et al, 2018). The composition of GOS includes multiple galactose units and a terminal glucose residue linked by β-glycosidic bonds (Ganzle, 2012; Vera et al, 2016). A recent definition of GOS is as a mixture of substances produced from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose and disaccharides comprising 2 units of galactose (Tzortis and Vulevic, 2009). The prebiotic properties of GOS are partly attributed to the presence of GOS-3 and GOS-4 (Panesar, Panesar, Singh, Kennedy, & Kumar, 2006; Gopal, Ramsay, Sullivan, & Smart, 2001; Vera et al, 2016) however, the bifidogenic properties of GOS have additionally been attributed to the disaccharides containing β1-6 linkages (Rodriguez-Colinas, 2013: Rodriguez-Colinas, 2013). GOS synthesised from cow's milk shares structural similarities with HMOs and is a common ingredient in formula milk for infants to stimulate the growth of *Bifidobacteria* and *Lactobacilli* (Ben et al, 2008). Microorganisms are predominantly used as a source of β-galactosidase used in the production of GOS although yeast, fungus, and plant sources are also used (Saqib, Akram, Halim, & Tassaduq, 2017).

GOS is incredibly bifidogenic and supplementation in adults has been shown to increase Bifidobacterium and lactobacilli numbers in faeces (Ito et al, 1990; Tanaka et al, 1983). Seven days supplementation of 10g/d Oligomate-50, GOS synthesised using β-galactosidase originating from *Aspergillus oryzae,* results in a two-fold increase in faecal *Bifidobacteria* and *Lactobacilli* numbers while total bacterial numbers are unchanged. This highlights the selectivity of GOS to target bifidobacteria and lactobascillus without influencing the gut microbiota as a whole. This selectivity is important as GOS can beneficially alter bacterial groups linked to health benefits without inadvertently effecting groups with undesirable effects. Compared to fructooligosaccharide (FOS) and maltodextrin GOS is more bifidogenic and lactobacilli promoting (Rycroft, Jones, Gibson, & Rastall, 2001). Within a batch culture fermentation model using human faecal bacteria GOS causes the largest increase in total bacterial counts, bifidobacteria and lactobacilli numbers. GOS was also shown by Rycroft et al (2001) to have the greatest effect on increasing total SCFAs, and in the individual production of acetate, propionate, and lactate. GOS therefore not only increases the growth of beneficial bacterial but additionally increases the activity of the microbiota leading to greater SCFA production.

Even further bacterial selectivity following GOS supplementation can be increased by using the bacterium of interest as the source of β-galactosidase used to produce GOS. This approach considers the glycosidase specificity of the target bacterium for which the level of expression and pattern of linkage specificity is likely unique (Tzortzis et al, 2005). An oligosaccharide mixture with a structure specific to the target bacterium would lead to faster hydrolysis by the glycosidases of the target bacterium and thus lead to species level targeting. Rabiu, Jay, Gibson, & Rastall (2001) assessed the growth rates of Bifidobacterium species in response to GOS produced by β-galactosidase from Bifidobacterium anguatum, B.bifidum BB-12, B.adolescentis ANB-7, B.infantis DSM-20088, and B.pseudolongum DSM-20099

within a fermentation batch culture model. Interestingly, increased growth rate of bacterial species paralleled there homologues GOS mixture. This means that GOS produced by β-galactosidase originating from B.bifidum BB-12 for example resulted in the greatest increases in B.bifidum growth rates. This study showed that the selectivity in gut bacterial growth can be increased by carefully selecting the source of β-galactosidase. Additionally, Rabiu et al. (2001) found that GOS synthesised from β-galactosidase from B.bifidum had an overall prebiotic effect equivalent of Oligomate-55. These studies highlight that species level targeting can be achieved by producing a GOS mixture synthesised by β-galactosidase originating from the target bacterial species. This is of major importance for creating a prebiotic that could be used as a therapeutic target for asthma. Due to many Bifidobacterium species and SCFAs having immune modulating capacities resulting in immune tolerance which can be effective in reducing airway inflammation in asthma (please see section 2.6.3. for more detail) utilizing β-galactosidase from Bifidobacterium species could have therapeutic potential in asthma and HIB.

This effect has been utilised in the synthesis of B-GOS, a GOS mixture produced through transglycosylation using a β-galactosidase enzyme derived from whole cell Bifidobacterium bifidum NCIMB 41171 isolated from human faeces (Tzortzis et al, 2005). This process generates oligosaccharides with a degree of polymerisation ranging in chain lengths between 2 and 9 carbohydrates. The most common degrees of polymerisation (DP) in B-GOS are ≤ DP 4 (DP 2 = 52; DP 3 = 26; DP 4 = 14) and saccharide linkages of β1-3 (26), β1-4 (23), and β1-6 (51) (Vulevic et al, 2008; Depient et al, 2008; Grimaldi, Swann, Vulevic, Gibson, & Costabile, 2016) (Table 2.4). In batch culture experiments *B.bifidum* growth rates and SCFA production were significantly higher in the presence of B-GOS compared to glucose, fructose, maltose, cellobiose, raffinose, xylooligosaccharide, GOS, and inulin (Tzortzis et al,

2005). This showed B.bifidum growth rates and metabolic activity to be specific to a GOS substrate generated using β-galactosidase originating from *B.bifidum*.

Table 2.4: Composition of galacto-oligosaccharide mixture (B-GOS) including the degree of polymerisation (DP) and saccharide linkages, as percentage of the galacto-oligosacchairde content used in the studies by Vulevic, 2008, Depient, 2008, and Williams, 2016.

	B-GOS (*n*)
DP	
DP 2	52
DP 3	26
DP 4	14
DP 5	8
Linkages	
β1-3	26
β1-4	23
β1-6	51
Average molecular weight (kDa)	496·8
Galacto-oligosaccharide content (%)	48

In young adults and the elderly B-GOS supplementation has been shown to be incredibly bifidogenic (Depeint et al, 2008; Vulvec et al, 2008). This novel B-GOS mixture was studied in 59 healthy adults (Depeint et al, 2008). In a randomise, double-blind, cross-over design Depeint et al. (2008) assessed the effect of B-GOS supplementation on faecal bacterial compositions in 59 healthy adults. Participants supplemented with B-GOS (0, 3.6, or 7g/d) or V-GOS (7g/d) for 7-days with a 7-day wash-out between treatments. Bifidobacterium populations and prebiotic index (PI) scores was increased with 3.6g/d B-GOS with further

increases found by 7g/d highlighting a dose-dependent response. *B. bifidum, B infantis, B.longum,* and *B. animalis* were found to have significantly higher growth rates in the presence of B-GOS compared to placebo. Compared to V-GOS another commercially available GOS product, B-GOS significantly increased Bifidobacterium numbers with *B.bifidum* and *B.longum* showing higher growth rates. *B.bifidum* has been shown to induce Treg cell development and function in the gut by modulating DCs (Hart et al, 2004) and is therefore an important species to potentially stimulate the gut lung axis. B-GOS is therefore very bifidogenic compared to other commercially available GOS products and possesses high selectivity. This study highlighted the bifidogenic effects of B-GOS and species level targeting for the first time within humans.

B-GOS has also be assessed in the elderly who have reduced levels of Bifidobacterium. Vulevic et al (2008) assessed the effect of 10 week of 5.5g/d supplementation of B-GOS on faecal bacteria composition and immune function. The study was a randomised double-blind placebo controlled cross-over design with participants completing a 10-week B-GOS and placebo trial with a 4-week wash-out period in between. B-GOS supplementation increased *Bifidobacterium* spp., *Lactobacillus-Enterococcus* spp., and the *C.coccoides–E.rectale* group compared to baseline and placebo. B-GOS additionally decreased *Bacteroides* spp., the *C. histolyticum* group, *E. coli*, and *Desulfovibrio* spp. This effect was even greater at 10 weeks compared to 5 weeks showing the benefit of chronic supplementation although this is likely to occur faster in a younger population were higher baseline Bifidobacterium numbers would be present. This is because richer microbiotas exhibit greater resistance to change upon increased dietary fibre intake (Tap et al, 2015). While composition changes were evident following B-GOS supplementation there was no change in total bacterial counts. Immune function was also enhanced including the phagocytosis and activity of natural killer cells. Additionally, B-GOS supplementation resulted in a higher concentration of the anti-

inflammatory cytokine IL-10 and a reduction in the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in blood plasma.

The effect of B-GOS on attenuate systemic inflammation was shown in participants with asthma and HIB (Williams et al, 2016). EVH test induced increase in TNF-α were abolished following 21 days B-GOS supplementation and resting CCL17 and CRP were significantly reduced compared to placebo. This corresponded with a 40% reduction of the peak fall in FEV_1 post EVH test showing B-GOS could attenuate markers of airway inflammation and airway hyper-responsiveness. Previous studies involving inulin ingestion in asthma participants have resulted in significant reductions in sputum percentage eosinophils, sputum lymphocytes count, total sputum cell counts, and sputum IL-8 concentrations compared to placebo treatments (McLoughlin et al, 2019; Halnes et al, 2017). The effect of B-GOS on airway inflammation has not been investigated previously, given its superior bifidogenic and fermentation properties compared to inulin (Tzortzis et al, 2005) it is likely to have a strong influence of the gut-lung axis. As such, the effect of B-GOS supplementation on airway inflammation in participants with asthma and HIB needs investigating.

The synthesis of B-GOS results in a mixture containing monosaccharides including free glucose and galactose, and lactose alongside GOS. These monosaccharides are undesirable within the B-GOS mixture as they are absorbed in the small intestine and therefore possess no prebiotic properties. The removal of these monosaccharide by purification methods can be used to produce B-GOS with a higher GOS content. Purification of B-GOS is achieved by fermenting the monosaccharides in the product with *S. cerevisiae.* In this process the monosaccharides are removed by S. *cerevisiae* through anaerobic glycolysis, converting monosaccharides into ethanol and CO_2 (Goulas, Tzortis, & Gibson, 2007). This process removes 92% of glucose and ~4% galactose whilst not affecting the numbers of degrees of polymerisation within GOS (Goulas et al, 2007). This process allowed Grimaldi et al (2016)

to assess the effect of a B-GOS mixture containing 65% GOS compared to a mixture containing 52% GOS. Within an anaerobic batch culture model 65% B-GOS resulted in a positive modulation of bacterial composition including increases in lactobacilli, and significantly increased SCFAs with the most pronounced effect on acetate. Compared to 52% B-GOS however, there was no difference in bacterial composition changes or SCFA production between the two substrates. The current (as of the year 2019) commercially available B-GOS mixture in dry powder form has an 80% GOS content (Table 2.5. The 80% B-GOS has been assessed in children with autistic spectrum disorder with 1.8g/d supplementation for six weeks associated with faecal *Bifidobacterium* spp., *Ruminococcus* spp., Lachnospiraceae family (*Coprococcus* spp., *Dorea formicigenerans*, *Oribacterium* spp.), *Eubacterium dolchum*, TM7–3 family and Mogibacteriaceae (Grimaldi et al, 2018). It is noteworthy that the gut microbiome of autistic children differs from non-autistic controls, being less diverse and exhibiting lower levels of Bifidobacterium (Li, Han, Dy, & Hagerman, 2017). Changes in gut Bifidobacterium number in response to prebiotic supplementation is confounded by participant's baseline levels with participants with lower baseline levels of Bifidobacterium exhibiting greater change (Kolida, Meyer, & Gibson, 2007; De Preter et al, 2008). Due to the lower levels of Bifidobacterium in children with autism the change seen in this population with 80% B-GOS cannot be extrapolated to a non-autistic adult population. Unfortunately, no data exists for the effect of 80% B-GOS supplementation on the gut microbiota in healthy adults, however, given that GOS in 80% B-GOS is synthesised in the same way as 48% B-GOS (Vulevic et al, 2008; Depient et al, 2008) it would be expected to produce equivocal or greater positive changes in gut microbial composition and metabolite production.

Table 2.5: Content of commercially available Bimuno-galactooligosaccharide (Bimuno daily) mixture in dry powder form as available in the year 2019.

B-GOS	Per 100g	Per sachet (3.65g)
Galactooligosaccharides (g)	79.7	2.9
Lactose (g)	11.3	0.4
Glucose (g)	6	0.2
Galactose (g)	0.5	0.02
Water/moisture (g)	4.5	0.2

Grams of each constitute in the per sachet (3.65g) column are rounded to 1 decimal place resulting in sum greater than 3.65g.

In conclusion, B-GOS exhibits prebiotic qualities that suggest it may have therapeutic potential in asthma and HIB. It possesses significant bifidogenic properties and additionally increases the production SCFAs. Both these changes have been implicated in inducing immune modulation that can potentially reduce airway inflammation. The one study assessing the effect of B-GOS in individuals with asthma and HIB reports that B-GOS can attenuate HIB by 40% and reduce systemic markers of airway inflammation (Williams et al, 2016). Due to B-GOS's prebiotic qualities that seem favourable for attenuating airway inflammation and the findings of Williams et al (2016) more research is warranted to establish the effect B-GOS on asthma and HIB, in particular the effects on airway inflammation.

2.8. Methods of measuring airway inflammation

To evaluate the effect of B-GOS supplementation on airway inflammation in asthma and HIB a method is required that allows for measurements to be taken during bronchoconstriction

post an EVH test. Multiple methods of measuring airway inflammation exist that have been implemented in patients with asthma including but not limited to bronchoscopy, sputum induction and FeNO. Bronchoscopy is an invasive technique in which a bronchoscope is inserted into the airways (Busse et al, 2005). Bronchoscopies can be highly informative as it allows an inside view of the lungs and allows for the collection of bronchoalveolar lavage fluid and airway biopsies (Busse et al, 2005). The technique can also allow samples to be taken from different regions of the respiratory tree. While highly informative the invasive nature of the technique is unsafe during HIB and following an EVH test when bronchoconstriction is present. In addition, due to safety reasons the technique needs to be performed by a well-trained medical practitioner which prevents it use outside a hospital setting and by those without the appropriate training (Busse et al, 2005).

Sputum induction is a relatively non-invasive method of sampling the airways which is conducted through the inhalation of a nebulised saline solution (isotonic or hypertonic) followed by coughing to bring up airway secretions (Weiszhar & Horvath, 2013). Differential cell counts of sputum samples is commonly used to phenotype asthma patients while sputum can also be used to measure inflammatory cytokines and reflect the lung microbiome (Weiszhar & Horvath, 2013; Marri, Stern, Wright, Billheimer & Martinez, 2013). While much less invasive than a bronchoscopy, sputum induction can provoke bronchoconstriction (Weiszhar & Horvath, 2013). This makes sputum induction unsafe following an EVH test or during HIB and therefore limits it ability to be used to measure airway inflammation during HIB and post an EVH test. Furthermore, the method can be time consuming, and requires sample processing shortly after induction (<2 hrs).

FeNO is a non-invasive measure of airway inflammation that is measured using a portable device. FeNO is easy to measure simply requiring a patient to exhale at a slow and constant rate through a FeNO device. While NO is present in everyone's lungs a greater production of

NO is measured in patients with eosinophilic asthma (Bjermer et al, 2014). FeNO is useful in the diagnosis of eosinophilic asthma, predicting ICS responsiveness in asthma patients and assessing whether ICS therapies requiring stepping up or down based upon elevated or reduced FeNO measures (Bjermer et al, 2014; Menzies-Gow, Mansur, & Brightling, 2020). While FeNO is non-invasive and easy to implement it is limited to reflecting eosinophilic airway inflammation and may lack usefulness if measuring airway inflammation in patients without this inflammatory phenotype. Furthermore, FeNO is reduced following exercise and spirometry in asthma patients potentially due to changes in airway calibre (Bjermer et al, 2014). This limits the utility of FeNO in assessing HIB, were FeNO values will be influenced by hyperpnoea, bronchoconstriction, and the frequent spirometry maneuverers required in the assessment of HIB.

Exhaled breath condensate (EBC) is another sample which can be collected non-invasively and may have potential for use in HIB research. EBC is an airway sample consisting of condensed water vapour with a small fraction of respiratory lining fluid droplets (Horvath et al, 2005). These respiratory droplets contain non-volatile molecules and biomarkers of airway inflammation. EBC samples are collected by directing an individual expired air across a cold surface, which results in surface condensation (Horvath et al, 2017). The EBC sample then be analysed through a variety of biochemistry techniques to measure inflammatory biomarkers. EBC has been utilised in EIB/HIB with significant increases in Cyst-LTs, RANTES, Endothelin-1 and 8-isoprostane concentrations and decreased EBC pH measured post compared to pre-exercise/EVH tests (Zietkowski et al, 2010; Zietkowski et al, 2007; Bikov et al, 2014; Tecklenburg-Lund et al, 2010; Mickleborough et al, 2013). A commercial EBC collection device called the The Respiratory Tube (RTube) is available and has the benefit of being cheap and portable in comparison to other devices (e.g. EcoScreen & Turbo-Dec). This is beneficial for use in EIB/HIB where frequent sampling is required, and field testing is

common. The RTube may therefore be a useful device to collect EBC to measure airway inflammation in individuals with asthma and HIB. There is however limit data available on the measurement of inflammatory cytokines in EBC collected with the RTube device. Due to the importance of inflammatory cytokines in the pathophysiology of asthma and HIB the efficacy of measuring inflammatory cytokines in EBC collected with the RTube device requires investigation. If effective the RTube would be a useful device to assess the effect of B-GOS supplementation on airway inflammation in individuals with asthma and HIB.

2.9. General summary and thesis aims

Asthma effects approximately 350,000,000 people worldwide and 5,300,000 people in the UK (GINA Report, 2020; Masoli et al, 2004). Approximately 50% of asthma patients have hyperpnoea-induced bronchoconstriction (HIB), defined by a transient narrowing of the airway following hyperpnoea (Sano et al, 1998; Lucia et al, 1999). In addition, a significant proportion of the general population without underlying asthma suffer from HIB (~10%) and in athletes the prevalence of HIB is exceptionally high (~35%) (Aguiar et al, 2018; Molphy et al, 2013; Dickinson et al, 2011; Parson et al, 2007; Burnett et al, 2016). Prevalence in athletes varies greatly between sports however this is often confounded by low cohort numbers and the use of different diagnostic criteria. HIB is underdiagnosed and undertreated partly due to the lack of association between respiratory symptoms and HIB (Dickinson et al, 2011). This lack of associations however has been assessed retrospectively. Removing the effect of memory and assessing respiratory symptoms *in situ* when the presence of bronchoconstriction can be documented may allow for an association to be established (Conner and Barret 2012). Furthermore, there is very few studies addressing any differences in the prevalence of HIB between male and female athletes (Parsons et al, 2007; Wilber et al,

2000). Therefore, the burden of HIB in individual sports and sexes requires further investigation.

Both asthma and HIB share similar pathophysiological features such as airway hyper-responsiveness (AHR) and airway inflammation that lead to limitations in expiratory airflow (Weiler et al, 2016). Many pharmacological treatments are available for the treatment of asthma and HIB with the most common prescribed treatments being inhaled corticosteroids (ICS) and β_2-agonists. These medications, however, are not curative, can induce tolerance leading to an increased risk of exacerbations and can cause undesirable side effects (Billington, 2017; Barnes, 2010). The search for novel non-pharmacological treatments that may improve asthma and HIB control and reduce the reliance on pharmacological therapies is warranted. The gut microbiota has long been linked with the host immune system and could affect immune responses at distal sites including the lung, a link defined as the gut-lung axis (Koh et al, 2016). Healthy gut bacteria and the metabolites they produce have been shown to influence immune maturation in such a way that can enhance immune regulation and dampen inflammation in the gut but also at distal sites such as the lung (Dang et al, 2019; Budden et al, 2017).

Dietary prebiotics defined as: ‘a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring a benefit upon host health’ (Gibson et al, 2010), are a method to beneficial influence the gut microbiota in a way that may affect the gut-lung axis. The prebiotic, Bimuno-galactooligossacharide (B-GOS), is renowned for inducing positive changes in gut bacterial composition and the production of short-chain fatty acids and is exceptional bifidogenic in comparison to other prebiotics (Tzortis et al, 2005; Depeint et al, 2008; Vulvec et al, 2008). In patients with asthma and HIB 21-days B-GOS supplementation has been shown to attenuate HIB by an astonishing 40% and attenuate systemic markers of airway inflammation

(Williams et al, 2016). The effects of B-GOS in individuals with asthma and HIB warrants further investigations, especially the effects of B-GOS on airway inflammation. Novel methods in measuring airway inflammation are required in HIB as common methods such as bronchoscopies, induced sputum and FeNO are not practical during the assessment of HIB. A sample called exhaled breath condensate (EBC) can be collected non-invasively using a device named the RTube. This method, if effective, would allow for the assessment of B-GOS supplementation on airway inflammation in participants with HIB and therefore needs investigating.

In relation to the gaps in the literature highlighted above this thesis aims to investigate the following research questions:

I. What is the prevalence of hyperpnoea-induced bronchoconstriction in field hockey athletes?

This research aimed to:

- Determine the prevalence of HIB in British University field hockey athletes and determine sex differences in HIB prevalence.
- Evaluate the associations between symptoms of dyspnoea and HIB assess the effect of different HIB diagnostics criteria on prevalence rates.

II. Can cytokines be measured in exhaled breath condensate collected with the RTube device from participants with asthma and hyperpnoea-induced bronchoconstriction?

This research aimed to:

- Determine whether the RTube device can collect exhaled breath condensate from participants with asthma and HIB in which cytokines can be measured.
- If effective this will allow for airway inflammation to be measured in response to B-GOS supplementation in participants with asthma and HIB.

III. What is the effect of 21-days supplementation with the prebiotic, Bimuno-galactooligosaccharide (B-GOS), on HIB severity, asthma control and airway inflammation?

This research aimed to:

- Determine whether B-GOS supplementation attenuates HIB severity and improves asthma controls.
- Assess whether B-GOS supplementation attenuates markers of airway inflammation in EBC.

Chapter 3 – General methods

3.1. Participants

Participants were recruited through adverts placed in Nottingham Trent University, local park run events and on local sports club's social media pages. All participants provided written informed consent to take part in each experimental study which were approved by the Nottingham Trent University Human Research Ethics Committee. Following informed consent each participant completed a self-reported health screen questionnaire to confirm participants met the inclusion and exclusion criteria. This was followed by anthropometric measurements (section 3.2. Anthropometric measurements). In the lead up to trails participants were asked to adhere to the restrictions outlined in section 3.3. In the lead up to experimental chapter 7 participants completed a 24-hour food diary prior to their first experimental visit which was then replicated for all subsequent visits.

3.2. Anthropometric measurements

Participants body mass was measured to the nearest 0.1 kg using calibrated electronic scales (SECA 877 Scale, SECA, Birmingham, UK) whilst wearing lightweight clothing and barefoot. Participant's height was measure with a portable stadiometer (SECA stadiometer, SECA, Birmingham, UK). Participants stood bare foot with heels together, arms by their side and in the Frankfurt plane. Participants were then instructed to take a maximal inhalation priort to the measurement (Eston & Reilly, 2013).

3.3. Participant restrictions prior to experimental trials

In the lead up to trials involving pulmonary function and EVH tests participants using asthma medication were asked to cease the use of medication for the durations outlined in Table 3.1

(Anderson & Kippelen, 2013; Weiler et al, 2016; Hull, Ansley, Price, Dickinson, & Bonini, 2016). All participants were additionally instructed not to partake in strenuous exercise for 24 hours prior to trials to prevent a refractory period which may interfere with the EVH test response (Argyros, Roach, Hurwitz, Eliasson, & Phillips, 1995), consume alcohol for 24 hours, or ingest caffeine for 8 hours prior to all trials as it is show attenuate the fall in FEV_1 following the EVH test (Duffy & Phillips, 1991).

Table 3.1: Medication restrictions for asthma and hyperpnoea-induced bronchoconstriction participants in the lead up to trials involving pulmonary function and the eucapnic voluntary hyperpnoea test. (Anderson & Kippelen, 2013; Weiler et al, 2016; Hull et al, 2016).

Medication	Time restriction (hours)
Short-acting β_2-agonists	8
Long-acting β_2-agonists	24
Inhaled corticosteroids	12
Inhaled corticosteroids + Long-acting β_2-agonists	24
Leukotriene receptor antagonists/modifiers	96
Leukotriene synthesis inhibitors	Standard = 12 Slow release = 16
Short-acting muscarinic antagonists	12
Long-acting muscarinic antagonists	72
Antihistamines	72

3.4. Pulmonary function – Dynamic spirometry

Pulmonary function was conducted in accordance with the ATS/ERS guidelines (Miller et al, 2005) using a spirometer (Pneumotrac; Vitalograph, Buckingham, UK) calibrated with a 3L syringe. Participants performed pulmonary function manoeuvres in a standing upright position whilst wearing a nose clip. Participants performed maximal flow-volume loops to

determine absolute and percentage predicted forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), peak expiratory flow rate (PEF), forced expiratory flow rate from 25-75% of FVC ($FEF_{25-75\%}$), and FEV_1/FVC ratios. Before manoeuvres participants were instructed to inhale and 'fill their lungs' maximally, place their mouth around the mouthpiece and expire and 'empty their lungs' as quickly as possible for seven seconds (or until a plateau in expired volume was observed). At seven seconds participants were instructed to rapidly inhale to full inspiration. During familiarisation visits participants were given a demonstration prior to practising manoeuvres. During experimental trials participants performed a minimum of three flow-volume loops for baseline pulmonary function measures. The two highest values for the measure of FEV_1 had to be within 0.150 L with the highest values taken for subsequent analysis. If participants could not achieve the reproducibility criteria within three manoeuvres or their final manoeuvres was their best, they performed further baseline manoeuvres up to a maximum of eight manoeuvres to prevent fatigue. Post EVH test participants performed flow-volume loops in duplicate with a reproducibility of 0.150L. The higher of the two values at each post EVH time interval was accepted for further analysis.

3.5. Eucapnic voluntary hyperpnoea (EVH) test

The EVH test was originally designed and validated by the US army as a surrogate to exercise testing to identify EIB (Eliasson et al, 1992; Hurwitz et al, 1995). It was subsequently adopted for identifying EIB in elite athletes and became the recommended test by the International Olympic Committee Medical Commission for confirming EIB in athletes. EVH tests are frequently used to identify EIB in athletes (Dickinson et al, 2011; Burnett et al, 2016), and has been used previously to assess the effect of dietary interventions

on hyperpnoea-induced bronchoconstriction (HIB) and EIB (Williams et al, 2016; Mickleborough et al, 2003).

The EVH test is used in experimental chapters 4, 5, and 7. The EVH test comprised 6-min of breathing at a target minute ventilation ($\dot{V}_E$) of 85% of predicted maximal voluntary ventilation (MVV) (baseline $FEV_1 \times 30$) (Anderson et al, 2001). Participants breathed through a mouthpiece connected to a two-way valve (model 2730; Hans Rudolph, Kansas City, MO) with the inspiratory port connected via corrugated tubing to a Douglas bag filled with a dry gas mixture (<2% humidity; 21% O_2, 5% CO_2, balance N_2, 21°C). The expiratory port was connected via corrugated tubing to a dry gas meter (Harvard Apparatus, Cambridge, UK) for determination of $\dot{V}_E$ (Figure 3.1). Participants received frequent coaching and verbal encouragement to achieve their target $\dot{V}_E$. An EVH test was considered valid if the mean $\dot{V}_E$ was ≥60% of predicted MVV (baseline $FEV_1 \times 21$), or a participant was HIB-positive despite $\dot{V}_E$ being <60% of predicted MVV. Pulmonary function was assessed in duplicate at 3, 6, 10, 15, 20, and 30 minutes after the EVH test, with the highest values at each time point used for subsequent analysis (Anderson et al, 2001). HIB was identified when the forced expiratory volume in 1-s (FEV_1) falls, relative to baseline, by ≥10% at two consecutive time points after EVH in accordance with the ATS guidelines (FI_{ATS}) (Parsons et al, 2013; Weiler et al, 2016). HIB was characterised as mild (≥10% - <30%), moderate (≥30% - <50%), and severe (≥50%). Diagnostics cut-offs of 13%, 15%, and 20% have been used to identify EIB and HIB previously (Weiler et al, 2016). The lower limit reference range for the fall in FEV_1 following an exercise challenge test in healthy controls is 7% while a fall in FEV_1 of 11% has been shown to be 100% specific in identifying subjects with EIB (Rundell et al, 2000; Eliasson et al, 1992). This suggests a ≥10% fall in FEV_1 is a clinically significant response to an EVH test in accordance with FI_{ATS} (Hurwitz et al, 1995). The smallest meaningful change for the fall in FEV_1 over 21 days is 164 mL (Williams et al, 2015). The ATS criterion was used to

identify HIB in chapters 4, 5, and 7, with additional criteria used in chapter 4 which are outlined in chapter 4.

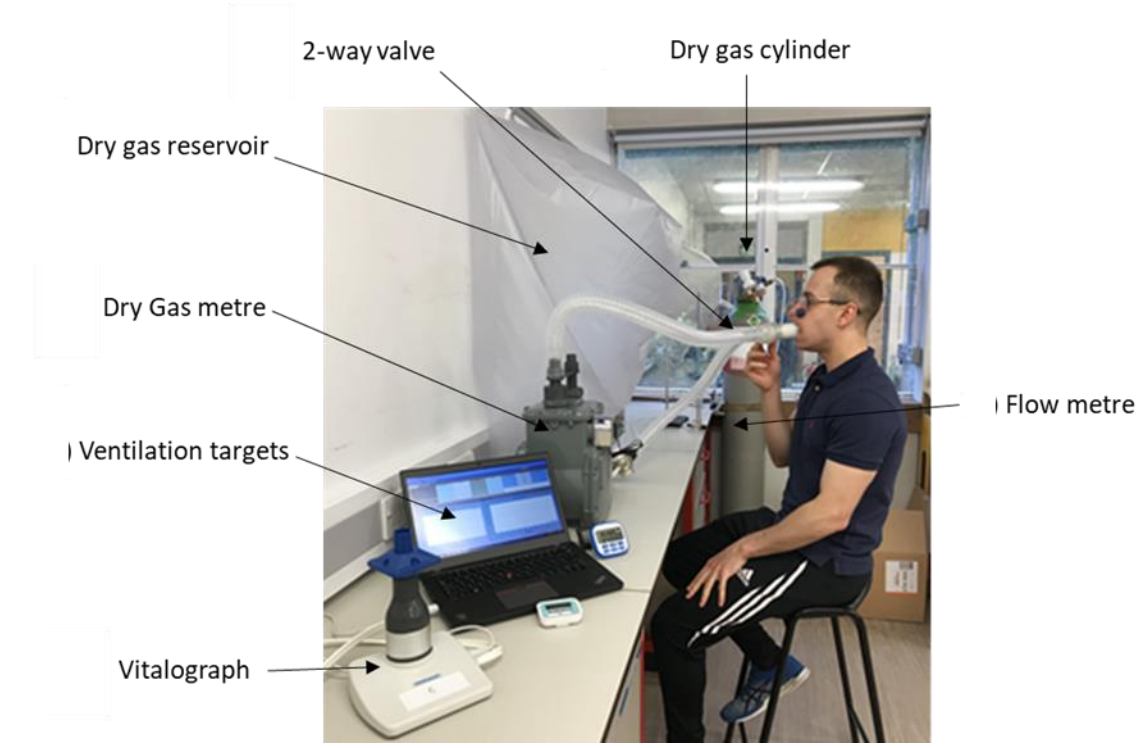

Figure 3.1: Eucapnic voluntary hyperpnoea set-up.

3.6. Exhaled breath condensate collection

Exhaled breath condensate was collected in accordance with the ERS guidelines (Horvath, Hunt, & Barnes, 2005; Horvath et al, 2017) using the RTube device (Respriatory Tube, COSMED, Oxfordshire, United Kingdom) (Figure 3.2 & Figure 3.3). Participants breathed into the RTube mouthpiece for 10 minutes whilst wearing a nose clip. The aluminium cooling sleeve was pre-cooled to -20°C and placed over the condensing chamber prior to sample collection. Following sample collection, the condensing chamber is removed and placed over an aluminium plunger which forces the salivary trap up and pools the condensate. The sample is then pipetted and aliquots for storage or immediate analysis (Figure 3.2). For the

measurement of EBC pH 400µl of EBC was pipetted into an Eppendorf and measured immediately using a using a Jenway 3510 pH meter. The remaining sample was stored at -80°C until subsequent analysis (for more information please see section 3.8. Analysis of inflammatory biomarkers**)**.

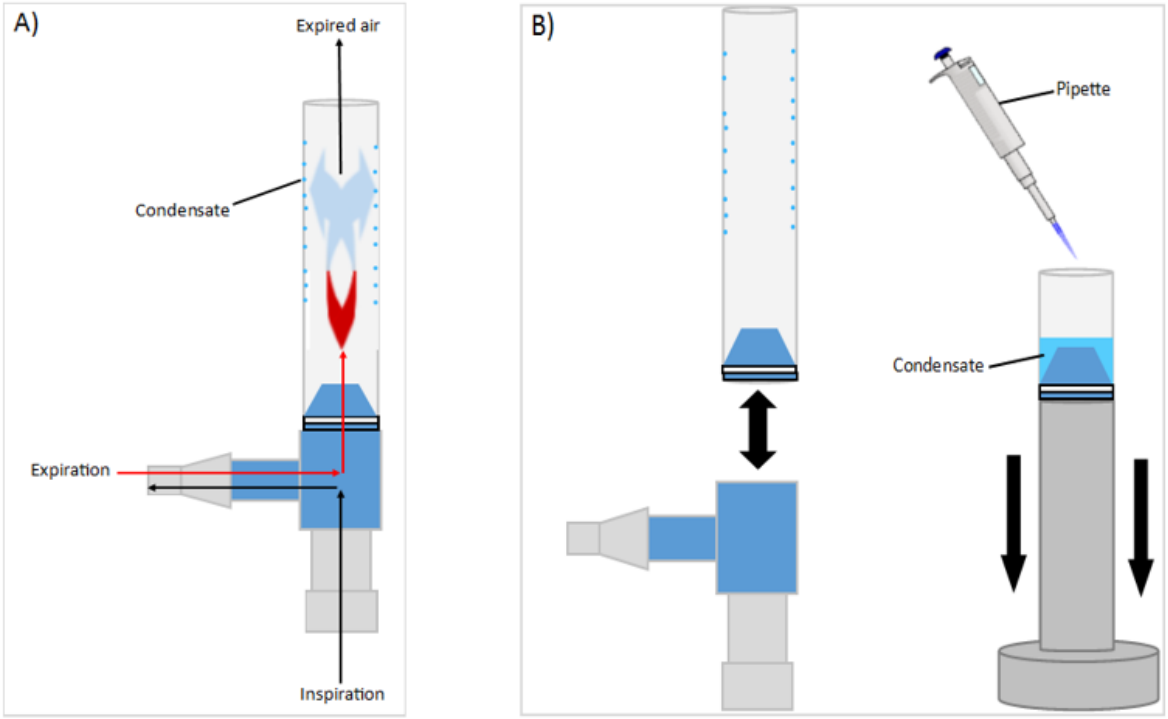

Figure 3.2: Participants expired air passes through the RTubes condensing chamber and condenses as it makes contact with the pre-cooled surface (A); after sample collection the condensing chamber is removed and placed over an aluminium plunger which forces the salivary trap up and pools the condensate. The sample is then pipetted and aliquots for storage or immediate analysis (B).

3.6.1. Temperature changes during EBC collection with the RTube device

Rapid increases from -20°C to 20°C have been reported with a standard 10-minute EBC sample collection using the RTube device (Soyer, Dizdar, Keskin, Lilly, & Kalayci, 2006). Assuming a standard laboratory air humidity of 40%, expired air at a temperature of 35°C has a dew point of approximately 13°C. This suggests that during large parts of a 10-minute collection the RTube temperature rises above the dew point to allow for surface condensation of expired air. Soyer et al (2006) however, measured temperature inside the condensing chamber in direct flow of participants expired air. The temperature rises they measured are therefore likely from their participants expired air. As surface condensation requires air to make contact with a cool surface (e.g. the RTubes condenser wall) it is the condenser surface temperature which is important in converting expired air into liquid condensate. As such we conducted a small pilot study assessing the temperature rises of the RTubes condensing chamber wall. Participants (n = 3) breathed into the RTube device (tidal breathing) for 10 minutes. A temperature monitor was attached to the condensing chamber wall to measure temperature throughout sample collection (Figure 3.3). Temperature data were analysed using a one-way repeated measures ANOVA and Bonferroni-adjusted paired T tests. Statistical significance was set at $p < 0.05$. Data were analysed using IBM SPSS Statistics V26.0 and presented as mean ± SD.

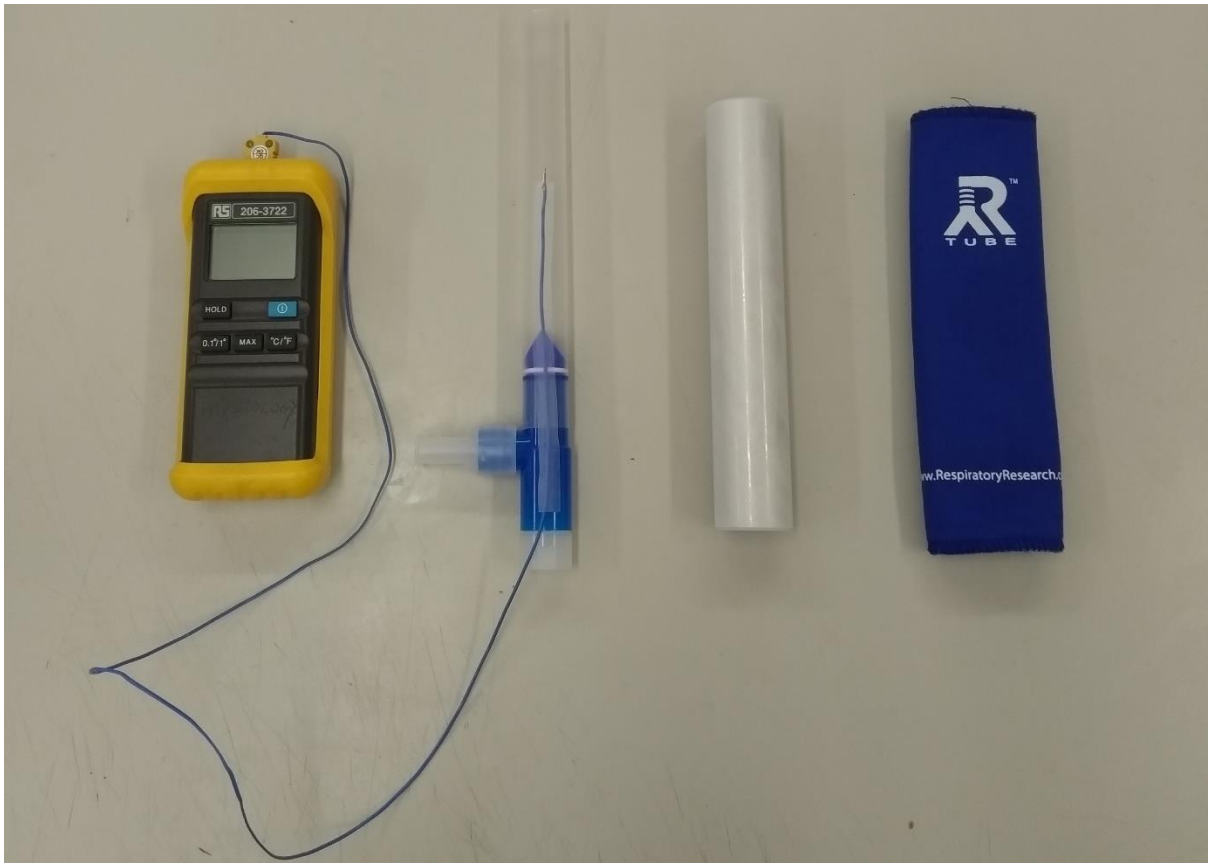

Figure 3.3: Set-up to measure the temperature of the RTubes condensing chamber. From left to right temperature monitor, RTube device, aluminium cooling sleeve, thermal sleeve. The temperature monitor was attached to the RTubes condensing chamber prior to sample collection. The aluminium cooling sleeve was cooled to -20°C prior to sample collection and place over the RTubes condensing chamber with the thermal sleeve placed over the aluminium cooling sleeve.

The surface temperature of the condensing chamber significantly increased over time (p = 0.003) (Figure 3.4). Temperature was significantly higher at 8 minutes (3.8 ± 0.6°C; p = 0.035), 9 minutes (4.8 ± 1.3°C; p = 0.015), and 10 minutes (6.6 ± 1.3°C; p = 0.015) compared to the start of sample collection (0 minutes: -20°C) (Figure 3.4).

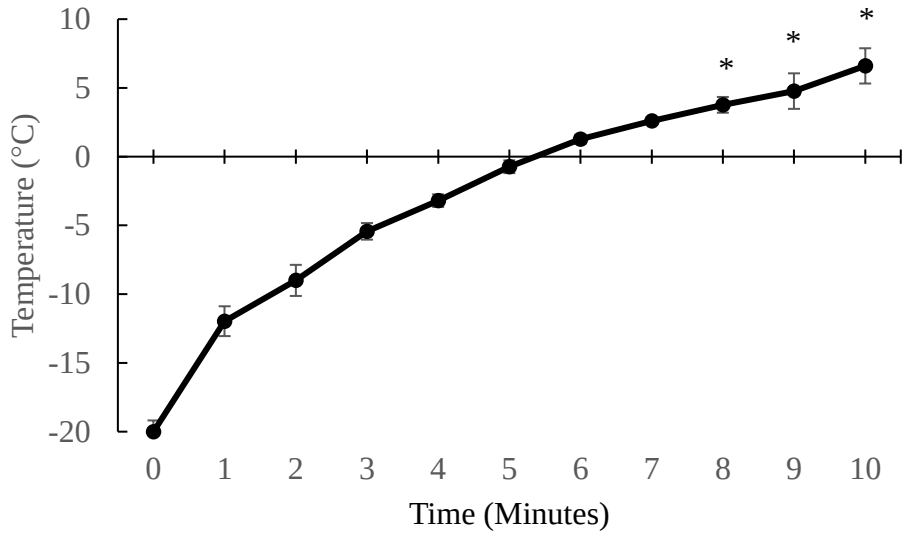

Figure 3.4: RTube condensing chamber surface temperature during a 10 minute exhaled breath condensate collection. (n = 3). *Significantly higher temperature compared to the start of sample collection (0 minutes) ($p < 0.05$).

While temperature increases are evident these are not as drastic as previously reported by Soyer et al (2006). Additionally, temperature remains below the dew point (13°C) in standard laboratory conditions throughout sample collection ensuring that EBC is being formed and collected during the whole duration of a 10-minute sample collection.

3.7. Blood sampling

Venous blood was drawn from a vein in the antecubital fossa region of the elbow using a 23-gauge butterfly needle (BD Vacutainer Safety Lok Blood Set 23g x 7" Tubing, Plymouth, UK). In chapter 5, 5mL of blood was drawn into a 5ml Ethylenediaminetetraacetic acid (EDTA) vacutainer (BD, Plymouth, UK) for the measurements of differential white blood cell (WBC) counts using a XS-1000i haematology analyser at rest. In chapter 7 during each experimental visit 15mL of blood was drawn pre and 30 minutes post EVH test. At each time point 5mL was drawn into a EDTA vacutainer for the measure of WBC counts, 5mL was

drawn into a lithium heparin vacutainer (BD, Plymouth, UK) for the measure of chemokine/cytokines in blood plasma, and 5mL into a serum separating tube (BD, Plymouth, UK) for the measure of chemokines/cytokines in blood serum. Immediately after sample collection vacutainers were inverted 8-times to ensure the sample had mixed with the vacutainers constitutes prior to subsequent steps. Lithium heparin vacutainers were immediately centrifuged for 15 minutes at 1,500g, the plasma layer was aliquoted and subsequently stored at -80°C until analysis. Serum separating tubes were left to stand for 30 minutes at room temperature and then centrifuged for 10 minutes at 2,000g, the serum layer was aliquoted and subsequently stored at -80°C until analysis. After blood sampling the butterfly needle was removed, and firm pressure was applied upon the puncture site to avoid haematoma.

3.8. Analysis of inflammatory biomarkers

3.8.1. Differential white blood cell (WBC) counts

Differential WBC counts were analysed using an automated point of care test haematology analyser (XS-1000i Haematology analyser). Differential WBC counts and percentages were presented for blood eosinophils, neutrophils, lymphocytes, monocytes, and basophils. Differential WBC counts were measured during familiarisation in experimental chapter 4 to characterise participants and experimental chapter 7 during day 0 and day 21 of the supplementation period, pre and post EVH test.

3.8.2. Enzyme-linked immunosorbent assays

Commercially available enzyme linked immune-sorbent assays (ELISAs) were used to determine concentrations of cytokines and eicosanoids from human blood serum, blood plasma, urine, and EBC samples. In experimental chapter 5 ELISAs were used to try and quantify IL-13 (Abcam, UK) and TSLP (Abcam, UK) in EBC samples. In experimental chapter 7 ELISAs were used to determine the concentration of Cysteine Leukotrienes (Cayman Chemicals, UK), 9α, 11β-PGF_2 (Cayman Chemicals, UK), and club cell 16 (Biovendor, UK) in urine samples and lipopolysaccharide binding protein (Hycult Biotech, Netherlands) in blood plasma samples. All ELISAs were performed under the manufacturer's instructions. The general protocol for determining cytokines and eicosanoids is outlined in Figure 3.5. Samples, reagents, and standards were prepared using the appropriate dilutions as instructed by the manufacturer. The standards and samples were introduced into each well containing the capture antibody and incubated at room temperature allowing for the antigen to bind with the capture antibody. The plate was then washed with wash buffer before adding the detection antibody. A further wash step was followed by adding the enzyme labelled antibody. The plate was then washed again before adding the substrate solution. Finally, the stop solution was added, and the plate was read immediately on a plate reader.

3.8.3. Bioplex multiplex assays

Commercially available bioplex multiplex assays (Bioplex, Bio-rad, UK) were used to determine the concentration of cytokines and chemokines in human blood serum and EBC. In experimental chapter 6 bioplex multiplex assays were used to determine the concentration of IL-5 and Tumour necrosis factor-alpha (TNF-α) in EBC. In experimental chapter 7 bioplex multiplex assays were used to determine the concentrations of eotaxin, monocyte

chemoattractant protein-1 (MCP-1), Regulated on Activation, Normal T Cell Expressed and Secreted/chemokine ligand 5 (RANTES/CCL5), TNF-α, and stem cell factors (SCF) in blood serum. Samples, capture beads, reagents and standards were prepared using the appropriate dilutions as instructed by the manufacturer. Initially the capture beads were dispensed into each well followed by the addition of standards and samples. The plate was incubated at room temperature allowing for antigens to attach to the capture beads. The plate was subsequently washed using a Bioplex Pro^{TM} Wash Station (Bioplex, Bio-rad, UK). Next the biotinylated detection antibody was added to each well and incubated at room temperature. The plate was subsequently washed before adding the streptavidin-PE reporter dye and incubated at room temperature. The plate was washed a final time before the beads were re-suspended and fluorescent sorting was performed using the Bioplex 200 Systems (Bioplex, Bio-rad, UK).

3.9. Self-reporting questionnaires

3.9.1. Multi-dimensional dyspnoea profile (MDP)

The MDP was devised by Banzett et al (2015) as a clinical and laboratory research tool to assess dyspnoea during a specific time or an activity and is designed to assess individual items and their relations to the time/activity investigated (Banzett, 2015). The MDP consists of 11 items evaluating sensory and affective dimensions of dyspnoea. The first item (A1) assesses the unpleasantness or discomfort of breathing on a scale ranging from 0 (“neutral”) to 10 (“unbearable”). The subsequent five items assess the intensity of sensory dimensions on a scale ranging from 0 (“none”) to 10 (“as intense as I can imagine”). The five items include, S1: my breathing requires muscle work or effort; S2: I am not getting enough air; S3: my chest and lungs feel tight or constricted; S4: my breathing requires mental effort or

concentration; and S5: I am breathing a lot. The final five items assess the intensity of affective dimensions of dyspnoea on a scale ranging from 0 (“none”) to 10 (“the most I can imagine”). Using this scale participants are asked to rate how there breathing makes them feel in relation to five emotions: depressed, anxious, frustrated, angry, and afraid. The MDP was used in experimental chapter 4 to assess dyspnoea post EVH test in university hockey players and getting participants to refer to dyspnoea during the period between three- and ten-minutes post EVH test. Within chapter 4 only sensory and not affective dimensions were assessed. Items were scored individually and as an “immediate perception domain score” (IPDS) calculated as the sum of A1 and S1-S5. The MDP is displayed in appendix 1.

3.9.2. Asthma control questionnaire 7-item (ACQ-7)

The ACQ-7 is a validated questionnaire designed by Juniper, O’byrne, Guyatt, Ferrie, & King (1999) to assess asthma control. When compared against the GINA guidelines for asthma control the ACQ-7 can accurately distinguish not well controlled asthma (ACQ-7 score: > 1) and well controlled asthma (ACQ-7 score >1). Optimal cut-offs to identify not well controlled asthma is set at a score ≥1.5 and well controlled asthma is set at a score ≤0.75 with a minimal important difference being a 0.5 change in score (Juniper et al, 2005). The questionnaire shows good test re-test reproducibility in stable asthma patients (intraclass correlation coefficient = 0.9). The ACQ-7 includes seven items which are scored on a 7-point scale (0 = never/no symptoms/none; 6 = all the time/very severe symptoms/totally limited). The first six items assess symptoms and rescue bronchodilator use and item seven assesses percentage predicted FEV_1. Participants retrospectively recall their symptoms/rescue inhaler use over the previous week, while having there FEV_1 assessed at the time of completing the questionnaire via spirometry. The ACQ-7 was used in experimental chapter 7 to assess

asthma control pre (day 0) and post (day 21) B-GOS or placebo supplementation. The ACQ-7 is displayed in appendix 2.

Chapter 4 – The prevalence of hyperpnoea-induced bronchoconstriction in university field hockey athletes: the effect of sex and diagnostic criteria on prevalence, and the association between hyperpnoea-induced bronchoconstriction and dyspnoea.

4.1. INTRODUCTION

Exercise-induced bronchoconstriction (EIB) defines a transient narrowing of the airways following an exercise challenge test which results in a ≥10% reduction in forced expiratory volume in 1 second from pre to post test (Weiler et al, 2016). Surrogate indirect challenges, such as the eucapnic voluntary hyperpnoea (EVH) test, are often used to support a diagnosis of EIB in athletes (Hull et al, 2016; Williams et al, 2015; Weiler et al, 2016). While used to screen athletes for EIB an EVH test specifically identifies hyperpnoea-induced bronchoconstriction (HIB). For clarity, the term EIB will be used throughout this chapter in reference to studies adopting an exercise-challenge test while HIB will be used in reference the studies adopting an EVH test for the screening of EIB. The prevalence of HIB and EIB is exceptionally high in athletes although there is high variability between sports (18-62%) (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016). Care is required however when making comparisons between studies and sports due to low cohort numbers, different proportions of each sex within cohorts, the use of difference challenge test and diagnostic criteria.

While studies have commonly assessed the prevalence of HIB and EIB in athletes using a large sample size ($n \geq 100$) individual sports often include very low participant numbers ($n \leq 15$) (Parsons et al, 2007; Dickinson et al, 2011). This hinders interpretation of HIB and EIB prevalence within individual sports due to sample size being too small to represent the population. As such, the prevalence of HIB and EIB in athletes of individual sports needs to be assessed in larger sample sizes. Additionally, while little data exists, sex differences for the prevalence of HIB and EIB in athletes have been reported, although the data are conflicting with higher (42% vs 38%) and lower (18% vs 26%) prevalence rates reported in males than females (Parsons et al, 2007; Wilber et al, 2000).

The American Thoracic Society (ATS) criterion for diagnosing HIB using the EVH test in athletes requires a ≥10% fall in FEV_1, relative to baseline, at two consecutive time points after an EVH test (Parson, 2013). Using this criteria Dickinson et al (2011) reported the prevalence of EIB in elite athletes at 34%. Higher prevalence's are found in cohorts of athletes using less stringent criteria of a ≥10% fall in FEV_1 at just one time point post EVH test (39-43%) (Parsons et al, 2007; Burnett et al, 2016). Indeed, differences in diagnostic criteria may influence prevalence by up to 18% (Koch et al, 2018). The lack of continuity in diagnostic criteria may distort actual prevalence rates, as such the effect of such differing criteria on prevalence rates in athletes warrants investigation.

Furthermore, HIB and EIB diagnosis is confounded by the poor diagnostic accuracy of self-reported respiratory symptoms in comparison to objective challenge tests such as exercise and EVH tests which results in high false-positive and false-negative diagnosis (~50%) (Ansley et al, 2012; Parsons et al, 2007). The poor diagnostic accuracy of self-reported symptoms is thought to be due to a weak relationship between respiratory symptoms and bronchoconstriction post exercise/hyperpnoea (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016). However, in studies previously assessing the diagnostic accuracy of respiratory symptoms, symptoms were assessed retrospectively with participants at rest and asymptomatic, rather than shortly after an exercise or EVH test when bronchoconstriction was present. This is problematic because the severity of EIB may relate poorly to respiratory symptoms that are assessed retrospectively and reliant on memory (Conner and Barret 2012). Conversely, momentary conscious respiratory symptoms are more likely to relate to the severity of bronchoconstriction if assessed *in situ*, i.e. shortly after an EVH test (Conner and Barret 2012). In addition, symptoms assessed in relation to previous habitual exercise which lacks a sufficient stimulus to provoke bronchoconstriction (e.g. exercise $\dot{V}_E$ <85% MVV) would lack validity.

HIB and EIB is underdiagnosed and undertreated (Dickinson et al, 2011; Burnett et al, 2016). The lack of treatment may reduce endurance exercise capacity and more importantly is a risk factor for exercise-induced related deaths (Jackson et al, 2018; DeJuilio, 2016; Becker et al, 2004). It is therefore important to understand the scale of the issue in individual sports and sexes, understand the influence of diagnostic criteria on prevalence rates, and the relationship between HIB and respiratory symptoms. Therefore, the aims of the present study were to: (I) assess the prevalence of EIB in university field hockey players and whether this differs between males and females; (II) assess the relationship between EIB and symptoms of dyspnoea measured *in situ*; and (III) assess the effect of different diagnostics criteria on the prevalence of EIB.

4.2. METHODS

4.2.1. Participants

Fifty-five British University field hockey players (age: 22 ± 4 years; height: 173 ± 9 cm; body mass: 72 ± 10 kg), training and competing 8 ± 3 hours per week and competing in British University Championships fixtures, provided written informed consent to participate in the study. The study was approved by the Nottingham Trent University Human Ethics Committee (approval number: 582) and all procedures conformed to the standard set by the Declaration of Helsinki.

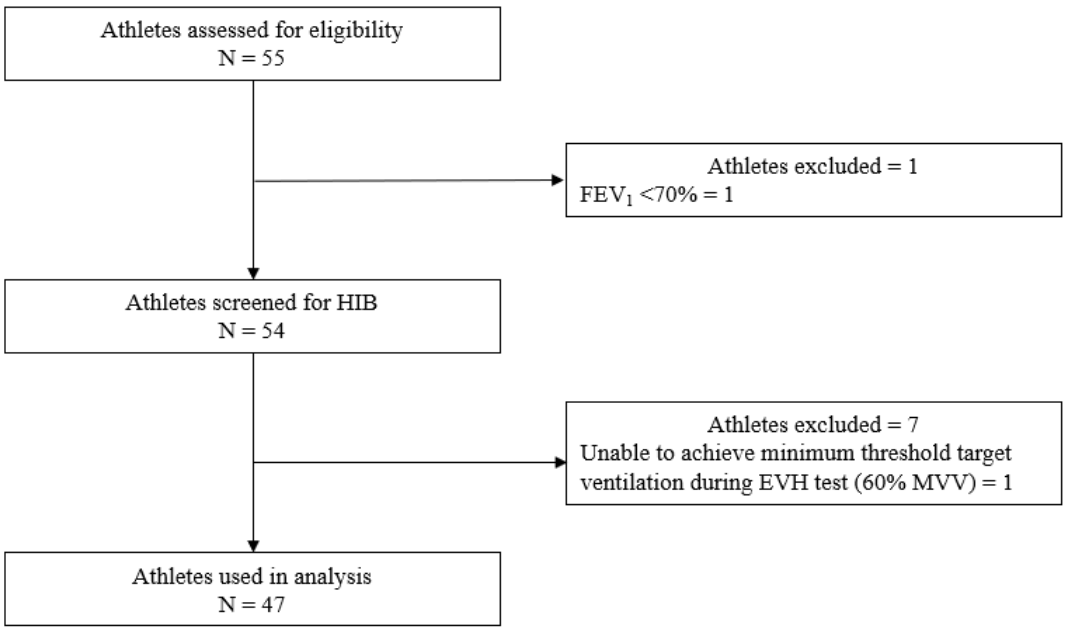

Figure 4.1. Participant flow through study.

4.2.2. Experimental design

Testing took place across four consecutive months (November to February). Participants attended the laboratory on one occasion to perform an EVH test preceded and followed by spirometry to evaluate the presence and severity of HIB. Ten minutes after the EVH test,

participants completed a multidimensional dyspnoea profile (MDP) (Banzett, 2016). Each participant stated whether they had current/previous diagnosis of asthma or EIB. A current diagnosis was classified as having up to date prescribed medication for the treatment of asthma/EIB, whereas a previous diagnosis was classified as previously receiving prescribed medication for the treatment of asthma/EIB, but which was not current. In the lead up to the laboratory visits participants adhered to the restriction guidelines set out in Chapter 3: General Methods (Section 3.3. Participants restrictions prior to experimental trials).

4.2.3. Pulmonary function and EVH test

Pulmonary function was assessed via spirometry. Pulmonary function and the EVH test were conducted in accordance with the procedures outlined in Chapter 3: General methods (Sections 3.4. Pulmonary function – Dynamic spirometry; & Section 3.5. Eucapnic voluntary hyperpnoea test). EIB was diagnosed under three separate fall index (FI) criteria (Table 4.1) to assess the effect of diagnostics criteria on prevalence rates.

Table 4.1: Fall index criteria used for diagnosing hyperpnoea-induced bronchoconstriction (HIB) following a eucapnic voluntary hyperpnoea test.

FI criteria	Calculation
FI_{ATS} (Parsons, 2013; Weiler, 2016).	The highest FEV_1 value of the two spirometry manoeuvres at each post-EVH test time point is selected. The difference for each is compared to baseline FEV_1 to define the FI. A positive test is defined by a FI ≥10% at two consecutive time points in line with the ATS guidelines.
$FI_{\geq 10\%}$ (Anderson, 2001).	The highest FEV_1 value of the two spirometry manoeuvres at each post-EVH test time point is selected. The difference for each is compared to baseline FEV_1 ti define the FI. A positive test is defined by a FI ≥10% at any one time point.
$FI_{\geq 10\%\text{-NORM}}$ (Hurwitz, 1995).	The highest FEV_1 value of the two spirometry manoeuvres at each post-EVH test time point is selected. The difference for each is compared to baseline FEV_1. The FI index is subsequently normalised to the achieved ventilation during the EVH test using the following equation: $(\frac{\text{Baseline FEV1} - \text{Post FEV1}}{\text{Baseline FEV1}}) \times (\frac{30 \times \text{FEV1}}{\text{Achieved VE}})$ A positive test is defined by a FI ≥10% at any one time point.

FI = Fall index; FEV_1 = Forced expiratory volume in 1 second; EVH = Eucapnic voluntary hyperpnoea test; ATS = American thoracic society.

4.2.4. Multi-dimensional dyspnoea profile

Participants completed a MDP (Banzett et al, 2016) specifically referring to highest levels of dyspnoea experienced between 3-10 minutes post EVH-test, which typically captures the peak fall in FEV_1 in HIB-positive (HIB^+) individuals (Dickinson et al, 2011; Williams et al. 2015). The MDP consists of 11 items evaluating sensory and affective dimensions of dyspnoea, although affective dimensions were not assessed in the present study. The first item (A1) assesses the unpleasantness or discomfort of breathing on a scale ranging from 0 ("neutral") to 10 ("unbearable"). The subsequent five items assess the intensity of sensory dimensions on a scale ranging from 0 ("none") to 10 ("as intense as I can imagine"). The five items include, S1: my breathing requires muscle work or effort; S2: I am not getting enough

air; S3: my chest and lungs feel tight or constricted; S4: my breathing requires mental effort or concentration; and S5: I am breathing a lot. Items were scored individually and as an "immediate perception domain score" (IPDS) calculated as the sum of A1 and S1-S5.

4.2.5. Statistical analysis

Participants were grouped according to HIB diagnosis (HIB^+ or HIB^-) and sex. Independent samples T-tests assessed between-group (HIB^+ vs. HIB^-; male vs. female) differences in baseline pulmonary function, average percentage of MVV achieved during EVH, peak fall in FEV_1, and MDP items. Receiver operator characteristic (ROC) curves were determined for MDP items that differed between HIB^+ and HIB^- groups. Stepwise multiple regression was performed using the peak fall in FEV_1 as the dependant variable and MDP items as candidate predictors. Mixed model repeated measures ANOVA assessed the effects of time (baseline and 3, 10, 15, and 20-min post-EVH test) on FEV_1, with a between-subjects factor of HIB diagnosis (HIB^+ vs. HIB^-). Significant main and interaction effects were followed by independent samples T-tests at each measurement point. Within-group changes in FEV_1 were assessed using one-way repeated measures ANOVA followed by Tukey's post-hoc test. A Cochran Q test followed by a McNemar post-hoc test assessed differences in the number of HIB^+ and HIB^- diagnoses based on each FI criteria. Statistical significance was set at $P < 0.05$, except for the McNemar test, which was set at $P < 0.0083$. For significant differences, 95% confidence intervals are presented. Effect sizes are presented as Cohen's d. Data were analysed using IBM SPSS Statistics V26.0 and presented as mean ± SD unless indicated otherwise.

4.3. RESULTS

One participant had a baseline FEV_1 <70% of predicted and therefore could not perform an EVH test. Seven participants did not achieve an average $\dot{V}_E$ ≥60% MVV while not having a ≥10% fall in FEV_1 from pre to post EVH test and were excluded from further analysis. Therefore, 47 participants (male = 27; female = 20) completed a valid EVH test. Of this cohort, 9% had a current asthma diagnosis, 17% had a previous asthma diagnosis, 0% had a current EIB diagnosis, and 6% had a previous GP diagnosis of EIB of which none were diagnosed via an objective challenge test. Sixty-seven percent (6/9) of HIB^+ participants were not on prescribed EIB/asthma medication. The percentage of current/previous diagnosis of asthma and EIB within each group is summarised in Table 4.2.

4.3.1. Baseline pulmonary function

Baseline pulmonary function (Table 4.2) was not different between HIB^+ and HIB^- groups (*p* = 0.27 – 0.85; *d* = 0.09 - 0.41). FEV_1 (% predicted) was lower in males (91 ± 10%) than females regardless of HIB status (97 ± 9%) (*p* = 0.032; 95% CI [-12, -0.6%]; *d* = 0.63). FVC (% predicted) was lower in males (94 ± 10%) than females (100 ± 10%) regardless of HIB status (*p* = 0.050; 95% CI [-11, -0.0004%]; *d* = 0.58).

Table 4.2: Baseline pulmonary function, shown as percentage of the predicted value, for HIB^+ and HIB^- groups, and male and female groups irrespective of HIB diagnosis.

	HIB^+	**HIB^-**	**Male**	**Female**
Percentage predicted FEV_1	91 ± 11	94 ± 10	91 ± 10*	97 ± 9
Percentage predicted FVC	95 ± 9	97 ± 11	94 ± 10*	100 ± 10
Percentage predicted FEV_1/FVC	95 ± 9	98 ± 7	97 ± 8	98 ± 7
Percentage predicted PEF	97 ± 15	98 ± 12	96 ± 13	100 ± 12
Percentage predicted $FEF_{25-75\%}$	84 ± 25	93 ± 20	88 ± 22	96 ± 19
Peak fall in FEV_1 (%)	-18 ± 3	-7 ± 3	-10 ± 6	-8 ± 3
Current asthma diagnosis (%)	33	3	15	0
Current EIB diagnosis (%)	0	0	0	0
Previous asthma diagnosis (%)	56	8	22	10
Previous EIB diagnosis (%)	11	5	4	10

HIB, hyperpnoea-induced bronchoconstriction; EVH, eucapnic voluntary hyperpnoea; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; PEF, peak expiratory flow; $FEF_{25-75\%}$, forced expiratory flow from 25 to 75% of FVC. Date are mean ± SD. *Difference between male and female groups ($P \leq 0.05$).

4.3.2. Pulmonary function after the EVH test

By design, the peak fall in FEV_1 was greater in the HIB^+ (-18 ± 3%) than HIB^- (-7 ± 3%) group ($p < 0.001$; 95% CI [-14, -10%]; $d = 2.23$) (Figure 4.2). For the fall in FEV_1, there were main effects of time and a group × time interaction ($p \leq 0.001$). The fall in FEV_1 was greater in the HIB^+ group than the HIB^- group at all-time points after EVH ($p \leq 0.001$; $d = 1.2$ - 2.1). In the HIB^+ group, FEV_1 was below baseline throughout recovery ($p \leq 0.006$; $d = 1.35$ – 1.88). In the HIB^+ group, peak falls in FEV_1 were observed at 3-min ($n = 4$), 6-min ($n = 2$),

10-min (n = 2), and 15-min (n = 1) after EVH. The prevalence of HIB for all participants, males, and females, was 19%, 30%, and 5% respectively.

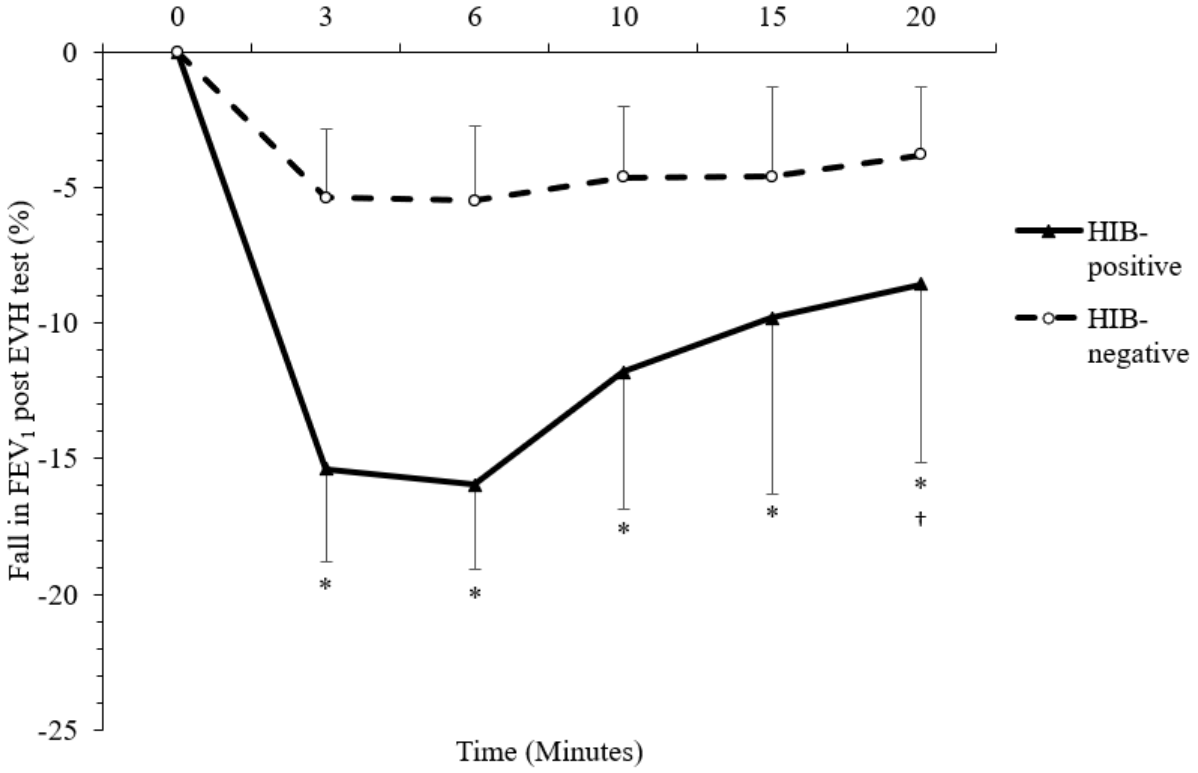

Figure 4.2: Fall in forced expiratory volume in 1 second (FEV_1) from baseline (0) following the eucapnic voluntary hyperpnoea test in HIB-positive (closed triangles) and HIB-negative (open circles) groups. *Different from baseline in HIB-positive group (P = <0.001 - 0.045). †Different from 3- and 6-min post-EVH in the HIB-positive group ($P \leq 0.002$).

4.3.3. Achieved ventilation during the EVH test

The average percentage achieved of MVV during the EVH test was 71%, ranging from 53-93%. $\dot{V}_E$ significantly increased throughout the EVH test ($p < 0.001$). $\dot{V}_E$ was lower at 1 minute compared to 2, 3, 4, 5, and 6 minutes ($p \leq 0.003$), 2 minutes compared to 5 and 6 minutes (p = 0.037 – 0.001), and 3, 4, and 5 minutes compared to 6 minutes (p = 0.028 – 0.001) (Figure 6.3). One HIB^+ participant had a $\dot{V}_E$ <60% MVV. Percentage achieved MVV was not different between HIB^+ (69 ± 10%) and HIB^- (72 ± 7%) groups (p = 0.35, d = 0.43), or male (72 ± 8% MVV) and female (71 ± 7% MVV) groups (p = 0.49; d = 0.19). The

%MVV achieved during the EVH test was not correlated with the peak fall in FEV_1 (r = 0.21; p = 0.15) (Figure 4.3).

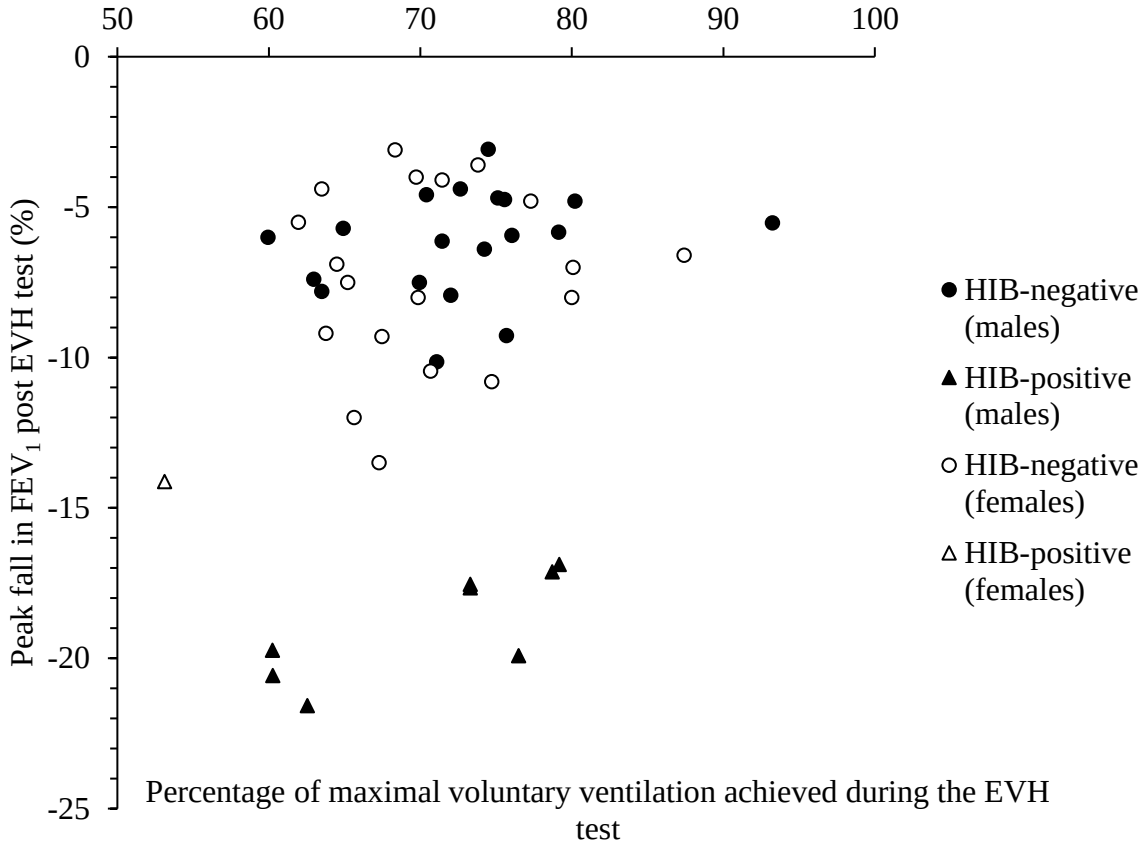

Figure 4.3: Peak fall in forced expiratory volume in 1 second (FEV_1) from pre to post eucapnic voluntary hyperpnoea (EVH) test in relation to the percentage of maximal voluntary ventilation achieved during the EVH test.

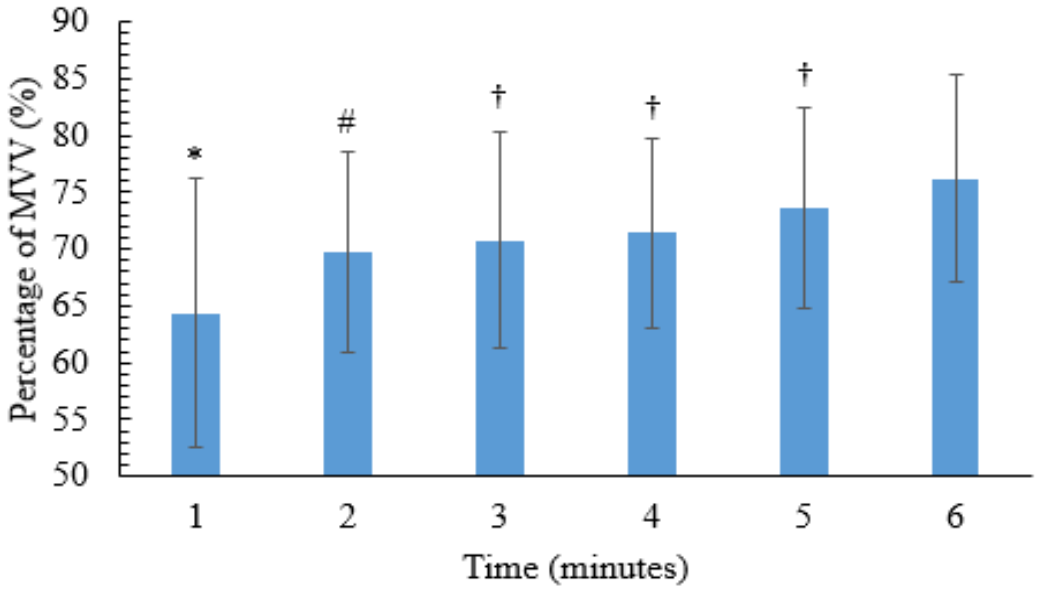

Figure 4.4: Percentage of maximal voluntary ventilation (MVV) achieved minute by minute during the eucapnic voluntary hyperpnoea test. *Difference from 2, 3, 4, 5, and 6 min (P ≤ 0.003). #Different from 5 and 6 min (P = 0.037 – 0.001). †Significant different from minute 6 (P ≤ 0.003).

4.3.4. Effect of diagnostic criteria on HIB prevalence

The prevalence of HIB^+ cases differed between FI criteria (χ^2 (3) = 14.818, P = 0.002) (Table 4.3). Prevalence was greater under $FI_{10\%\text{-}NORM}$ than FI_{ATS} (P = 0.004). No differences in the prevalence of HIB^+ cases were found between FI_{ATS} and $FI_{\geq 10\%}$ (P = 0.063), and between $FI_{\geq 10\%}$ and $FI_{10\%\text{-}NORM}$ (P = 0.219).

Table 4.3: Percentage of hyperpnoea-induced bronchoconstriction (HIB) positive cases based on three different fall index (FI) criteria for all (N = 47), males (N = 27), and females (N = 20).

	All	**Male**	**Female**
FI_{ATS}	19 (9/47)	30 (8/27)	5 (1/20)
$FI_{\geq 10\%}$	30 (14/47)	33 (9/27)	25 (5/20)
$FI_{10\%\text{-}Norm}$	38 (18/47)*	44 (12/27)	30 (6/20)

*Different from FI_{ATS} (P = 0.004). Ratios of HIB^+/total group number within each group are shown in brackets.

4.3.5. Symptoms of dyspnoea after EVH

The IPDS (sum of all questions) was higher in the HIB^+ (15 ± 9) than the HIB^- (6 ± 8) group (P = 0.031; 95% CI [2, 15]; d = 1.1). Similarly, A1 (unpleasantness or discomfort of breathing) was higher in the HIB^+ (5 ± 2) than the HIB^- (1 ± 2) group (P = 0.001; 95% CI [1, 4]; d = 0.72). S1-S5 items were not different between groups (P = 0.06 – 0.82; d = 0.09 - 0.92). ROC analyses produced significant area under the curves for A1 (AUC = 0.843; P = 0.002; 95% CI [0.727, 0.959]) and IPDS (AUC = 0.778; P = 0.011; 95% CI [0.635, 0.92]) (Figure 4.4). The maximal combined sensitivity and specificity cut-off for detecting HIB corresponded to scores of A1 = 3 (sensitivity = 1; specificity = 0.63; positive prediction value = 53%; negative prediction value = 100%) and IPDS = 7 (sensitivity = 0.89; specificity = 0.66; positive prediction value = 44%; negative prediction value = 96%).

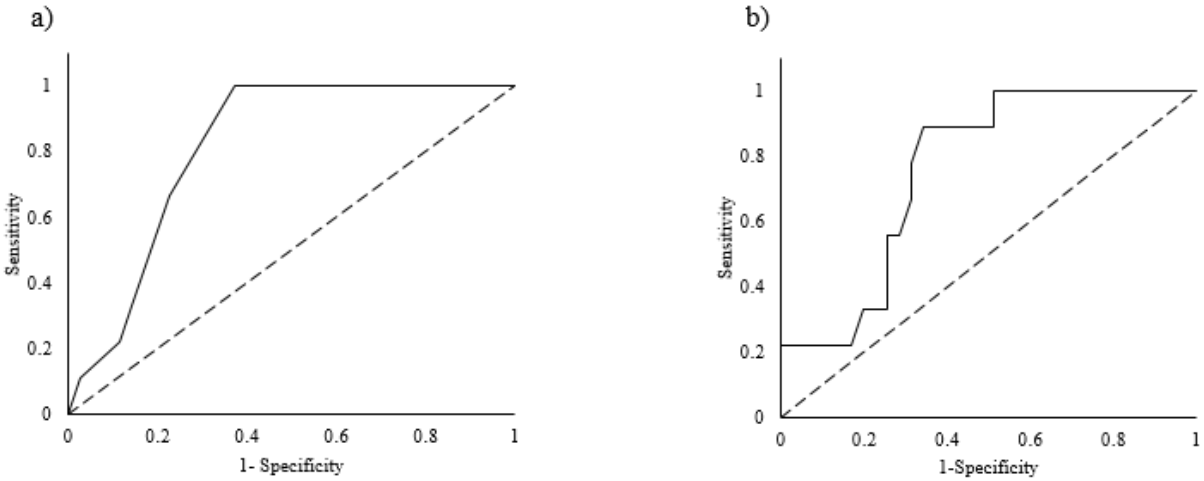

Figure 4.5: Receiver operating characteristics curves for multi-dyspnoea profile items A1 (a) and IPDS (b).

The peak fall in FEV_1 after EVH correlated negatively with IPDS (r = -0.31; P = 0.044) and A1 (r = -0.45; P = 0.003) scores. Multiple regression analysis of the peak fall in FEV_1 revealed item A1 to be the only contributing factor. The A1 score was higher in males (3 ± 2) than females (1 ± 2) (P = 0.011; 95% CI [0.4, 3.0]; d = 0.52), whereas other items did not differ between sex (P = 0.068 – 0.64; d = 0.35 - 0.87).

4.4. DISCUSSION

4.4.1. Main findings

The main findings of the present study were threefold: (I) prevalence of HIB in British University field hockey players was 19% and greater in males (30%) than females (5%); (II) symptoms of dyspnoea assessed after the EVH test were higher in HIB^{+} participants and sensitive in detecting HIB diagnosis; and (III) prevalence of HIB ranged from 19-38% depending on the diagnostic FI criteria used.

4.4.2. Prevalence of HIB and sex differences

The present study comprised, to date, the largest cohort of field hockey players screened for HIB. The prevalence of HIB in our cohort of field hockey players (19%) supports that HIB is prevalent in competitive athletes (Parsons et al, 2005). The prevalence of HIB was, however, lower than that reported in other university athletic populations containing mixed sexes (39-43%) (Burnett et al, 2016; Parsons et al, 2007). This is due, in part, to previous studies using $FI_{\geq 10\%}$ to diagnose HIB, which is less conservative than the FI_{ATS} criteria and therefore results in higher prevalence rates (Koch et al, 2018). Indeed, using $FI_{\geq 10\%}$ in the current study yielded a 30% prevalence rate which is broadly similar to the previous reports. Previous studies (Burnett et al, 2016; Parsons et al, 2007) screening for HIB used athletes from different sports, which also affects prevalence rates (Parsons et al, 2005). Compared to the present study, Dickinson et al (2011) reported a higher prevalence of HIB (38%), based on FI_{ATS}, in 21 elite hockey players (sex not reported). Higher prevalence may be explained, in part, by the higher training frequencies / intensities / volumes performed by elite hockey players, which exacerbates the chronic mechanical stress on the airways that can result in airway epithelial cell damage and increased risk of developing HIB and EIB (Kippelen & Anderson,

2013). The elite hockey players also achieved a higher %MVV during EVH (79% vs. 71% in the present study); however, a relationship between the %MVV achieved and the fall in FEV_1 was not observed in the present study or the study of Dickinson et al (2011). Interestingly, compared to the present study, the prevalence of HIB (based on FI_{ATS}) was also higher (28%) in male professional footballers (Jackson et al, 2018); however, when compared to the males only group in the present study, the prevalence of HIB was strikingly similar (30%), which suggests that sex also affects the prevalence of HIB.

In the present study, the prevalence of HIB in field hockey players was lower in females (5%) than males (30%). Parsons et al. (2007) reported similar HIB prevalence rates (based on $FI_{\geq 10\%}$) between male (42%) and female (38%) university athletes across 22 sports, although these did not include field hockey which might partly explain this discrepancy. It might also be explained partly by differences in HIB diagnostic criteria. In support, if the diagnosis of HIB in the present study is based on $FI_{\geq 10\%}$, the prevalence in males (33%) and females (25%) is more comparable . In contrast to the present study, the prevalence of EIB was higher in female (26%) than male (18%) elite winter sport athletes (Wilber et al, 2000). An explanation for this discrepancy is unclear but might be related to sport-specific differences such as environmental conditions (Parsons et al, 2005) and/or the type of challenge (exercise vs. EVH). It is also noteworthy that HIB and EIB screening in females is confounded by fluctuations in sex hormones during the menstrual cycle, which affects lung function in women with and without asthma (Farha et al, 2009). Additionally, the peak fall in FEV_1 following an exercise test is greater during the mid-luteal phase (-17.4%) of the menstrual cycle when salivary progesterone levels are significantly higher compared to the mid-follicular phase (-12.8%) (Stanford et al, 2006). Asthma symptoms may also worsen during the pre or perimenstrual phase of the menstrual cycle, whereas oral contraceptives may alleviate symptoms (Jung et al. 2018). Females with mild HIB may therefore fluctuate

either side of the diagnostics threshold depending upon the phase of the menstrual cycle they are tested on and the use of contraception. Therefore, a limitation of the present study, and previous studies investigating HIB and EIB prevalence in females, is that menstrual cycle phase and contraceptive therapies were not documented / controlled. Nevertheless, our results suggest that the prevalence of HIB, when diagnosed using FI_{ATS}, is lower in female compared to male university field hockey players.

4.4.3. Dyspnoea and HIB

Symptoms of dyspnoea, namely the "unpleasantness or discomfort of breathing" (A1) and the sum of A1 and scores relating to the intensity of sensory dimensions (IPDS), were higher in HIB^+ than HIB^- participants. Interestingly, these symptoms had a sensitivity and specificity comparable to a methacholine challenge (Anderson et al, 2009). Previous studies have reported that only ~50% of individuals with HIB and EIB report respiratory symptoms and that respiratory symptoms are not associated with HIB and EIB (Dickinson et al, 2011; Parsons et al, 2007; Burnett et al, 2016; Ansley et al, 2012; Jackson et al, 2018). In addition, the dyspnoea-12 questionnaire has been shown to fail in differentiating HIB^+ and HIB^- athletes although question Q4 (I have difficulty catching my breath) and Q11 (My breathing makes me agitated) in the D-12 are the most influential in ruling in a diagnosis of HIB (Price et al, 2019). However, these studies administered questionnaires with participants at rest and asymptomatic, rather than after an exercise or EVH challenge when bronchoconstriction is present. Additionally, some sports may lack the ventilation demands and environmental stimuli to induce EIB in EIB^+ individuals. This is exemplified in swimmers were HIB prevalence assessed using an EVH test is high (55%) but the prevalence following a field swimming challenge is very low (3%) (Castricum et al, 2010). Symptoms recall from

previous habitual exercise would lack validity if the stimulus was insufficient to induce bronchoconstriction (e.g. exercise $\dot{V}_E$ <85% MVV); and retrospective self-report measures that rely on memory are poorly tied to momentary biological processes (Conner and Barret 2012). Therefore, a strength of the present study is that the MDP was administered shortly after the EVH test and was therefore more likely to connect momentary conscious respiratory symptoms with documented bronchoconstriction (Conner and Barret 2012). This may explain why the MDP items IPDS and A1 were both associated with the post-EVH fall in FEV_1 and were highly sensitive and specific to HIB in this cohort. These results therefore suggest that respiratory symptoms, when assessed *in situ*, have a strong association with HIB diagnosis. Interestingly, A1 had a 100% negative prediction value (NPV) to the post-EVH fall in FEV_1 and, therefore, from a practical perspective this offers a useful tool for coaches / practitioners and athletes to rule out, *in situ*, the likelihood that HIB is/was present and the need for further objective testing.

There is a need to develop and validate an EIB/HIB diagnostic questionnaire. The development of such a questionnaire will require qualitative approaches to identify symptoms and pattern of symptoms that distinguish EIB/HIB positive and negative athletes. Price et al (2016) used semi-structured interviews in endurance athletes previously screen for HIB and found the location and recovery time of symptoms following exercise to be the best distinguishes features between HIB^+ and HIB^- athletes. HIB^+ athletes typically present symptoms lower down the respiratory tree in the chest/lung in comparison to HIB^- athletes that suffer from exercise dyspnoea who present with symptoms in the larynx region. Recovery duration for symptoms usually presents in the 2-10 minutes range for HIB^+ athletes while 10-60 minutes is common for HIB^- athletes presenting with dyspnoea (Price et al, 2016). Future work should focus on merging the methods of the present study and Price et al (2016) by conducting interviews with positive and negative responder's post exercise

challenges and EVH tests when bronchoconstriction can be documented to identify symptoms and pattern of symptoms that distinguish positive and negative responders. This may identify symptoms which are closely tied to the momentary biological process of HIB that can be used to devise such a questionnaire. Furthermore, greater field-based testing needs to be conducted on EIB-positive individuals to assess whether they frequently suffer with bronchoconstriction following their respective sports. If certain EIB/HIB-positive athletes rarely suffer with bronchoconstriction following their respective sport, then EIB/HIB-specific symptoms will not develop and will not be detected by such a questionnaire.

4.4.4. Conclusion

In conclusion, the prevalence of HIB in university field hockey players was 19% and greater in males than females. Dyspnoea symptoms, assessed *in situ*, were associated with HIB and had high NPVs and may therefore offer a useful tool to for ruling out the presence of HIB or the need for further objective testing. Finally, the prevalence of HIB depends on the FI criteria employed, thus previous studies that did not use FI_{ATS} may have overestimated the prevalence of EIB.

Chapter 5 - The efficacy of the RTube device to collect exhaled breath condensate for the measurement of inflammatory cytokines in adults with hyperpnoea-induced bronchoconstriction and asthma.

5.1. INTRODUCTION

Hyperpnoea-induced bronchoconstriction (HIB) is the transient narrowing of the airways following an EVH test defined by a ≥10% drop in forced expiratory volume in 1 second (Weiler et al, 2016; Parsons, 2013). HIB is present in approximately 50% of asthma patients and shares similar pathophysiological characteristics with asthma including airway hyper-responsiveness and inflammation which are orchestrated by the release of lipid mediators and cytokines within the airways (Sano et al, 1998; Weiler et al, 2016; Parsons, 2013). Airway hyper-responsiveness in HIB is measured easily and non-invasively using spirometry, however the ability to measure airway inflammation is more troublesome due to the nature of techniques. Specifically, although bronchoscopies, sputum induction and FeNO provide in depth sampling of the airways these samples require techniques that are impractical following an EVH test when HIB is present. In the presence of HIB, bronchoscopies and sputum induction are unsafe while the influence of exercise and spirometry on FeNO concentrations prevents its utility following an EVH test (Busse et al, 2005; Weiszhar & Horvath, 2013; Bjermer et al, 2014). Additionally, the collection of multiple samples in quick succession is not practical with such techniques. This limits the ability to investigation the effect of novel treatments on airway inflammation in individuals with HIB.

The collection of exhaled breath condensate (EBC) is a novel non-invasive technique that has potential for use in HIB research. EBC is an airway sample consisting primarily of condensed water vapour with a small fraction of respiratory lining fluid droplets (Horvath et al, 2005). These respiratory droplets contain non-volatile molecules and biomarkers of airway inflammation. EBC samples are collected by directing an individual expired air across a cold surface, which results in surface condensation (Horvath et al, 2017). This condensate is collected and subsequently analysed using biochemistry techniques to quantify the concentration of biomarkers. The Respiratory Tube (RTube) is a commercially EBC

collection device that has previously been used to collect EBC for the measure of inflammatory biomarker (Kazani et al, 2013; Tahan, Eke, Bicici, Saraymen, & Akar, 2016; Vaughan et al, 2003). Concentrations of leukotriene B_4 (LTB_4) cysteinyl leukotrienes (Cyst-LTs) and lipoxin A_4 (LXA_4) have been measured in EBC collected with the RTube device, with higher resting concentrations found in asthma patients compared to healthy controls (Kazani, et al, 2013; Tahan et al, 2016). Additionally, the collection of EBC with the RTube device identified an increased LXA_4/LTB_4 ration with increased asthma severity. The measurement of Cyst-LTs however, was below the lower limits of detection in 62% of samples (Kazani et al, 2013) Furthermore, EBC pH has been linked to asthma status with more acidic pH found in asthma patients compared to healthy controls and a reduction in EBC pH in patients with acute asthma (Aldakheel et al, 2016; Hunt et al, 2000). EBC pH is additionally shown to reduce from pre to post an exercise challenge test in participants with EIB (Bikov et al, 2014). The measurement of EBC pH collected using the RTube device is reproducible (Vaughan et al, 2013). The RTube is therefore a potentially useful research tool to measure airway inflammation involved in asthma and HIB.

Leukotrienes undergo significant degrade in EBC samples within a few weeks, in contrast cytokines are stable in EBC for up to 1 year (Ohanian, Zimmerman, & Debley, 2010; Robroeks et al, 2007). There is however limited data on the ability to detect cytokines in EBC collected using the RTube device, while cytokines have been measured using other commercially available and self-constructed devices. In asthma patients and healthy controls interleukin-4 (IL-4), IL-5, IL-6, IL-8, IL-10, IL-13, thymic stromal lymphopoietin (TSLP), and tumour necrosis factor-alpha (TNF-α) have been measured in EBC collected using the Ecoscreen device and self-constructed devices using enzyme-linked immunosorbent assays (ELISA) and multiplex assays (Matsunga et al, 2006; Tufvesson & Bjermer, 2005; Gluck, Rymarczyk, Kasprzak, & Rogala, 2016; Robroeks et al, 2010). The pathophysiological

importance of cytokines in orchestrating airway inflammation in asthma and HIB make them important to measure in EBC, and therefore the ability of the RTube device to collect EBC with measurable cytokines requires evaluation. IL-13 and TSLP have been measured in substantial concentrations in asthma patients and healthy controls previously (Tufvesson et al, 2005; Matsunaga et al, 2006; Gluck et al, 2016). IL-13 is released from Th2 cells and type-2 innate lymphoid (ILC2) cells and plays a pivotal role in AHR by initiating airway smooth-muscle contraction and goblet cell hyperplasia (Wills-Karp et al, 1998; Grunig et al, 1998). TSLP is an airway epithelial derived cytokine involved orchestrating type-2 inflammatory airway responses (Ziegler et al, 2013; Nguyen, Vanichsarn, & Nadeau, 2010). TSLP is released from airway epithelial cells in response to epithelial damage and osmotic stress, as such, it is released from airway epithelial cells at higher concentrations in asthma patients with exercise-induced bronchoconstriction (EIB) compared to asthma patients without EIB and healthy controls (Lai et al, 2014). The ability to measure IL-13 and TSLP in EBC collected using the RTube device could subsequently provide insight into the effect of novel treatments to attenuate airway inflammation in asthma and HIB.

The aim of the present study was to therefore evaluate whether IL-13 and TSLP can be measured in EBC samples collected using the RTube device in participants with asthma and HIB. The study additionally looks to map presumed changes in EBC pH during the course of bronchoconstriction and recovery from pre to 2 hours post an EVH test. By testing the efficacy of the RTube device to collect EBC with measurable cytokines we can assess whether it can be used to evaluate the effect of B-GOS supplementation on airway inflammation.

5.2. METHODS

5.2.1. Participants

Nine adult males with mild to moderate asthma, assessed using the British Thoracic Society Guidelines, and HIB were assessed for eligibility to take part in the study and provided written informed consent. Participant characteristics are summarised in table 5.1. Study inclusion criteria required participants to have a doctor's diagnosis of asthma and a ≥10% reduction in forced expiratory volume in 1 second at two consecutive time-points from pre to post EVH test (Weiler et al, 2016; Parsons, 2013). The study was approved by the Nottingham Trent University Human Ethics Committee, and all procedures were conducted in accordance with the Declaration of Helsinki.

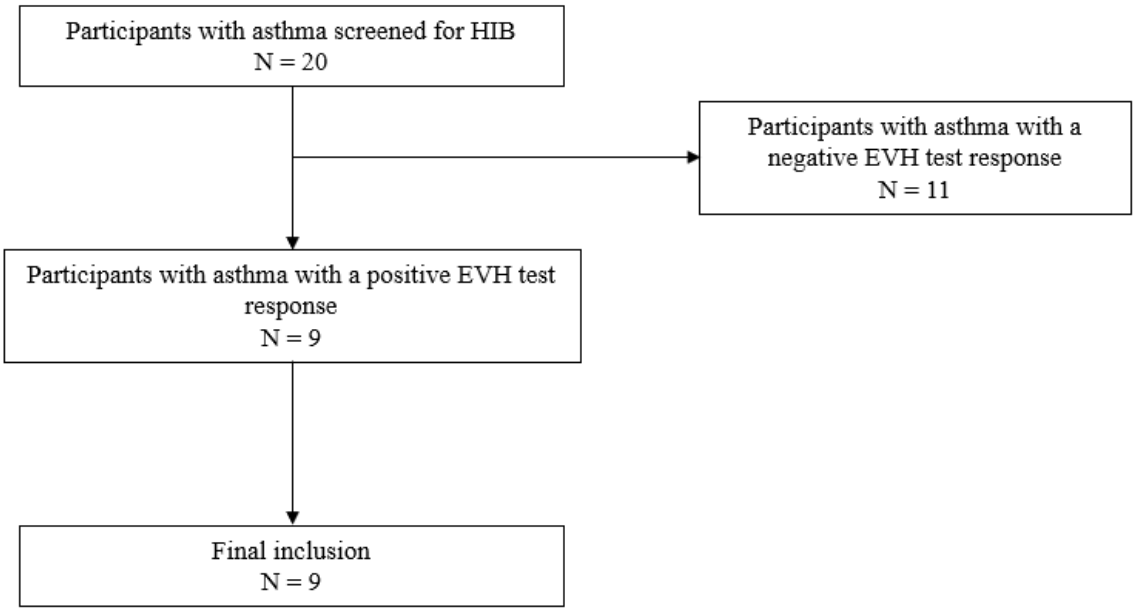

Figure 5.1. Participant flow through study.

Table 5.1: Demographics, differential white blood cell counts, baseline pulmonary function, and EVH test response characteristics of participants.

Characteristics	Outcome
Demographics	
Age (years)	22 ± 4
BMI (kg/m^2)	23.8 ± 2.6
Differential white blood cell count (10^9 cells/L)	
Eosinophils (%)	0.28 ± 0.16 (4.5 ± 3.15)
Neutrophils (%)	3.5 ± 5 1.01 (3 ± 7.9)
Basophils (%)	0.03 ± 0.02 (0.5 ± 0.3)
Lymphocytes (%)	2.12 ± 0.36 (33.2 ± 6.7)
Monocytes (%)	0.58 ± 0.15 (8.8 ± 1.1)
ICS use (%)	89
SA β_2-agonists use (%)	100
Percentage predicted baseline pulmonary function	
FEV_1	93 ± 14
FVC	99 ± 10
PEF	100 ± 25
$FEF_{25-75\%}$	77 ± 27
HIB severity	
Mean maximum percentage fall in FEV_1	-31 ± 14
Severe HIB cases	2/9
Moderate HIB cases	2/9
Mild HIB cases	5/9

BMI = body mass index; ICS = inhaled corticosteroids; SA β_2-agonists = Short-acting β_2-agonists; FEV_1 = force expiratory volume in one second; FVC = forced vital capacity; PEF = peak expiratory flow; $FEF_{25-75\%}$ = forced expiratory flow at 25 to 75% of force vital capacity.

In the lead up to trials participants adhered to the restriction guidelines set out in Chapter 3: General Methods (Section 3.3. Participant restrictions prior to experimental trials).

5.2.2 Study design

Participants completed a part familiarisation visit followed by a single experimental trial one week later. During the familiarisation visit participants were familiarised with spirometry and the EVH test, the familiarisation visit was additionally used to screen for the presence of HIB in accordance with the ATS guidelines (Parsons, 2013). During familiarisation, a baseline 5 ml blood sample was also collected in an EDTA vacutainer for the measure of differential white blood cell counts.

5.2.3. Experimental trial

Initially, a baseline EBC sample was collected prior baseline pulmonary function measurements. Participants subsequently completed an EVH test with pulmonary function reassessed at 3, 6, 10, 20, 30, 60 and 120 minutes post EVH test. An EBC samples was collected at 20, 60 and 120 minutes post EVH test.

5.2.4. Pulmonary function and EVH test

Pulmonary function was assessed via spirometry. Pulmonary function and the EVH test were conducted in accordance with the procedures outlined in Chapter 3: General methods (Sections 3.4. Pulmonary function – Dynamic spirometry; & Section 3.5. Eucapnic voluntary hyperpnoea test).

5.2.5. Exhaled breath condensate

EBC samples were collected using the RTube device (Respriatory Tube, COSMED, Oxfordshire, United Kingdom) in accordance with the procedures outlined in Chapter 3: General methods (Section 3.6. Exhaled breath condensate collection). A 400µL aliquot of EBC was used to immediately measure EBC pH at each time interval. Further aliquots were frozen at -80°C until analysis.

5.2.6. IL-13, TSLP, and EBC pH analysis

IL-13 and TSLP concentrations in EBC samples were quantified using ELISAs (Abcam, UK). The lower limits of detection for the assays were 0.15pg/mL for IL-13 and 3pg/mL for TSLP. The intra-assay coefficient of variation was 3.97% for IL-13 and 3.41% for TSLP. All samples were analysed in duplicate on one 96-well plate for both assays. EBC pH was measured in non-deaerated EBC samples using a Jenway 3510 pH meter.

5.2.7. Statistical analysis

All data is presented as mean ± standard deviation. Changes in FEV_1 and EBC pH measured from pre to post EVH test were assessed using a one-way repeated measures analysis of variance (ANOVA) followed by Bonferroni multiple comparisons post-hoc test. For significant differences, 95% confidence intervals are presented. No statistical analysis was conducted on TSLP and IL-13 concentrations due to a lack of detection in EBC samples.

5.3. RESULTS

5.3.1. Pulmonary function

The mean peak fall in FEV_1 from pre to post test was -31.2 ± 13.9% ($p < 0.001$; 95% CI [-42.5, -20.8%]) (Figure 5.2 & Figure 5.3). FEV_1 was reduced from baseline to 3 (-29.9 ± 8%; $p = 0.008$; 95% CI [-7.62, -51.92]) and 6 minutes (-27.6 ± 13.4%; $p = 0.011$; 95% CI [-5.83, -49.44]) post EVH-test (Figure 5.2). No significant fall in FEV_1 was present at 10, 20, 30, 60, and 120 minutes post EVH test compared to baseline ($p \leq 0.15$) (Figure 5.2).

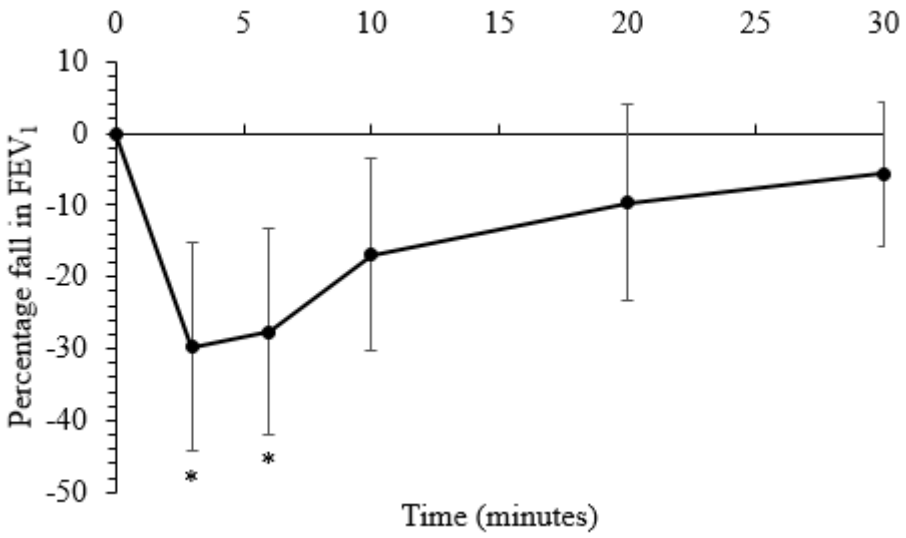

Figure 5.2: Percentage fall in FEV_1 from pre to 3, 6, 10, 20, and 30 minutes post EVH test. *Significant difference from baseline (0 minutes) ($p = 0.008 – 0.011$).

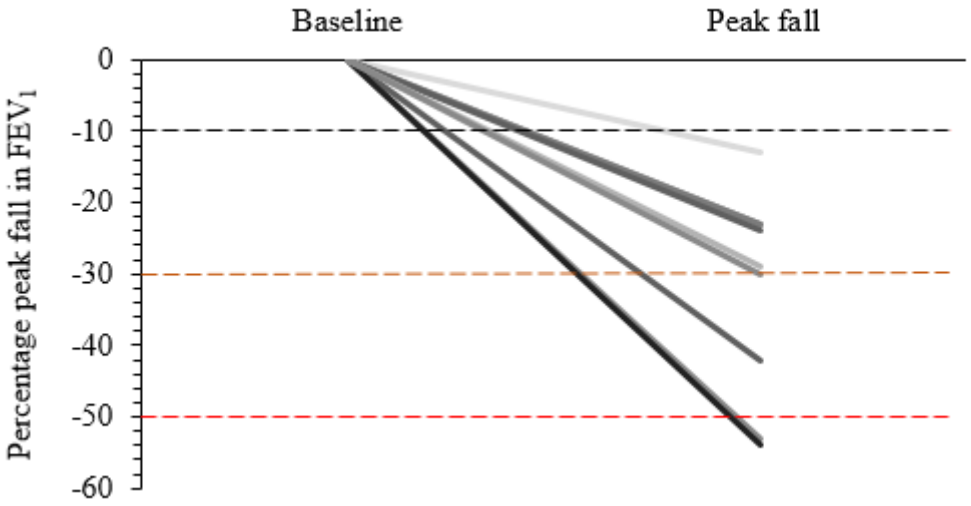

Figure 5.3: Peak fall in FEV_1 from pre to post EVH-test. Each line represents an individual participant. Dashed lines represent classifications of mild (black to orange), moderate (orange to red) and severe (below red) hyperpnoea-induced bronchoconstriction.

Significant peak reductions from baseline for other pulmonary function measures were presents for FVC (-19.1 ± 9.2%; p < 0.001), PEF (-30.4 ± 11.1%; p < 0.001), FEV_1/FVC (-17.4 ± 10.5%; p = 0.002), and $FEF^{25-75\%}$ (-46 ± 19.7%; *p* < 0.001).

5.3.2. Exhaled breath condensate IL-13 and TSLP concentrations

IL-13 was detectable in one of 36 samples (2.7%) but the concentration was too low for reliable interpretation. TSLP was undetectable in all samples.

5.3.3. Exhaled breath condensate pH

EBC pH was not significantly different from baseline at any time points post EVH-test ($p \leq 0.98$) (Figure 5.4).

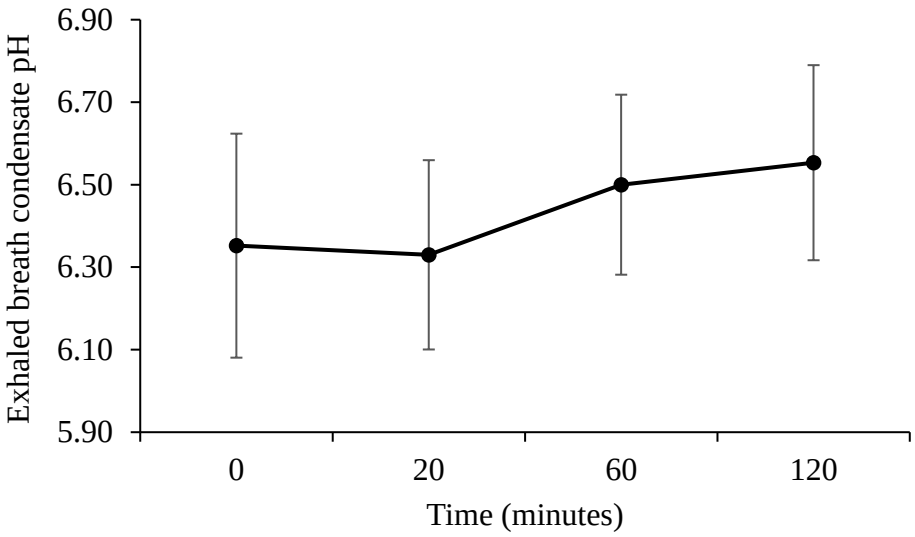

Figure 5.4: Exhaled breath condensate pH from baseline (0 minutes) to 20, 60, and 120 minutes post EVH-test.

5.4. DISCUSSION

5.4.1. Summary of key findings

The mean fall in FEV_1 from pre to post EVH-test was moderate (31%) in severity, while 2/9 (22%) experienced severe bronchoconstriction (≥ 50% drop in FEV_1). However, despite evident HIB, IL-13 and TSLP were undetectable in EBC samples. EBC pH did not change from to post EVH test in non-deaerated samples.

5.4.2. IL-13 and TSLP concentrations

IL-13 in EBC has been previously measured using the Ecoscreen at concentrations ranging between 5-20 pg/ml in mild asthma patients, and 4 pg/ml in healthy controls (Tufvesson et al, 2005; Matsunaga et al, 2006). IL-13 in EBC has also been measured in paediatric asthma patients and healthy controls using glass condenser systems at concentrations of 80.8 pg/ml and 75.7 pg/ml, with high detection rates of 97 and 100% for asthma and control participants (Roebroeks et al, 2010). The significantly higher IL-13 concentration in the paediatric cohort may be related to a greater asthma severity as 49% of the paediatric cohort had poor asthma control. Moreover, the pathophysiology of childhood and adult asthma is not the same, and an optimized glass condenser system may provide greater recovery of IL-13 in EBC than the Ecoscreen. Regardless, even the lower concentrations found in asthma and control participants in the above-mentioned studies are above the lower limits of detection of the assay used in the present study (0.15 pg/ml).

A TSLP concentration of 39 pg/ml has also been measured, via ELISA, in EBC samples collected using the EcoScreen from patients with asthma (Gluck et al, 2016). This concentration is also well above the minimum detection limit of the assay used in the current study (3 pg/ml). Additionally, Gluck et al (2016) reported that only 2 of 53 participants had a

TSLP concentration in EBC that was below the minimum detection limit of the assay (4.935 pg/ml). Compared to the participants in the present study, those tested by Gluck et al (2016) had a greater asthma severity: 32% of patients were classified as uncontrolled under the GINA recommendations, whereas 43% were on high doses of budesonide and therefore were likely to have marked airway inflammation. Interestingly, in the Gluck et al (2016) study, subgroups of controlled asthma patients and healthy controls had TSLP concentrations in EBC of 41 pg/ml and 13 pg/ml, respectively, which are also well above the limit of detection for the assay used in the present study. This suggests that the lack of detection of TSLP in EBC in the present study is probably not due to the degree of asthma severity.

The baseline blood eosinophil counts of 0.28×10^9/ L suggests that the participants in the present study had airway eosinophilia. A blood eosinophil count above $\geq 0.27 \times 10^9$/L has been shown to be highly predictive of sputum eosinophilia (≥ 3%) (Wagner et al, 2015). Given the close relationship between blood and sputum eosinophilia as predictors of the type-2-high asthma phenotypes it is surprising that type-2 cytokines such as TSLP and IL-13 were not detectable within the EBC of our cohort. In addition, EBC samples were taken post EVH-test with all participants experiencing HIB, with and 2/9 participants experiencing severe bronchoconstriction (≥ 50% drop in FEV_1). As such, the lack of IL-13 and TSLP detection in EBC samples is unlikely to be due to no presence within the airways of the present study participants.

Following an EVH test in participants with HIB, cysteine leukotrienes and adenosine in EBC can increase ~2-fold (Tecklenburg-Lund et al, 2010; Csoma et al, 2005). TSLP is released from airway epithelial cells upon epithelial damage, a process involved in the pathogenesis of both exercise-induced bronchoconstriction (EIB) and HIB that is induced by osmotic and luminal wall shear stress (Lai et al, 2013). TSLP release is also greater in airway epithelial cells from patients with EIB compared to asthma patients without EIB and healthy controls

upon such stresses (Lai et al, 2013). Concomitantly, IL-13 plays a pivotal role in AHR by initiating airway smooth-muscle contraction (Wills-Karp et al, 1998; Grunig et al, 1998). While TSLP and IL-13 have only been measured in the asthma without HIB phenotype, and not the asthma with HIB phenotype, it would be expected that these would mirror other airway inflammatory biomarkers and increase post EVH test as both are linked to the pathophysiology of HIB and bronchoconstriction.

To summarise, the lack of detection of IL-13 and TSLP in EBC in the present study contrasts previous studies reporting substantial concentrations of both cytokines in asthma patients and healthy controls. These discrepancies may be explained by between-study differences in the employed EBC collection device (RTube vs. Ecoscreen), which may affect the validity of cytokine measurement in EBC.

5.4.3. EBC pH

EBC pH was unchanged after the EVH test (20 – 120 mins). This concurs with Mickleborough et al (2013), the only other study to assess EBC pH pre to post EVH test, who found no change in non-deaerated EBC pH from pre to post EVH test in participants with asthma and HIB. Baseline EBC pH was lower in the present study compared to Mickleborough et al (2013) (6.35 vs 6.6), which may be explained by differences in EBC collection device. Specifically, Mickleborough et al (2013) used the Ecoscreen device, which has been shown to result in an EBC pH ~0.3 higher than the pH of EBC collected using the RTube device (Prieto et al, 2007). EBC pH was also lower than previously reported with EBC collection using the RTube device (7.55) (Soyer et al 2006). This is likely due to differences in sample processing with samples in the present study not deaerated compared to deaerated samples in previous reports. While there is no current consensus on which method

is best (Horvath et al, 2017) deaerating EBC samples by bubbling with an inert gas reduces the carbon dioxide (CO_2) partial pressure (Rosias, 2012). This should reduce the cofounding effect of CO_2 on sample pH and possibly result in the pH of EBC being more reflective of the respiratory lining fluid. However, deaeration does not completely remove CO_2 from EBC and may influence EBC composition (Effros et al, 2012; Grob, Aytekin, & Dweik, 2008). CO_2 can be accounted for by loading the sample with CO_2 whilst periodically measuring pH and CO_2 tension (PCO_2) simultaneously using a blood gas analyser. EBC pH can then be determined for a given PCO_2 using regression analysis (Horvath et al, 2017). A limitation of the present study is using non-deaerated samples instead of analysing samples for pH using the CO_2 loading technique.

5.4.4. Hypothesis on the lack of IL-13 and TSLP detection

There is evidence to support that the ability to detect biomarkers in EBC may depend on the collection device used. Total protein content of EBC is approximately 2-fold lower when EBC is collected with the RTube compared to the Ecoscreen (Lui et al, 2007; Czebe et al, 2008). Additionally, Czebe et al (2008) reported a cyst-LTs concentration of 66 pg/ml in EBC samples collected with the Ecoscreen from healthy participants, whereas cyst-LTs were not detected in any EBC samples collected with the RTube. Similar observation was made by Soyer et al (2006) who reported cyst-LT concentrations almost 10-fold lower in EBC samples collected with the RTube compared to the Ecoscreen (21.6 pg/ml vs 205.4 pg/ml). Similarly, lower concentrations of eotaxin were found in EBC collected with RTube compared to the Ecoscreen (11.7 pg/ml vs 17 pg/ml) (Soyer et al, 2006). Therefore, when compared with the Ecoscreen, the RTube underperforms in collecting EBC with measurable levels of protein and airway inflammatory biomarkers. Previous studies measuring IL-13 and TSLP in EBC

have utilised the Ecoscreen for EBC which may explain why they were able to measure these biomarkers.

Temperature may contribute to the RTubes poor performance in collection EBC with measurable biomarkers. The temperature within the RTubes condensing chamber has been reported to rise from -20°C to 20°C during a 10-minute EBC collection, whereas the Ecoscreen maintains -15°C throughout EBC collection (Soyer et al, 2006). The higher temperatures experienced by the RTube may limit condensation if the temperature of the chamber increases above the dew point. Moreover, a greater temperature may also affect the stability of temperature sensitive biomarkers. However, a limitation of the study by Soyer et al (2006) is that because temperature was measured within the condensing chamber rather than in/on the condenser wall surface, the measured temperature rise may be partly attributed to the participants expired air.

The surface temperature of the condensing chamber is more informative as this is where surface condensation occurs and where biomarkers are trapped until the sample is pooled and stored. Our data from Chapter 3: General Methods (Section 3.6. Exhaled breath condensate collection) shows that the condensing chamber surface temperature only increases to 7°C during a 10-minute EBC collection and remains below 0°C for the first 5 minutes. The temperature/duration stability of TSLP and IL-13 in EBC samples is unknown but in blood plasma IL-13 is stable if refrigerated (~4°C) for up to 4hrs (Simpson, Kaislasuo, Guller, & Pal, 2020). It is thus possible that the low temperatures (≤ 7°C) and short exposure (5 – 10 minutes) would not cause significant degradation of IL-13 and TSLP within EBC samples collected with the RTube. In addition, protein content of EBC collected with the RTube is not different when the cooling sleeve is cooled to -20°C or -70°C prior to collection (Czebe et al, 2008). Chamber surface temperature during a standard EBC collection with the RTube as used in the present study (-20°C, 10 minutes) does not therefore affect protein recovery.

Collectively, this suggests that rises in chamber temperature are unlikely to contribute to the lack of IL-13 and TSLP detection in EBC collected using the RTube device.

EBC device material, which may comprise glass, silicone, or polypropylene, may also affect biomarker recovery (Rosias et al, 2006; Rosias et al, 2008). Polypropylene, which is a plastic from which the RTube device is constructed, results in lower recovery of albumin (40%) and lower concentrations of 8-isoprostane (0.5 pg/ml) compared to glass (75%, 3.0 pg/ml) and silicone (40%, 9.2 pg/ml) (Rosias et al, 2006). Substantial adsorption of various proteins to plastic ware is evident (Bratcher & Gaggar, 2013; Goebel-Stengel, Stengel, Tache, & Reeve, 2011). Polypropylene possesses non-polar and hydrophobic properties, which can destabilize proteins leading to conformational changes and protein surface adsorption (Anand, Sharma, Dutta, Kumar, & Belfort, 2010). Below a critical protein concentration adsorption can leave a sample void of protein (Weikart, Breeland, Taha, & Maurer, 2019). The dilution of respiratory lining fluid within EBC is estimated between 2,000 and 10,000-fold (Horvath et al, 2005), which means that even small amounts of protein adsorption could drastically reduce protein/biomarker recovery. In addition, during sample collection and removal an aluminium sleeve/plunger is placed over/inserted into the RTubes condensing chamber. The separation of aluminium and polypropylene along the triboelectric series suggests polypropylene may become positively charged if friction is generated between the two materials (Diaz & Felix-Navarro, 2004). If the condensing chamber became positively charged, this would enhance adsorption of negatively charged proteins. TSLP and IL-13 have an isoelectric point of 9.75 and 8.69, making both negatively charged at the pH range of EBC found in the present study (6.33 – 6.55).

While device material seems a likely contributor to poor biomarker recovery sample analysis techniques may also be influential. While not specific to IL-13 and TSLP, direct comparison between a radioimmunoassay (RIA) and enzyme immunoassay (EIA) for the measure of

thromboxane A_2 in EBC have been made (Huszar et al, 2005). Detection rates for thromboxane A_2 are 100% with RIA compared to 75% for EIA. While both IL13 and TSLP have been previously measured in EBC using ELISAs (Tufvesson et al, 2006; Gluck et al, 2016), as with the present study, the ELISA manufacturers are different. The influence of this is unknown but may affect the detection of IL-13 and TSLP in EBC samples.

5.4.5. Conclusion

In conclusion, IL-13 and TSLP in EBC collected with the RTube from participants with asthma and HIB were not detected by ELISAs. RTube device material and/or analysis techniques may explain the lack of biomarkers detection. Caution is therefore warranted if using the RTube device to collect EBC samples for the measurement of IL-13 and TSLP. The next section assesses potential techniques to improve biomarker recovery in EBC collected with the RTube device with focus primarily on attempts to reduce protein adsorption and increasing sample concentration.

Chapter 6 – Exhaled breath condensate sample collection and analysis: method development and optimisation

6.1. Introduction

In chapter 4, it was not possible to detect IL-13 or TSLP in EBC samples collected with the RTube device and analysed using ELISAs. Device material (polypropylene) may contribute to poor biomarker recovery by enhancing protein adsorption, and/or analysis techniques may have been inadequate for the measurement IL-13 and TSLP in EBC samples. The high dilution of EBC with water vapour was noted as a barrier to biomarker detection in EBC samples collected via any device. These factors are potential barriers to measuring cytokines in EBC samples collected with the RTube device. We have tried to tackle these issues by undertaking a pilot study to optimise the collection and analysis of EBC samples collected using the RTube device.

Proteins readily adsorb to solid surfaces due to their amphiphatic nature. Substantial adsorption of proteins to plastics is evident (Bratcher et al, 2013; Goebel-Stengel et al, 2012). The RTube condenser chamber is made of the plastic polypropylene which additionally possesses non-polar and hydrophobic properties which contribute to protein surface adsorption (Anand et al, 2010). Protein adsorption to the RTube condensing chamber would reduce protein recovery in EBC samples and drastically reduce concentrations within such a diluted sample. Surface protein adsorption to plastics can be reduced by coating plastic ware with bovine serum albumin (BSA) which reduces non-specific binding of target proteins (Bratcher et al, 2013; Goebel-Stengel et al, 2012).

Coating polypropylene with 1% BSA greatly diminishes the binding of surfactant protein-D (SP-D) (Bratcher et al, 2013). While the reduced adsorption of SP-D cannot be directly transferred to other proteins it suggests that protein adsorption can potentially be inhibited by coating the RTube condenser with 1% BSA before sample collection. BSA in combination with Tween-20 may additionally improve detection rates of cytokines with the RTube device

(Tufvesson et al, 2005). Tween-20 is a non-ionic detergent frequently used to reduce adsorption particularly to hydrophobic surfaces, such as polypropylene. Coating the RTube with BSA and Tween-20 may therefore be an effective way to improve biomarker recovery.

The type of assay used to analyse biomarkers in EBC samples may also affect biomarker recovery. High heterogeneity in the use of commercially available assays to measure biomarkers in EBC makes it difficult to identify assay kits with high efficacy. Multiplex immunoassays are commonly used to measure multiple biomarkers in EBC (Sack et al, 2006; Schumann et al, 2006; Rosias et al, 2008; Robroeks et al, 2009; Stiegel, Pleil, Sobus, Morgan, & Madden, 2015). They show good inter-study reproducibility for the detection of biomarkers and have been used to measure cytokines from EBC collected using different devices (Sack, 2005; Rosias, 2008; Robroeks et al, 2010; Stiegel, 2014). Both IL-5 and TNF-α were selected for measurement in this pilot study as both had been measured using a liquid bead array multiplex system by Luminex Corporation and had been measured in EBC at >95% detection rates in both asthma and healthy control participants (Rosias et al, 2008; Robroeks et al, 2010). The use of these pre-tested assays and cytokines can give confidence knowing that if IL-5 and TNF-α are represent in EBC samples they will be measureable.

Another important consideration is that EBC in an incredibly diluted biological sample. Over 99% of EBC is estimated to be water vapour containing fragments of non-volatile molecules presumed to originate from respiratory lining fluid as respiratory droplets. These are generated during the breathing cycling and are subsequently trapped by condensing water vapour (Horvath et al, 2005). The ability to increase biomarker concentrations with EBC samples therefore requires either a greater production and exhalation of respiratory lining fluid droplets or the removal of water vapour from the sample. Dehydration of EBC samples can be easily achieved by vacuum centrifuge and has been used previously to increase EBC concentrations 8 to 10-fold (Tufvesson et al, 2005; Sack et al, 2005).

Respiratory droplet formation can be increased by manipulating the breathing cycle and respiratory flow rates. The bronchiole fluid film burst model suggests respiratory droplets are formed during breathing cycles by continuous bronchiole closure and reopening during expiration and inhalation, which results in the amalgamation and subsequent bursting of respiratory lining fluid. This bursting creates respiratory lining droplets which leave the airways during exhalation (Johnson & Morawska, 2009). Manipulating the breathing pattern may therefore facilitate greater bronchiole closure, respiratory droplet formation and transportation out of the airways.

Deep exhalations result in a 4 to 6-fold increase in exhaled particles with exhalation to residual volume (RV) increasing concentrations between 2 and 18-fold compared to functional residual capacity (Johnson & Morawska, 2009; Almstrand et al, 2010). Additionally, breath holds of 5s and 10s at RV increase exhaled particle concentrations 63% and 110%, respectively, due to a time delay in bronchiole closure (Holmgren et al, 2012). Furthermore, breath holds at TLC of 5s and 10s reduce concentrations by 37% and 55%, respectively (Holmgren et al, 2012). Breathing cycles from TLC to RV enhancing exhaled particle production 70-fold compared to tidal breathing (Fabian, Brain, Houseman, Gern, & Milton, 2011). A breathing cycle incorporating breathing from TLC to RV with rapid inhalations, breath holds at RV, and no breath hold at TLC could therefore be adopted during EBC collection to increase the production of respiratory droplets and potentially increase the concentration of EBC samples.

This pilot study looks to optimise the methods of EBC collection with the RTube device and subsequent sample processing. The present pilot study assesses the effect of coating the RTube device with 1% BSA and 0.01% Tween-20 prior to sample collection and concentrating EBC samples by vacuum centrifugation on the measurement of IL-5 and TNF-α analysed using multiplex assay.

6.2. METHODS

6.2.1. Participants

Seven male participants took part, four healthy controls and three individuals with a doctor's diagnosis of asthma. Participant's characteristics are summarised in table 6.1.

Table 6.1: Asthma and healthy control participant's characteristics.

Characteristics	**Asthma**	**Control**
Age (years)	28 ± 5	26 ± 4
Height (cm)	181 ± 3	183 ± 6
Weight (kg)	89 ± 14	75 ± 4
FVC (% Predicted)	97 ± 9	100 ± 4
FEV_1 (% Predicted)	80 ± 9.4	101 ± 7
PEF (% Predicted)	96 ± 9	113 ± 6
$FEF^{25-75\%}$ (% Predicted)	50 ± 4.24	107 ± 15.6
Severe asthma (ratio)	2/3	N/A

BMI = body mass index; FEV_1 = force expiratory volume in one second; FVC = forced vital capacity; PEF = peak expiratory flow; $FEF_{25-75\%}$ = forced expiratory flow at 25 to 75% of force vital capacity. Severe asthma assessed in accordance with the British Thoracic Society guidelines.

In the lead up to trials participants adhered to the restriction guidelines set out in Chapter 3: General Methods (Section 3.3. Participants restrictions prior to experimental trials

6.2.2. Experimental trial

Participants completed baseline pulmonary function. Two EBC samples were subsequently collected with each sample separated by 5 minutes. In a randomised order one sample was collected in an RTube coated with 1% BSA and 0.01% Tween20 with the other collected in an uncoated RTube. Following sample collection, samples were separated into two aliquots. One aliquot was stored immediately at -80°C until analysis, whereas the second aliquot was dried in a vacuum centrifuge, stored at -20°C, and re-suspended in 60µL of assay buffer (8-fold concentration) ready for analysis. All samples collected in the coated RTube were handled and stored in plastic ware that were also coated. This led to four conditions in which EBC were collected and treated:

1. Collection in an uncoated RTube, not concentrated.
2. Collected in an uncoated RTube, concentrated.
3. Collected in a coated RTube, not concentrated.
4. Collected in a coated RTube, concentrated.

6.2.3. Pulmonary function

Pulmonary function was assessed via spirometry. Pulmonary function was conducted in accordance with the procedures outlined in Chapter 3: General methods (Sections 3.4. Pulmonary function – Dynamic spirometry).

6.2.4. Exhaled breath condensate collection

EBC samples were collected with the RTube device. Participants adopted a specific breathing cycle involving a four second inhalation from RV to TLC followed by an immediate seven second exhalation back to RV. At RV participants maintained a breath hold for 4 seconds before continuing the breathing cycle. The breathing cycle was controlled using an audio metronome. Participants maintained this breathing cycling until they had expired 100L of air, which has been shown to improve the reproducibility of biomarkers in EBC compared to standardisation based on the duration of sample collection (Reinhold & Knobloch, 2009). The RTube was connected to a dry gas meter using a 1 metre length of corrugated tubing to monitor expired volume.

6.2.5. Dehydration of exhaled breath condensate samples

EBC samples were dehydrated via vacuum centrifugation. For cytokine measurements, the pellet was re-suspended in 60µL of assay buffer which caused an 8.3-fold increase in concentration.

6.2.6. Coating of the RTube device

The RTube was coated using a protocol previously optimized to reduce protein adsorption by BSA (Bratcher et al, 2013). Briefly, BSA and Tween-20 were reconstituted in deionized water at a concentration of 1% and 0.01%, respectively. The RTube condenser was filled with this solution and left for 24-hours at room temperature prior to EBC collection.

6.2.7. Analysis of IL-5 and TNF-α

IL-5 and TNF-α were assayed using a multiplex immunoassay (Bioplex, Bio-rad, UK). The lower limits of detection for IL-5 and TNF-α were 0.89pg/ml, and 1.13pg/ml. All samples were analysed on one 96-well plate.

6.3. RESULTS

6.3.1. Exhaled breath condensate IL-5 and TNF-α concentrations

IL-5 and TNF-α concentrations were below the assays lower limit of detection for all EBC samples in healthy controls and participants with asthma.

6.4. DISCUSSION

IL-5 and TNF-α were undetectable in EBC samples collected using the RTube device in participants with asthma and healthy controls. Importantly, both cytokines were undetectable in EBC despite the samples being concentrated 8.3-fold, and surface protein adsorption being reduced by pre-coating the RTube device in 1% BSA and 0.01% Tween-20 for 24 hours prior to EBC collection.

Both IL-5 and TNF-α have previously been measured in EBC collected from health adults, healthy children, and children with asthma (Sack et al, 2005; Rosias et al, 2009; Robroeks et al, 2010; Stiegel et al, 2014). Rosias et al (2009) reported TNF-α concentration in EBC of 38.8 pg/ml in children with asthma, and 41.7 pg/ml in healthy controls. They additionally found IL-5 concentrations in EBC of 17.5 pg/ml in children with asthma, and 16.7 pg/ml in healthy controls. Similarly, Robroeks et al (2010) reported TNF-α concentrations in EBC of 13.2 pg/ml in children with asthma, and 24.6 pg/ml in healthy controls. They additionally found IL-5 concentrations in EBC of 9.8 pg/ml in children with asthma, and 13.2 pg/ml in healthy controls. Detection rates in both studies for TNF-α and IL-5 were above 95% for both asthma and healthy control participants and neither study concentrated samples. Both these studies used the same analysis platform (liquid bead array multiplex assay; Luminex Corporations) as the present study. Interestingly, all previous studies measuring TNF-α and IL-5 have used the Ecoscreen for EBC collection. . Therefore, the lack of detection in the present study using an analysis platform successfully used previously to detect TNF-α and IL-5 suggests that the device used in the present study (RTube) is responsible for lack of biomarker detection.

Coating collection devices with BSA and Tween-20 improves cytokine concentrations in EBC and reduce surface adsorption of proteins to polypropylene, which is the material used for the condensing chamber of the RTube (Tuffvesson et al, 2005; Sack et al, 2005; Bratcher et al, 2013). The loss of cytokines through surface adsorption has been documented at 10.6% in the Ecoscreen device which can be reduced to 1.8% through coating the device with 1% BSA (Sack et al, 2005). In the present study 1% BSA and 0.01% Tween-20 was did not result in TNF-α or IL-5 detection in EBC samples collected with the RTube. The condensing chamber of the Ecoscreen is coated with Teflon. Cytokine adsorption may well occur to a greater degree in the RTube than the Ecoscreen due to the differences in condenser material, which coating with BSA and Tween20 cannot overcome.

It is well established that proteins adsorb to polypropylene and at very low concentrations protein adsorption can devoid a sample of protein (Bratcher et al, 2013). EBC is a very diluted biological sample such that the total protein content of EBC samples collected with the RTube device has been reported at 2.65 μg/ml, which is lower than the reference range in blood (6-8 g/dl) (Czebe et al, 2008). In the present study samples were concentrated >8-fold however, this was post sample collection which does not affect protein concentrations of EBC within the RTube device. While protein adsorption to the RTube device is a possible culprit it was not measured in the present study and therefore can only be suspected. In the case that protein adsorption is affecting the measurement of cytokines with the RTube, coating the device with surfactants may be a potential method to test to reduce protein adsorption in the future. This is because surfactant-polypropylene interactions are greater than protein-polypropylene interactions (Duncan, Lee, & Warchol, 1995).

The lack of biomarker detection in EBC could also be due to the open-ended design of the RTube device. Open-ended devices allow expired air to pass through and out of the device with subsequent loss of large amounts of uncondensed expired air (Rosias et al, 2012). It is

estimated that in open-ended devices only 30% of expired air is condensed, dependant on the condensing chambers surface area (Rosias et al, 2010). Closed-ended devices allow recirculation of uncondensed air allowing for a greater yield. As such, EBC volume is >2-fold higher when breath recirculation is applied to a device compared to no breath recirculation (Rosias et al, 2010). Breath recirculation does not however effect cytokine concentrations (Rosias et al, 2010). In the RTube device however, the inner surface of the condensing chambers is exposed to ambient air. This may allow surface condensation of ambient air and partial saturation of the condensing chamber with non-exhaled condensate prior to a participant's sample collection. This could limit the subsequent condensation of the participants expired air and result in the dilution of the participants EBC with pre-existing condensed ambient air.

6.4.1. Conclusion

In conclusion, TSLP, IL-13, IL-5, and TNF-α are undetectable in EBC collected with the RTube device. In addition, coating the RTube in 1% BSA and 0.01% Tween20, alongside concentrating samples 8.3-fold did not result in detectable levels of IL-5 and TNF-α. It is suspected that the RTube device material and design reduce its ability to collect EBC with measurable levels of TSLP, IL-13, IL-5, and TNF-α, all of which have been measured with other devices using the same analysis platforms. Care must be taken to ensure high compatibility between the biomarkers of interest and the EBC collection device used. While EBC pH is easily measured with the RTube we recommend researchers to exercise caution when using the RTube device for the measurement of cytokines in EBC. Collection of EBC using the RTube device is therefore not a suitable method to measure airway inflammation in the assessment of B-GOS supplementation in chapter 7.

Chapter 7 – The effect of prebiotic, Bimuno-galactooligosaccharide, supplementation on pulmonary function, asthma control, and systemic inflammation in participants with asthma and hyperpnoea-induced bronchoconstriction.

7.1. INTRODUCTION

Asthma is a respiratory disease accompanied by reversible expiratory airflow limitation and characterised by symptoms of wheezing, shortness of breath, and chest tightness that vary over time in their presence and intensity (GINA Report, 2020). Asthma drastically limits daily activity and has a negative effect on quality of life of those who suffer from the disease. Approximately 8% of the UK population have asthma (~5,280,000) of which 200,000 are estimated to suffer with serve asthma requiring the highest doses of medication to control their asthma or remains uncontrolled while being treated with high dose medication (BTS/SIGN asthma guidelines 2019). In addition, asthma treatment costs the NHS one billion pounds per year while on average one person per day dies from asthma in the UK (Asthma UK accessed 18/09/2020). As such asthma is a major burden to patients and the health care system.

The pathophysiology of asthma is commonly characterised by airway hyper-responsiveness (AHR), airway inflammation, and airway remodelling. AHR is an increased sensitivity of the airways to external triggers (e.g. allergens, irritants, exercise) which do not typically affect the airways of healthy individuals but can induce bronchoconstriction in those with asthma (Vernon et al, 2012). Approximately 40% of asthma patients suffer from eosinophilic airway inflammation (≥3% sputum eosinophils), 20% from neutrophilic airway inflammation (≥60% sputum neutrophils), while 40% have a paucigranulocytic airway inflammatory profile characterised by normal sputum white blood cells counts and percentages (Schleich et al, 2013; Ntontsi et al, 2017; Simpson et al, 2006). Both AHR and airway inflammation share a bidirectional relationship resulting in the initiation and exaggeration of each process by the other (Chung, 2000). If left uncontrolled features of airway remodelling such as airway smooth muscle (ASM) hypertrophy, sub-basement membrane thickening, airway angiogenesis and epithelial disruption can ensure (Chiappara et al, 2001). This can enhance

AHR and airway inflammation during future exposure to triggers thereby exacrbating expiratory airflow limitation and symptoms and increasing the risk of asthma exacerbations. Controlling these pathophysiological features is therefore a primary focus of asthma treatment.

For over 40 years the mainstay of asthma treatment has involved the use of inhaled corticosteroids (ICS) and short and long acting β_2-agonists (Chu & Drazen, 2005). ICS are a maintenance therapy which act by switching off activated inflammatory genes, which reduces the number and activation state of inflammatory cells in the airways helping to control airway inflammation (Barnes, 2010). B_2-agonsits act on β_2 adrenoceptors located on ASM cells resulting in ASM relaxation which helps to resolve bronchoconstriction (Billington, 2017). Despite their effectiveness frequent use of β_2-agonist is an indicator of poor asthma control and can lead to worsening bronchoconstriction and mast cell degranulation following allergen exposure (Swystun et al, 2000) while corticosteroid use can have undesirable side effects (Barnes, 2010). Furthermore, a poor response/no response to ICS is seen in severe asthma patients. New therapies therefore need investigating which may help reduce to use of ICS and β_2-agonists in asthma patients and target alternative pathways to improve asthma control in severe asthma patients.

The gut microbiota has been recognised as a potential therapeutic target in combatting inflammatory disorders such as asthma via the gut-lung axis (Dang et al, 2019; Budden et al, 2017). Gut microbes can interact directly with the gut-associate lymphoid tissue via pattern recognition receptors and indirectly by the production of metabolites to influence immune maturation both locally and at distal body sites. Multiple strains of Bifidobacterium and Lactobascillus have been shown to modulate intestinal dendritic cells to promote the maturation of T regulatory (Treg) cells and attenuate the development of inflammatory T helper 2 (Th2) cells resulting in enhanced regulation of inflammation (Baba et al, 2008; Smits

et al, 2005; Mohamadzadeh et al, 2005; Hoarau et al, 2006; Hart et al, 2004). Treg cells matured in such a manner can subsequently translocating to the airways helping to control airway inflammation (Narvarro et al, 2011; Strickland et al, 2011). The short chain fatty acids (SCFAs) acetate, propionate and butyrate produced by the gut microbiota additionally promote the maturation of Treg cells via G-coupled protein receptor signalling and histone deacetylase inhibition (Koh et al, 2016). In addition to influencing immune maturation at the site of the gut SCFAs are adsorbed into the circulation thereby inducing immunomodulation at distal sites such as the lung and bone marrow. SCFA can alter bone marrow haematopoiesis of DCs and macrophage precursors resulting in anti-inflammatory DC and macrophage phenotypes superseding the airways (Trompette et al, 2018). Favourably altering the composition of the gut microbiome and increasing its production of SCFAs may therefore have therapeutic potential to control airway inflammation in asthma.

Altering the gut microbiota can be achieved through supplementation with dietary pro and prebiotics. Dietary probiotics are live microorganisms ingested primarily in the form of capsules or probiotic yoghurts which when ingested in adequate amounts alter the gut microbial composition and confer a health benefit to the host (Martin & Langella, 2019). Probiotics have been assessed as a treatment for asthma in children by Gutowski et al (2011). In a between and matched groups design forty-six children (age: 4-10 years) received daily dose of either a probiotic Trilac capsule (1.6 × 109 lactic acid bacteria cells: Lactobacillus acidophilus – 37.5%, Bifidobacterium bifidum – 37.5% and Lactobacillus delbrueckii subsp. bulgaricus – 25%) or placebo for 12 weeks. Children receiving Trilac benefited from improved pulmonary function, reduced bronchodilator use and asthma exacerbations compared to the placebo group. Additionally, HLA-DR expression on monocytes decreased and CD8CD45RA^{+} lymphocytes decreased in PBMC in children receiving Trilac compared to placebo. To date no probiotic studies have been conducted in adult asthma patients. Whilst

probiotics beneficially alter the gut microbiota the production of SCFAs by the gut microbiota primarily relies on the fermentation of insoluble dietary fibre in the large intestine (Macfarlane and Macfarlane, 2012; Koh et al, 2016). In this case prebiotics are arguably more effective as they alter the composition of the gut microbiota similar to probiotics but additionally act as a substrate to increase the activity and metabolite production of the gut microbiota via fermentation.

Dietary prebiotics are defined as a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring a benefit upon host health (Gibson et al, 2010). The most recognised and well-studied dietary prebiotics are inulin type fructans, lactulose, and trans-galactooligosaccharides (T-GOS) (Gibson et al, 2010). To date only two studies have been performed to evaluate the effect of prebiotics in adult human asthma patients. McLoughlin et al (2019) conducted a randomised, double-blinded, placebo controlled cross-over trial in 17 adults with stable asthma who consumed 12g/d of inulin or placebo for 7 days. Asthma control questionnaire 6-item scores were significantly reduced with inulin (0.3) compared to placebo (0.8). Sputum percentage eosinophils were significantly reduced with inulin (1.5%) compared to placebo (2.3%). Furthermore, following inulin supplementation HDAC9 gene expression was significantly reduced in sputum cells alongside increases in *Anaerostipes* bacteria in faecal samples, a SCFA producing genus (Riviere et al, 2016). SCFAs have HDAC inhibitory abilities (Smith et al, 2013; Park et al, 2015) while HDAC9 down regulates Treg cell function. This suggests a potential mechanism by which inulin ingestion increases SCFA production, enhancing anti-inflammatory mechanisms leading to reduced sputum percentage eosinophils and improved asthma control.

Williams et al (2016) conducted a double-blind, randomised, placebo controlled cross-over study in ten adults with mild to moderate asthma and hyperpnoea-induced

bronchoconstriction to evaluate the effect of 21 days supplementation with 11g/d of Bimuno-galactooligosaccharide (B-GOS) (48% GOS content). B-GOS significantly attenuated hyperpnoea-induced bronchoconstriction (HIB) by 40% comparison to placebo. B-GOS supplementation also abolished the increases in TNF-α observed following an EVH test. In addition, resting serum concentrations of CCL17 and CRP were significantly reduced with B-GOS compared to placebo. In mice models of asthma GOS supplementation has been shown to attenuate HDM-induced airway eosinophilia and dampen markers of type-2 inflammation in the airways (Verheijden et al, 2015a; Verheijden et al, 2015b; Verheijden et al, 2015c; Verheijden et al, 2016; Verheijden et al, 2018). The protective effect of GOS is partly Treg cell dependant as anti-CD25 antibodies abolish the protective effect of GOS (Verheijden et al, 2016). This suggests GOS supplementation acts on mechanisms which regulate inflammation helping to attenuate airway inflammation in asthma. Furthermore, GOS in combination with budesonide is more effective in attenuating type-2 inflammation in lung homogenates supernatant from HDM-challenged mice than either treatment alone (Verheijden et al, 2018). As such GOS could be an affective adjunct treatment to current ICS therapies to improve asthma control.

GOS's are produced by the enzymatic activity of β-galactosidase on lactose. B-GOS is produced from the enzymatic activity of β-galactosidase derived from the bacteria B.bifidum NCIMB 41171 (Tzortis, 2005). The utilisation of β-galactosidase from B.bifidum NCIMB 41171 in the manufacturing process gives B-GOS unparalleled selectivity in increasing the Bifidum genus and allows for species targeting which promotes increased B.bifidum growth rates compare to other GOS products, FOS and inulin in faecal batch culture models, alongside enhanced SCFA production (Tzortis et al, 2005). In the elderly 5.5g/d of B-GOS (48% GOS content) for 5-weeks significantly increases healthy faecal bacterial groups including *Bifidobacterium* spp., *Lactobacillus-Enterococcus* spp., and the *C. coccoides*–

E. rectale group compared to baseline and placebo treatment (Vulevic et al, 2008). In healthy young adults, 3.5g/d and 7g/d of B-GOS (48% GOS content) for 1-week significantly increases the prebiotic index, a score that quantifies the prebiotic effect, and increases faecal bifidobacteria numbers compared to baseline and placebo (Depeint et al, 2008). B-GOS is therefore very bifidogenic and possesses high selectivity in inducing increases in bifidobacteria species and the ability to increase microbial activity leading to greater SCFA production. These qualities alongside the findings by Williams et al (2016) suggest B-GOS has the potential to induce immunomodulation along the gut-lung axis which may help to reduce airway inflammation in asthma and improve asthma control and attenuate HIB.

Research in the therapeutic potential of dietary prebiotics to dampen systemic and airway inflammation and treat asthma is compelling but data is currently lacking from human trials. Thus, the aim of the present study was to evaluate the effect of 21 days supplementation with 3.65g/d of commercially available B-GOS (80% GOS content) on asthma control, pulmonary function, and the severity of HIB using a double-blind, between groups, placebo controlled design. Secondly it was to investigate the effect of B-GOS on systemic markers of airway inflammation. Systemic markers of airway inflammation to be measured included differential white blood cell counts (WBC) including eosinophils, neutrophils, lymphocytes, monocytes and basophils. Blood serum cytokines and chemokines concentrations including, IL-13, thymic stromal lymphopoietin (TSLP), eotaxin-1/chemokine ligand 11 (CCL11), Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (CCL5), monocyte chemoattractant protein-1 (MCP-1), and stem cells factor (SCF). Finally, exhaled breath condensate (EBC) pH.

7.2. METHODS

7.2.1. Participants

Thirteen participants with a doctor's diagnosis of asthma, and hyperpnoea-induced bronchoconstriction as defined by a ≥10% fall in FEV_1 at two consecutive time points following an initial EVH screening test took part in the study. All participants were on steps 1-3 of the British Thoracic Society stepwise guidelines. The study was conducted in accordance with the guidelines in the Declaration of Helsinki, and all procedures were approved by the Nottingham Trent University Human Ethics Committee (Approval no. 581). All participants provided written informed consent.

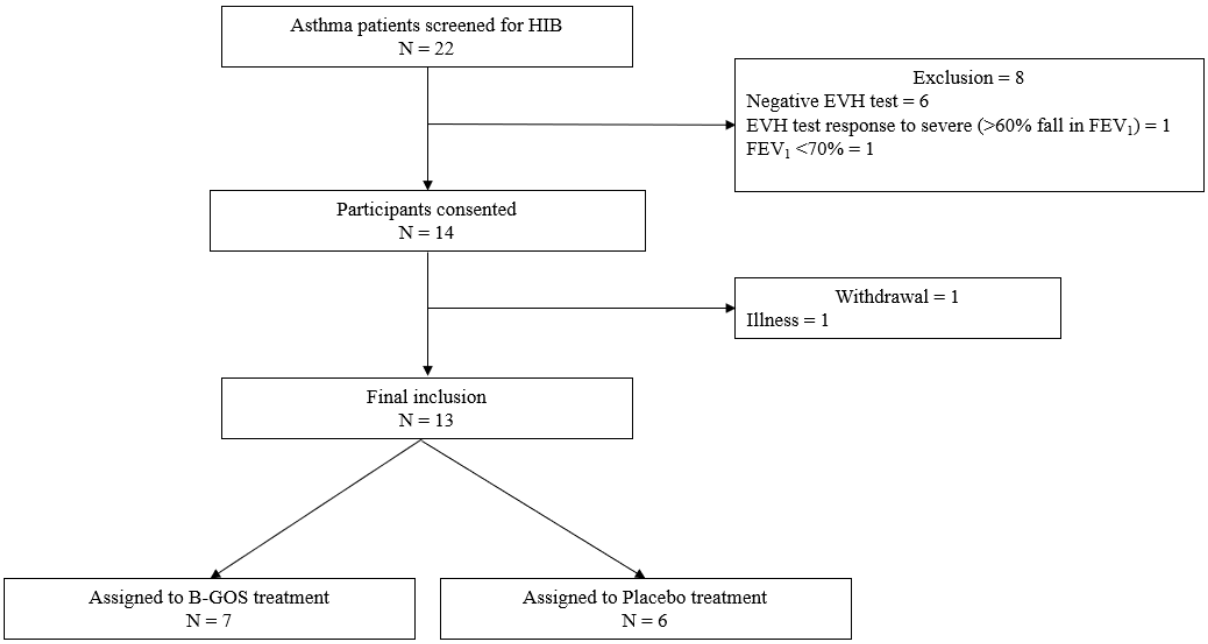

Figure 7.1. Participant flow through study.

Table 7.1: Participant's characteristics of the B-GOS and Placebo groups.

Participants characteristics	**B-GOS group (N = 7)**	**Placebo group (N = 6)**
FEV_1 (L)	4.15 ± 0.89	4.23 ± 0.9
% predicted	90 ± 14	99 ± 9
FVC (L)	5.53 ± 1.04	5.07 ± 0.95
% predicted	102 ± 10	101 ± 12
FEV_1/FVC	0.75 ± 0.07	0.83 ± 0.07
% predicted	88 ± 8	98 ± 8
PEF (L/s)	9.67 ± 1.92	9.41 ± 2.93
% predicted	101 ± 21	101 ± 22
$FEF_{25-75\%}$ (L/s)	3.49 ± 1.11	4.26 ± 1.45
% predicted	73 ± 23	92 ± 25
HIB severity: peak fall in FEV_1 (%)	-18.2 ± 7.4	-19.6 ± 9.6
Blood eosinophil counts (10^9 cell/L)	0.26 ± 0.2	0.21 ± 0.09
% blood eosinophils	3.5 ± 2.2	4 ± 1.7
Blood neutrophil counts (10^9 cell/L)	4.25 ± 1.49	2.9 ± 0.39
% blood neutrophils	60.3 ± 6.2	54 ± 6.1
Blood lymphocytes counts (10^9 cell/L)	1.91 ± 0.34	1.72 ± 0.35
% blood lymphocytes	28.3 ± 4	32.4 ± 7.5
Blood monocyte counts (10^9 cell/L)	0.52 ± 0.15	0.49 ± 0.1
% blood monocytes	7.7 ± 2.41	9.18 ± 1.74
Blood basophil counts (10^9 cell/L)	0.02 ± 0.00	0.03 ± 0.00
% blood basophils	0.3 ± 0.1	0.5 ± 0.1
Short-acting β_2-agonist use (%)	100	100
Inhaled corticosteroid use (%)	86	83

FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; PEF: peak expiratory flow rate; $FEF_{25-75\%}$: forced expiratory flow at 25-75% of FVC.

7.2.2. Experimental design

The study adopted a between groups, double-blind, placebo-controlled design. Participants were assigned to receive either 3.65g/d of B-GOS (80% GOS content) (Clasado Ltd) (N = 7) or placebo (maltodextrin) (Myprotein, UK) (N = 6) for 21 days. At days 0, 14, and 21 participants performed an EVH test with pulmonary function assessed pre and post. Venous blood samples were also collected pre and 30 minutes post EVH test, with exhaled breath condensate (EBC) samples collected pre and 20 minutes post-test. Asthma control was assessed at baseline during day 0 and 21 using the 7-item Asthma Control Questionnaire (ACQ7).

7.2.3. Pulmonary function and EVH test

Pulmonary function was assessed via spirometry. Pulmonary function and the EVH test were conducted in accordance with the procedures outlined in Chapter 3: General methods (Sections 3.4. Pulmonary function – Dynamic spirometry; & Section 3.5. Eucapnic voluntary hyperpnoea test).

7.2.4. Asthma control questionnaire 7-item (ACQ7)

Participants were asked to recall their experiences during the previous week in response to 6 questions (night-time waking, symptoms on waking, activity limitation, shortness of breath, wheeze, and rescue short-acting β_2-agonist use) on a 6-point scale (0 = no impairment; 6 = maximum impairment). A 7th question was calculated from the participants percentage predicted pre-bronchodilator FEV_1 (0 = > 95%; 6 = < 50%). Further details on ACQ7 can be found in Chapter 3: General methods (Section 3.9.2. Asthma control questionnaire 7-item)

7.2.5. Differential white blood cell counts

White blood cell counts (WBCs) and the percentage of blood eosinophils, neutrophils, leukoctyes, basophils, and monocytes were determined using a haematology analyser (XS-1000i, Sysmex, Germany). WBCs were measured on day 0 and day 21 pre and post EVH-test.

7.2.6. Systemic markers of airway inflammation

Serum IL-13 and TSLP were measured on day 0 ad day 21, pre EVH-test and determined using enzyme linked immunosorbent assays (ELISAs) (Abcam, UK). Further details of these procedures are outlined in Chapter 3: General methods (Section 3.8.2. Enzyme-linked immunosorbent assays). The lower limits of detection for the assays were 0.15pg/mL for IL-13 and 3pg/mL for TSLP. The intra-assay coefficient of variation was 1.9% for IL-13 and 1.7% for TSLP. All samples were analysed in duplicate on one 96-well plate for both assays.

Serum eotaxin, chemokine lingand-5 (CCL5), monocyte chemoattractant protein-1 (MCP-1) and stem cell factors (SCF) were measured on day 0 ad day 21 pre and post EVH-test and determined using a multiplex immunoassay (Bio-plex 200; Bio-Rad laboratories limited). Further details of these procedures are outlined in Chapter 3: General methods (Section 3.8.3. Bioplex multiplex assays).

7.2.7. Exhaled breath condensate

EBC samples were collected using the RTube device (Respriatory Tube, COSMED, Oxfordshire, United Kingdom) in accordance with the procedures outlined in Chapter 3: General methods (Section 3.6. Exhaled breath condensate collection). EBC pH was measured

immediately at each time interval in 400µL of non-deaerated EBC samples using a Jenway 3510 pH meter.

7.2.8. Statistical analysis

Data was analysed using statistical software package SPSS for Windows version 26 (SPSS Inc., Chicago, IL, USA). Data were initially assessed for normality using Shapiro-Wilks's test. For data measured pre and post EVH test (e.g. WBC counts and percentages, and serum CCL11, CCL5, MCP-1, and SCF concentrations) baseline data (day 0) for both groups was pooled and analysed using Paired Samples T test if data were normally distributed or using Wilcoxon Signed Ranks test if data were not normally distributed to assess the effect of time (pre vs. post EVH test). If there was no time effect only baseline data (e.g. pre EVH) were subsequently analysed. In normally distributed data with no baseline time effect data were analysed using a two-way mixed model repeated measures ANOVA to assess the effect of day (day 0, day 14, day 21), with a between subject factor of group (B-GOS vs. Placebo). Significant main and interaction effects were followed by one-way repeated measures ANOVAs to assess the effect of day and Paired Samples T test to assess difference between days within groups, and by Independent Samples T test to assess differences on each day between groups. If there was no time effect in non-normally distributed data baseline data (e.g. pre EVH) was subsequently analysed using a Friedman repeated measures test. Significant main effects were assessed using Wilcoxon Signed Ranks test to assess changes within groups between days and Mann-Whitney U tests to assess difference between groups on each day. If Paired Sampled T tests on baseline pooled data revealed a significant effect of time (pre vs. post EVH) then all data was subsequently analysed using a three-way mixed model repeated measures ANOVA to assess the effect of time (Pre EVH test, post EVH test)

and day (day 0, day 14, day 21), with a between subject factor of group (B-GOS vs. Placebo). Significant main effects of time were further explored using Paired Samples T test within each group at each day.

Peak fall in FEV_1, ACQ-7, and pulmonary function data (absolute & percentage predicted) was initially assessed for normality using Shapiro-Wilks's test. Normally distributed data was assessed using a two-way mixed model repeated measures ANOVA to assess the effect of day (day 0, day 14, day 21), with a between subject factor of group (B-GOS vs. Placebo). Significant main and interaction effects were followed by one-way repeated measures ANOVAs to assess the effect of day and Paired Samples T test to assess differences between days within groups, and by Independent Samples T test to assess differences on each day between groups. If data was not normally distributed, then data was analysed using a Friedman repeated measures test. Significant main effects were further explored using Wilcoxon Signed Ranks test to assess changes within groups between days and Mann-Whitney U tests to assess difference between groups on each day.

Pearson correlations were conducted on pooled baseline data for blood monocyte, neutrophil and basophil counts, and blood serum CCL11 concentrations. Cohen d effect sizes are present were appropriate with $d = 0.2$ a small effect size, $d = 0.5$ a medium effect size, and $d = 0.8$ a large effect size (Cohen 1988). Statistical significance was set at $p \leq 0.05$, all data are presented as mean ± SD.

7.3. RESULTS

7.3.1. EVH test response

7.3.1.1. Peak fall in FEV_1

Friedman repeated measures test revealed no significant change in the peak fall in FEV_1 from day 0 (-18.2 ± 7.4%) to day 14 (-18.7 ± 5.6%) and day 21 (-14.4 ± 8.4%) in the B-GOS group (p = 0.565) or day 0 (-19.6 ± 9.6%) to day 14 (-15.8 ± 11.5%) and day 21 (16.2 ± 9.6%) in the placebo group (p = 0.135). Mann-Whitney U test revealed that the peak fall in FEV_1 was not significantly different on day 0 (p = 0.650), day 14 (p = 0.182), and day 21 (p = 0.841) between B-GOS and placebo groups (Figure 7.2).

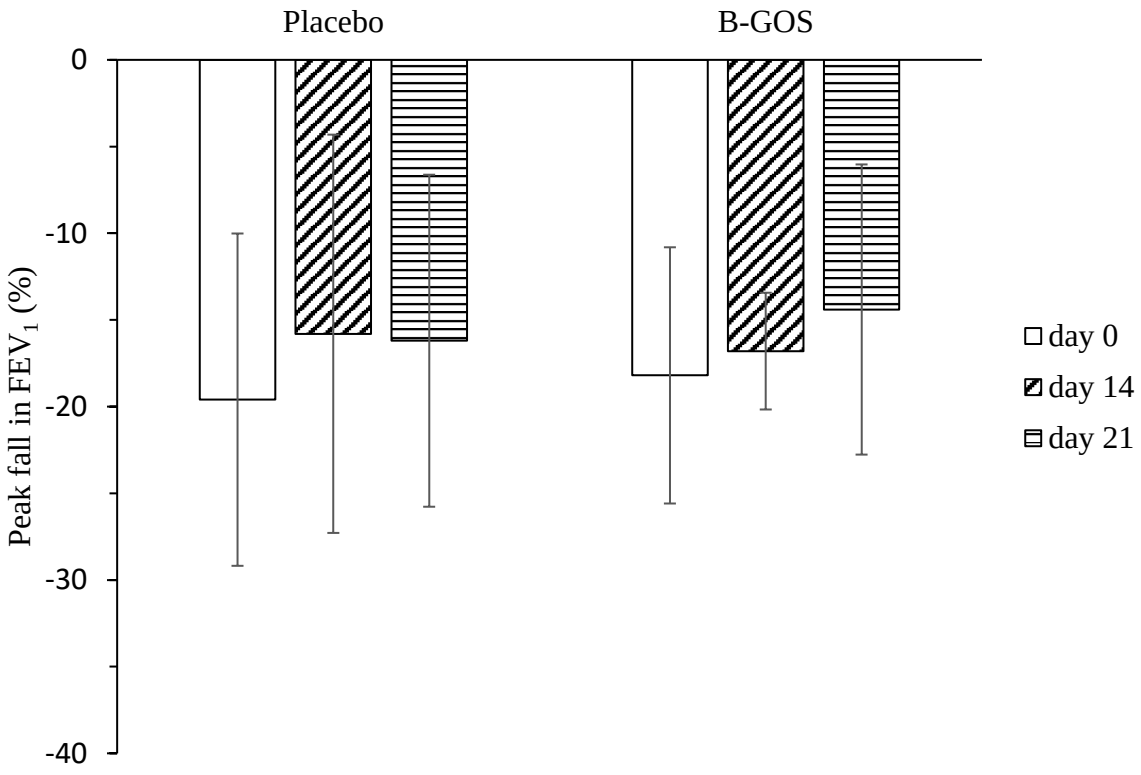

Figure 7.2: Peak fall in FEV_1 from pre to post EVH test in Placebo (Maltodextrin) and Bimuno-galactooligosaccharide (B-GOS) treatment groups at days 0, 14, and 21 during the supplementation period. Values are mean ± SD.

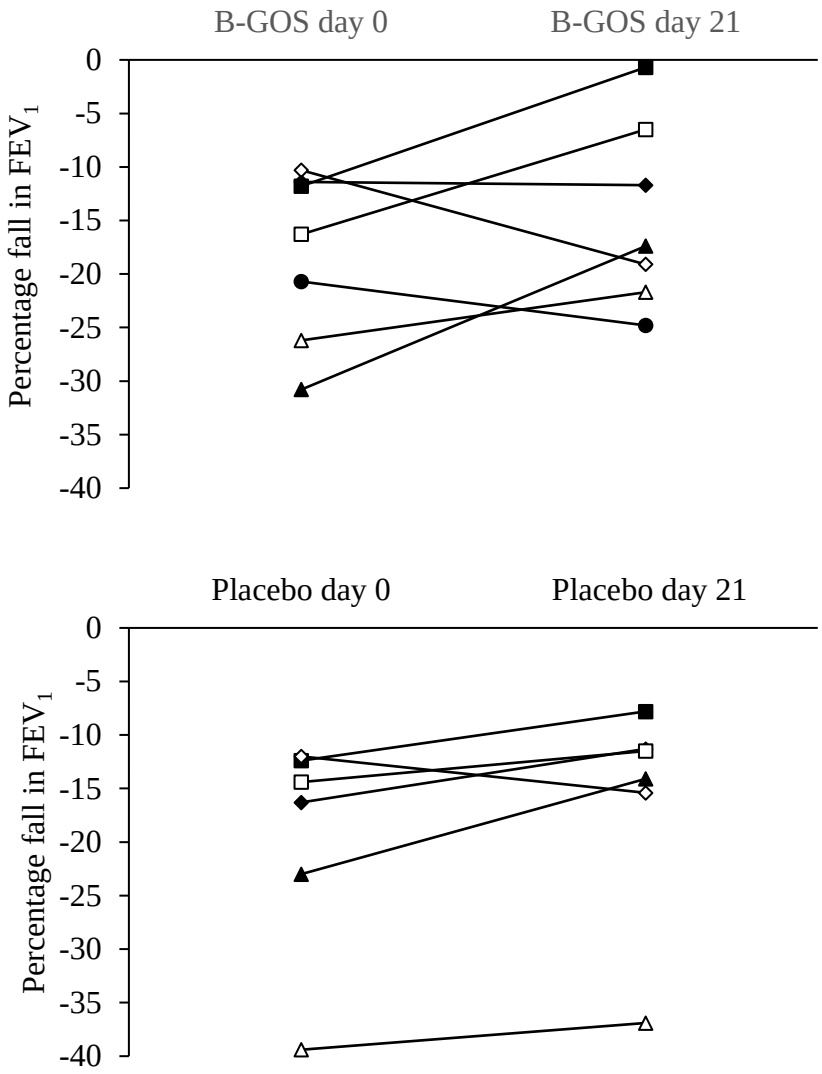

Figure 7.3: Individual data for the peak fall in forced expiratory volume in 1 second (FEV_1) after the eucapnic voluntary hyperpnoea test at day 0 and day 21 in the B-GOS (top) and Placebo (bottom) groups.

7.3.1.2. Achieved ventilation and percentage of maximal voluntary ventilation during EVH tests

Mixed model repeated measures ANOVA revealed no effect of day (p = 0.90; d = 0.002) but a trend for a day × group interaction effect (p = 0.078; d = 0.26) for the average $\dot{V}_E$ achieved during EVH test. Mixed model repeated measures ANOVA revealed no effect of day (p =

0.274; d = 0.11) but a significant day × group interaction effect (p = 0.036; d = 0.28) for percentage of target ventilation achieved during the EVH test. Independent paired sample T-test revealed no significant difference between B-GOS and placebo group for percentage of target ventilation achieved during the EVH test on day 0 (83.6 ± 10.7 vs 87.1 ± 9.9 %) (p = 0.561; 95% CI: -9.19, 16.04), day 14 (86.2 ± 11.3 vs 88.7 ± 12.7 %) (p = 0.714; 95% CI: -12.4, 17.46), or day 21 (90.6 ± 9.9 vs 85.2 ± 8.6 %) (p = 0.320; 95% CI: -16.62, 5.94) (Figure 7.4). The co-efficient variation in average MVV during EVH was 5.1%.

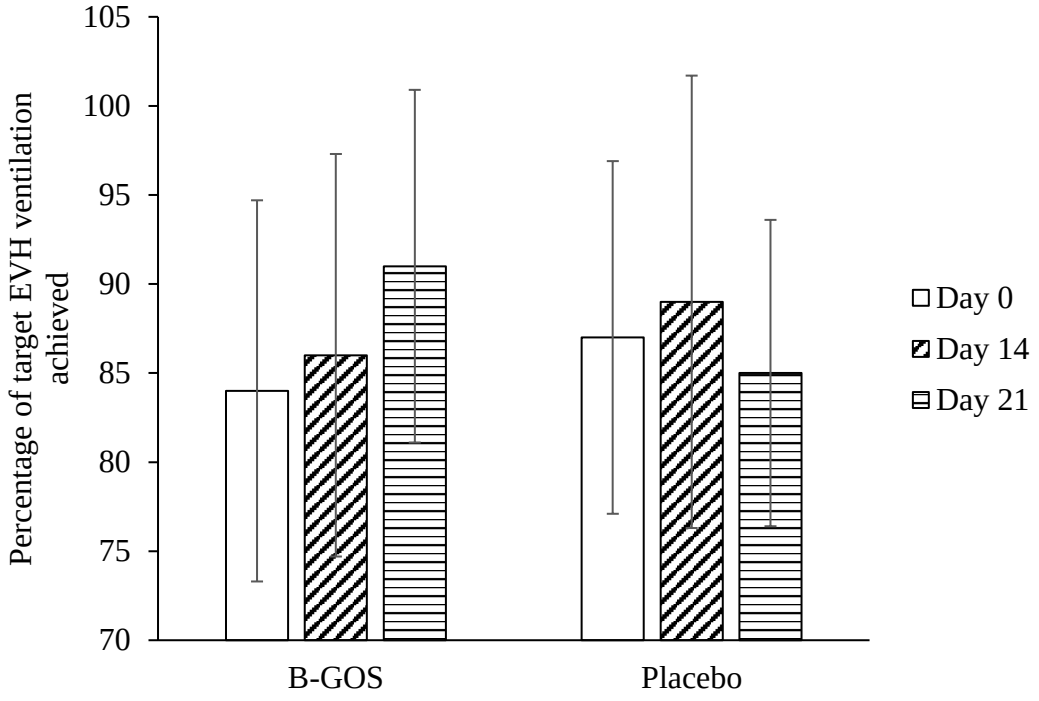

Figure 7.4: Percentage of target ventilation achieved during the eucapnic voluntary hyperpnoea test on day 0, day 14, and day 21 in the B-GOS and Placebo group. Values are mean ± SD.

7.3.2. Asthma control questionnaire 7-item scores

A mixed model repeated measures ANOVA revealed no day (p = 014; d = 0.19) or day × group interaction (p = 0.60; d = 0.03) for ACQ-7 scores. ACQ7 was unchanged in the B-GOS

group from day 0 (1.12 ± 1.09 a.u.) to day 21 (0.88 ± 0.77 a.u.) (p = 0.222). Similarly, ACQ7 was unchanged in the placebo group from day 0 (0.50 ± 0.54 a.u.) to day 21 (0.38 ± 0.4 a.u.) (p = 0.40) (Figure 7.5).

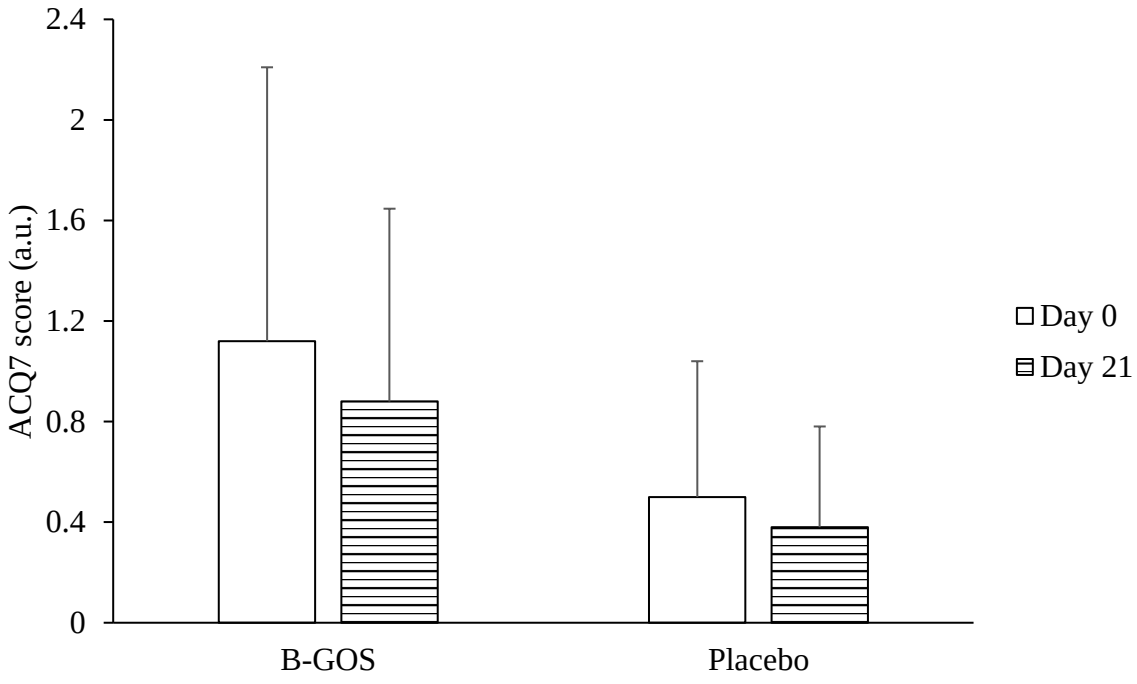

Figure 7.5: Asthma control questionnaire-7 scores in Bimuno-galactooligosaccharide (B-GOS) and Placebo (Maltodextrin) groups at day 0 and 21 during the supplementation period. Values are mean ± SD.

7.3.3. Pulmonary function

7.3.3.1. Forced expiratory volume in 1 second (FEV_1)

Mixed model repeated measures ANOVA revealed no effect of day or day × group interaction for percentage predicted FEV_1 ($p \geq 0.5$) (Table 7.2). For absolute FEV_1 there was an effect of time (p = 0.046; d = 0.25). One-way ANOVAs revealed a trend for a day effect for absolute FEV_1 in the B-GOS group (p = 0.077; d = 0.35). Bonferroni-adjusted pairwise comparisons revealed no significant differences between day 0 (4.15 ± 0.96 L) and day 14

(4.07 ± 1.03 L) (p = 0.389; 95% CI: -0.071, 0.234), and between day 0 and day 21 (4.00 ± 1.07 L) (p = 0.129; 95% CI: -0.042, 0.336). There was no effect of day in the placebo group for absolute FEV_1 (p = 0.223; d = 0.22).

7.3.3.2 Forced vital capacity (FVC)

Mixed model repeated measures ANOVA revealed an effect of day (p = 0.03; d = 0.27) but no day × group interaction (p = 0.632; d = 0.04) for percentage predicted FVC (Table 7.2). One-way ANOVAs revealed no effects of day for B-GOS (p = 0.065; d = 0.37) and placebo groups (p = 0.263; d = 0.201). For absolute FVC there was an effect of day (p = 0.005; d = 0.38) but no day × group interaction (p = 0.150; d = 0.16). One-way ANOVAs revealed an effect of day in the B-GOS group (p = 0.019; d = 0.49) but not the placebo group (p = 0.092; d = 0.38). Paired samples T-tests revealed that in the B-GOS group absolute FVC was significantly reduced from day 0 to day 14 (p = 0.038; 95% CI: 0.052, 0.264) but not from from day 0 to day 21 (p = 0.057; 95% CI: -0.006, 0.289). There was no difference in absolute FVC in the B-GOS group between day 14 and day 21 (p = 0.863; 95% CI: -0.054, 0.063).

7.3.3.3. Peak expiratory flow rate (PEF)

Friedman repeated measures test revealed no significant change in the percentage predicted PEF from day 0 to day 21 in B-GOS (p = 0.772) or placebo groups (p = 0.738) (Table 7.2). Mann-Whitney U test revealed that the percentage predicted PEF was not significantly different on day 0 (p = 0.811), day 14 (p = 0.764), and day 21 (p = 0.972) between B-GOS and placebo groups. Mixed model repeat measures ANOVA reveal no effect of day (p = 0.771; d = 0.02) or day × time interaction (p = 0.711; d = 0.03) for PEF.

7.3.3.4. Forced expiratory volume from 25% to 75% of FVC ($FEF_{25-75\%}$)

Mixed model repeated measures ANOVA revealed no effect of day ($p = 0.330$; $d = 0.11$) or day × group ($p = 0.262$; $d = 0.13$) interaction for percentage predicted $FEF_{25-75\%}$ (Table 7.2). For absolute $FEF_{25-75\%}$ there was no effect of day ($p = 0.30$; $d = 0.11$) or day × group interaction ($p = 0.271$; $d = 0.12$).

7.3.3.5. FEV_1/FVC ratio

Mixed model repeated measures ANOVA revealed no effect of day ($p = 0.864$; $d = 0.01$) or day × group ($p = 0.491$; $d = 0.06$) interaction for percentage predicted FEV_1/FVC ratio (Table 7.2). For absolute FEV_1/FVC ratio there was no effect of day ($p = 0.90$; $d = 0.01$) or day ×group interaction (p = 0.473; d = 0.07).

Table 7.2: Pulmonary function on day 0, day 14, and day 21 in the B-GOS and Placebo group. Values are mean ± SD.

Baseline pulmonary function	**B-GOS**			**Placebo**		
	Day 0	**Day 14**	**Day 21**	**Day 0**	**Day 14**	**Day 21**
FEV_1 (L)	4.15 ± 0.89	4.07 ± 0.95	4.00 ± 0.99	4.23 ± 0.9	4.16 ± 0.95	4.13 ± 0.87
% predicted	90 ± 14	88 ± 16	88 ± 17	99 ± 9	97 ± 14	96 ± 10
FVC (L)	5.53 ± 1.04	5.39 ± 1.03	5.38 ± 1.02	5.07 ± 0.95	5.07 ± 0.98	4.98 ± 0.94
% predicted	102 ± 10	99 ± 10	96 ± 7	101 ± 12	101 ± 11	99 ± 10
FEV_1/FVC	0.75 ± 0.07	0.76 ± 0.09	0.74 ± 0.08	0.83 ± 0.07	0.82 ± 0.10	0.83 ± 0.09
% predicted	88 ± 8	88 ± 10	87 ± 9	98 ± 8	97 ± 10	98 ± 10
PEF (L/s)	9.67 ± 1.92	9.58 ± 1.78	9.60 ± 1.59	9.41 ± 2.93	9.36 ± 3.2	9.59 ± 3.19
% predicted	101 ± 21	100 ± 21	100 ± 16	101 ± 22	101 ± 25	103 ± 24
FEF (L/s)	3.49 ± 1.11	3.40 ± 1.32	3.24 ± 1.46	4.26 ± 1.45	4.15 ± 1.63	4.22 ± 1.58
% predicted	73 ± 23	71 ± 27	67 ± 27	92 ± 25	91 ± 31	92 ± 29

FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; PEF: peak expiratory flow rate; FEF: forced expiratory flow at 25-75% of FVC.

7.3.4. Differential white blood cell counts

7.3.4.1. Blood eosinophils

At baseline a paired samples T-test revealed blood eosinophil counts did not change from pre (0.24 ± 0.17 10^9/L) to 30 minutes post EVH test (0.2 ± 0.13 10^9/L) (p = 0.234; 95% CI: -0.029, 0.108). Mixed model repeated measures ANOVA revealed no day (p = 0.921; d = 0.01) or day × group interaction (p = 0.751; d = 0.03) for resting blood eosinophil counts. Wilcoxon signed ranks test revealed blood eosinophil percentage did not change from pre (3.72 ± 2.14%) to 30 minutes post EVH test (2.86 ± 1.8%) (p = 0.075). Friedman repeated measures test revealed no significant change in resting blood eosinophil percentages from day 0 to day 14 and day 21 in B-GOS (day 0: 3.5 ± 2.2%; day 14: 3.6 ± 2.2%; day 21: 4.2 ± 4%) (p = 0.851) or placebo groups (day 0: 4 ± 1.7%; day 14: 3.1 ± 1.8%; day 21: 2.5 ± 2%) (p = 0.260).

7.3.4.2. Blood neutrophils

At baseline a paired samples T-test revealed blood neutrophil counts significantly increased from pre (3.64 ± 1.38 10^9/L) to 30 minutes post EVH test (4.37 ± 1.46 10^9/L) (p = 0.002; 95% CI: -1.12, -0.35; d = 0.52) (Figure 7.6). There was not a significant correlation between the change in blood neutrophil counts and the peak fall in FEV_1 at baseline (p = 0.823; r = 0.077). Mixed models repeated measure ANOVAs revealed a time effect for blood neutrophil counts ($p < 0.001$). Paired samples T-tests revealed that blood neutrophil counts increased from pre to post EVH test on day 0 (Pre = 4.25 ± 1.49; Post = 4.91 ± 1.66 10^9/L) and day 21 (Pre = 4.62 ± 1.88; Post = 5.04 ± 1.77 10^9/L) within the B-GOS group ($p \leq 0.016$). Blood neutrophil counts did not change from pre to post EVH test on day 14 in the B-GOS group and any day in the placebo group ($p \geq 0.055$). There was no day or day × time × treatment

interaction for blood neutrophil counts ($p \geq 0.210$). At baseline a paired samples T-test revealed blood neutrophil percentage did not change from pre (57.4 ± 7.2%) to 30 minutes post EVH test (59.5 ± 7.5%) ($p = 0.19$; 95% CI: -5.39, 1.24). Mixed model repeated measures ANOVA revealed no effect of day ($p = 0.271$; $d = 0.13$) or day × group ($p = 0.51$; $d = 0.072$) interaction for resting blood neutrophil percentages.

7.3.4.3. Blood lymphocytes

At baseline a paired samples T-test revealed blood lymphocytes counts did not change from pre (1.82 ± 0.38 10^9/L) to 30 minutes post EVH test (1.99 ± 0.33 10^9/L) ($p = 0.152$; 95% CI: -0.41, -1.55). Mixed model repeated measures ANOVA revealed no day ($p = 0.30$; $d = 0.12$) or day × group interaction ($p = 0.90$; $d = 0.01$) for resting blood lymphocytes counts. At baseline a paired samples T-test revealed blood lymphocytes percentage did not change from pre (30.1 ± 6.5%) to 30 minutes post EVH test (28.5 ± 6.8%) ($p = 0.344$; 95% CI: -2.03; 5.28). Mixed model repeated measures ANOVA revealed no day ($p = 0.232$; $d = 0.15$) or day × group interaction ($p = 0.90$; $d = 0.01$) for resting blood lymphocytes percentages.

7.3.4.4. Blood monocytes

At baseline a paired samples T-test revealed blood monocyte counts significantly increased from pre (0.51 ± 0.14 10^9/L) to 30 minutes post EVH test (0.63 ± 0.19 10^9/L) ($p = 0.004$; 95% CI: -0.20, -0.05; $d = 0.71$) (Figure 7.6). There was not a significant correlation between the change in blood monocyte counts from pre to post EVH test and the peak fall in FEV_1 at baseline ($p = 0.469$; r = 0.24). Mixed models repeated measure ANOVAs revealed a time effect for blood monocyte counts ($p < 0.001$). Blood monocytes counts increased from pre to post EVH test on day 0 and 14 in the placebo groups and day 21 in the B-GOS group ($p \leq$

0.044). Blood monocyte counts did not change from pre to post EVH test on day0 and 14 in the B-GOS group and day 21 in the placebo group ($p \geq 0.229$). There was no effect of day or day × time × treatment interaction for blood monocyte counts ($p \geq 0.269$). At baseline a paired samples T-test revealed blood monocyte percentage did not change from pre (8.4 ± 2.4%) to 30 minutes post EVH test (8.8 ± 2. %) ($p = 0.297$; 95% CI: -1.21, 0.41). Mixed model repeated measures ANOVA revealed no effect of day ($p = 0.682$; $d = 0.04$) or day × group ($p = 0.121$; $d = 0.21$) interaction for resting blood monocyte percentages.

7.3.4.5. Blood basophils

At baseline a paired samples T-test revealed blood basophil counts significantly increased from pre (0.02 ± 0.01 10^9/L) to 30 minutes post EVH test (0.03 ± 0.01 10^9/L) ($p = 0.046$; 95% CI: -0.01257, -0.00015; $d = 0.65$) (Figure 7.6). There was not a significant correlation between the change in blood basophil counts pre to post EVH test and the peak fall in FEV_1 at baseline ($p = 0.931$; r = 0.03). Mixed model repeated measures ANOVA revealed no day, or day × time × treatment interaction effect for blood basophil counts ($p \geq 0.11$). Wilcoxon signed ranks test revealed blood basophil percentage did not change from pre (0.37 ± 0.16%) to 30 minutes post EVH test (0.38 ± 0.2%) ($p = 0.713$). Friedman repeated measures test revealed no significant change in resting blood basophil percentages from day 0 to day 14 and day 21 in B-GOS (day 0: 0.3 ± 0.2%; day 14: 0.3 ± 0.1%; day 21: 0.3 ± 0.1%) ($p = 0.8$) or placebo groups (day 0: 0.5 ± 0.1%; day 14: 0.5 ± 0.3%; day 21: 0.6 ± 0.4%) ($p = 0.41$).

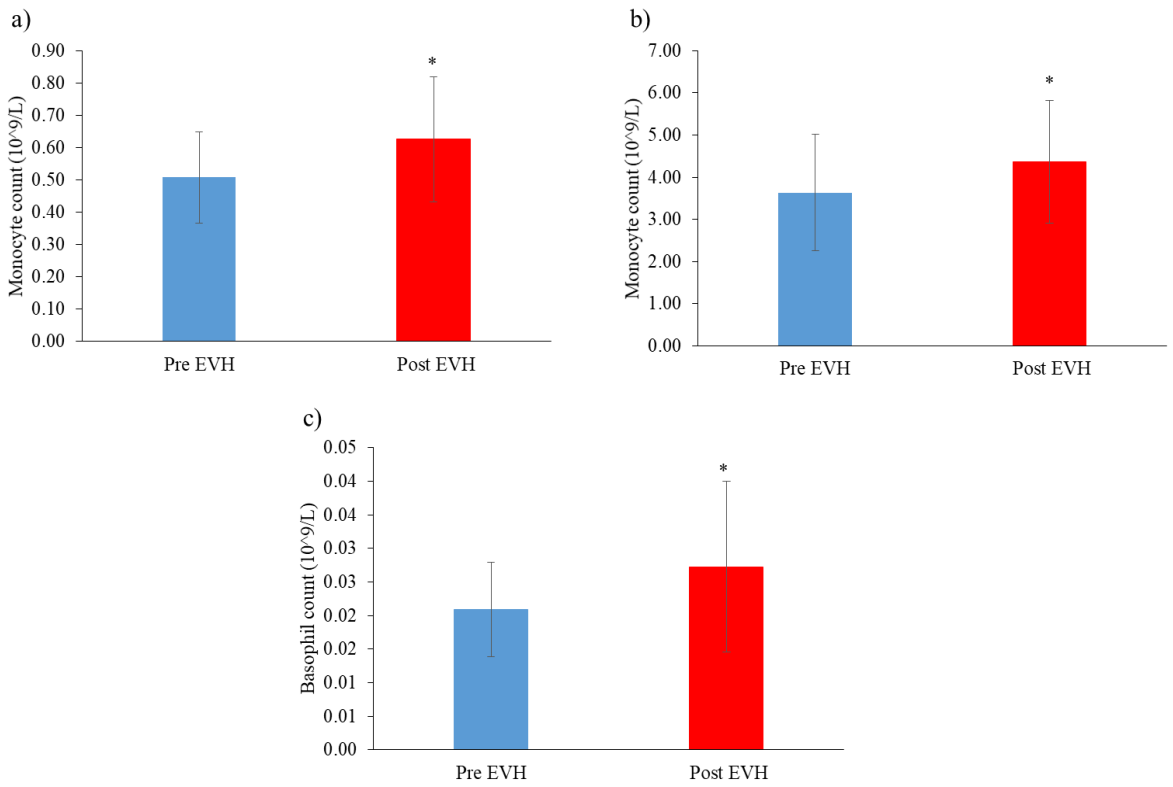

Figure 7.6: Pooled groups data for baseline (day 0) blood monocytes (a), neutrophils (b), and basophil counts (c) from pre to post eucapnic voluntary hyperpnoea (EVH) test. *Significant difference from Pre EVH time point (p = 0.002 - 0.046). Values are mean ± SD.

7.3.5. Serum cytokines and chemokines concentrations

7.3.5.1. Serum interleukin-13 (IL-13) concentrations

Mixed model repeated measure ANOVA revealed no effect of day (p = 0.411; d = 0.069) or a day × group interaction (p = 0.289; d = 0.112) for serum IL-13 concentrations. Serum IL-13 concentration was unchanged in the B-GOS groups from day 0 (9 ± 8 pg/ml) till day 21 (9 ± 8 pg/ml) (p = 0.821). Similarly, serum IL-13 concentrations were unchanged in the placebo group from day 0 (15 ± 15 pg/ml) till day 21 (14 ± 13 pg/ml) (p = 0.350).

7.3.5.2. Serum thymic stromal lymphopoietin (TSLP) concentrations

Mixed model repeated measure ANOVA revealed no effect of day (p = 0.199; d = 0.159) or a day × group interaction (p = 0.684; d = 0.017) for serum TSLP concentrations. Serum TSLP concentration was unchanged in the B-GOS groups from day 0 (250 ± 224 pg/ml) till day 21 (242 ± 217 pg/ml) (p = 0.532). Similarly, serum TSLP concentrations was unchanged in the placebo group from day 0 (158 ± 140 pg/ml) till day 21 (144 ± 132 pg/ml) (p = 0.210).

7.3.5.3. Serum eotaxin concentrations

At baseline, a paired samples T-test revealed serum eotaxin concentrations significantly increased from pre (43.7 ± 13.3 pg/ml) to 30 minutes post EVH test (51.7 ± 12 pg/ml) (p = 0.006; 95% CI: -13.004, -2.92; d = 0.63). There was a significant negative correlation between the change in serum CCL11 from pre to post test and the peak fall in FEV_1 at baseline (p = 0.046; r = -0.640). Mixed model repeated measures ANOVA revealed no time effect (p = 0.057; d = 0.383) and no effect for day (p = 0.171; d = 0.221) or a day × time (p = 0.245; d = 0.164), day × groups (p = 0.529; d = 0.051) or a day × time × groups interaction effect (p = 0.316; d = 0.13).

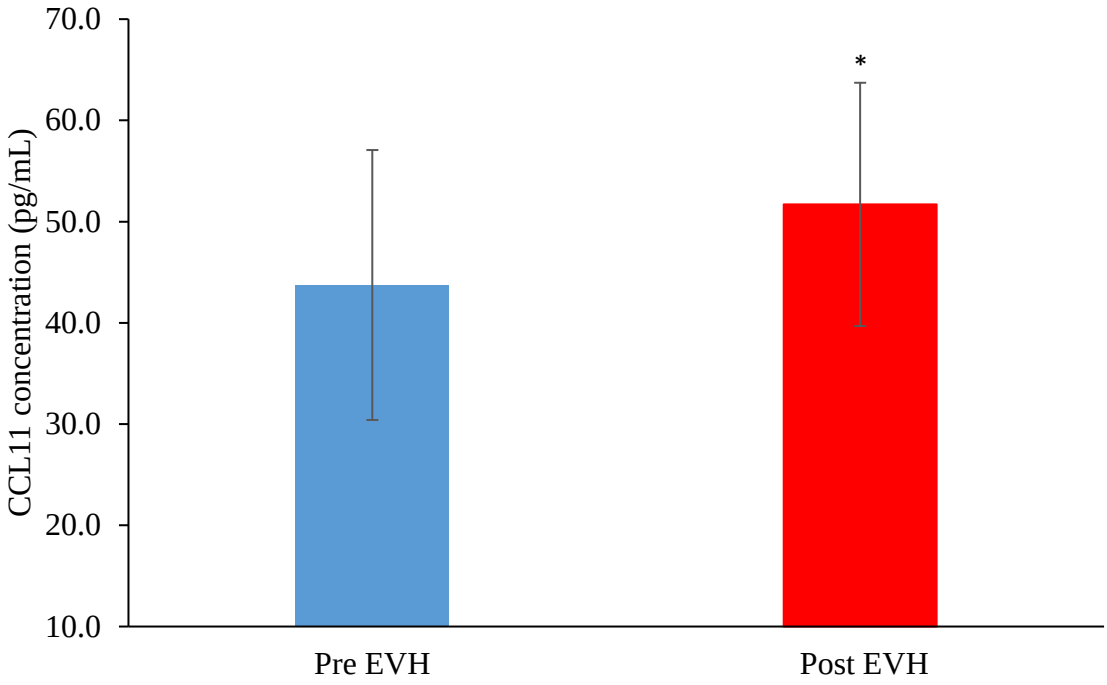

Figure 7.7: Pooled groups data for baseline (day 0) blood serum chemokine ligand-11 (CCL11) concentrations from pre to post eucapnic voluntary hyperpnoea (EVH) test. *Significant difference from Pre EVH time point (p = 0.006). Values are mean ± SD.

7.3.5.4. Serum monocyte chemoattractant protein-1 (MCP-1) concentrations

At baseline, a paired samples T-test revealed no change in serum MCP-1 concentrations from pre (36.5 ± 17.6 pg/ml) to 30 minutes post EVH test (37.3 ± 17.7 pg/ml) (p = 0.594; 95% CI: -4.26; 2.59). Mixed model repeated measures ANOVA revealed no effect of day (p = 0.340; d = 0.11) or day × group interaction effect (p = 0.934; d = 0.001) for resting blood serum MCP-1 concentrations.

7.3.5.5. Serum chemokine ligand 5 (CCL5) concentrations

At baseline, a paired samples T-test revealed no change in serum CCL5 concentrations from pre (10337 ± 3014 pg/ml) to 30 minutes post EVH test (12075 ± 1942 pg/ml) (p = 0.162;

95% CI: -4363.5; 888.09; *d* = 0.65). Mixed model repeated measures ANOVA revealed no effect of day (*p* = 0.302; *d* = 0.18) or day × group interaction effect (*p* = 0.328; *d* = 0.16) for resting blood serum CCL5 concentrations.

7.3.5.6. Stem cell factor (SCF) concentrations

At baseline a paired samples T-test revealed no change in serum SCF concentrations from pre (64 ± 24.8 pg/ml) to 30 minutes post EVH test (65.5 ± 19.3) (*p* = 0.877; 95% CI = -22.56; 19.58). Mixed model repeated measures ANOVA revealed no effect of day (*p* = 0.532; *d* = 0.051) or day × group interaction effect (*p* = 0.468; *d* = 0.068) for resting blood serum SCF concentrations.

7.3.6. Exhaled breath condensate pH

Wilcoxon signed ranks test showed no significant change in EBC pH from pre (5.7 ± 0.4 a.u.) to 30 minutes post EVH test (5.9 ± 0.3 a.u.) (*p* = 0.069). Friedman repeat measures test revealed a significant effect of day for resting EBC pH in the placebo group (*p* = 0.042) but not the B-GOS group (*p* = 0.276). Wilcoxon signed ranks tests revealed resting EBC pH was significantly higher at days 14 (5.8 ± 0.3 a.u.; *p* = 0.046) and 21 (5.8 ± 0.3 a.u.; *p* = 0.028) compared to day 0 (5.6 ± 0.4 a.u.).

7.4. Discussion

7.4.1. Main findings

Twenty-one days supplementation with 3.65g/d of B-GOS (80% GOS content) did not alter the severity of HIB, lung function, asthma control, or systemic markers of airway inflammation in adults with mild to moderate asthma and HIB. Additionally, B-GOS supplementation did not alter EBC pH. Blood monocytes (Δ 24%), neutrophil (Δ 20%) and basophil (Δ 50%) counts, and blood serum eotaxin concentrations (18%) significantly increased from pre to 30 minutes post EVH test in adults with mild to moderate asthma and HIB.

7.4.2. Pulmonary function response to B-GOS

B-GOS supplementation did not significantly affect the severity of HIB. This finding contrasts Williams et al (2016) who found 21 days supplementation of 5.5g/d B-GOS significantly attenuated HIB by 40% in a similar cohort of participants with asthma and HIB. One possible reason for this is the low sample size in the B-GOS group (N = 7) which could cause a type II error if the study is not statistically powered (Jones, 2004). The within-participants standard deviation for the fall in FEV_1 after an EVH test in participants with HIB is 100 mL, while the minimum perceptible improvements in FEV_1 in adults with asthma is 230 mL (Williams et al, 2015; Santanello, Zhang, Seidenberg, Reiss, & Barber, 1999). With power = 0.9 and $\alpha = 0.05$ an α prior sample size calculator estimated a sample size of seven would be required to detect a 230 mL change in the fall in FEV_1 after EVH. While sample size in the present study was therefore large enough issues with the reproducibility of the EVH response in participants with milder HIB (~-10% post-test fall in FEV_1) have be raised by Price, Ansley, & Hull (2015). The baseline mean peak fall in FEV_1 was notably milder in

the present study cohort (B-GOS group: -18.2%; Placebo group: -19.6%) when compared to the Williams et al (2016) cohort (-27%) with three of the seven participants in the B-GOS group having baseline mean peak fall in FEV_1 milder than -12%. The current cohorts HIB may therefore be too mild to respond to B-GOS.

In addition, Price et al (2015) assessed the reproducibility of the EVH test using the same protocol as the present study, whereby $\dot{V}_E$ is measured using a dry gas metre and this information is verbally relayed back to the participant to inform them on how close they are to their target ventilation. In Williams et al (2016) they used a breath-by-breath analyser to measure V_E which provides the additional benefit of providing live, on screen, real time V_E to the participant. This method may make the participant more aware of their ventilation in relation to their target and allows them to adjust their ventilation appropriately. Indeed, the breath-by-breath protocol elicits exceptional day to day reproducibility in the bronchoconstrictive response to EVH in asthma patients with HIB over 21 and 70 days even in mild cases of HIB (~-10% fall in FEV_1) (Williams et al, 2015). Compared to the dry gas metre method the breath-by-breath EVH protocol may therefore possess superior reproducibility by allowing participants to more accurately achieve their target V_E. Indeed, in the present study we observed a day*group interaction for the %MVV achieved during the EVH test. The use of the dry gas metre EVH method in the present study particularly in participants with mild HIB, may have therefore lacked the reproducibility required to assess the effect of B-GOS supplementation on in a sample size of seven, which may have resulted in a type II error.

In addition to sample size and EVH test reproducibility, B-GOS dosage and composition in the present study differed compared to that used by Williams et al (2016). Participants in the present study consumed one 3.65g/d sachet of B-GOS (80% GOS) resulting in a total GOS consumption of 2.92g/d. Participants in Williams et al (2016) study consumed two 5.5g/d

sachets of B-GOS (48% GOS) (11g/d of B-GOS) each day resulting in a total GOS consumption of 5.28g/d. In 44 elderly subjects 5.5g/d of B-GOS (48% GOS content) was shown to significantly increase healthy bacterial groups including *Bifidobacterium* spp., *Lactobacillus-Enterococcus* spp., and the *C. coccoides–E. rectale* group and reduces less favourable bacteria groups including *Bacteroides* spp., the *C. histolyticum* group, *E. coli*, and *Desulfovibrio* spp. in faecal samples compared to baseline and placebo treatment after 5-week with further changes at 10-weeks (Vulevic et al, 2008). However, gut bifidobacteria decline with age and it is speculated that individuals with higher baseline bifidobacteria counts exhibit resistance to further increases in bifidobacteria and SCFAs in response to GOS supplementation (Kolida et al, 2007; De Preter et al, 2008). This is noteworthy as faecal bacteria was not measured in the present study and therefore it is not known whether participants had a high/low baseline bifidobacteria which may have affected their responsive to B-GOS supplemetation. In adults (average age: 34 years), Depeint et al (2008) assessed the effect of 3.6g/d and 7g/d supplementation of B-GOS (48% GOS content) for seven days on faecal bacterial numbers. Both doses significantly increased bifidobacteria compared to baseline and increased the bifidobacterial population ratio. The increase in bifidobacteria portion of faecal bacteria however was positively related (r^2 = 0.753) to the B-GOS dose. Additionally, 7g/d resulted in a significantly higher prebiotic index compared to baseline, placebo, and 3.6g/d. This suggests that the prebiotics activity and bifidogenic characteristics of B-GOS are dose dependant.

B-GOS is formed through the enzymatic activity of β-galactosidase, derived from the bacteria B.bifidum NCIMB 41171, on lactose (Tzortzis et al, 2005). This process produces GOS with degrees of polymerisation (DP) primarily ≤ DP 4 (DP 2 = 52, DP 3 = 26, DP 4 = 14, DP 5 = 8) and β1-3 (26), β1-4 (23), and β1-6 (51) saccharide linkages (Vulevic, 2008; Grimaldi, 2016). In addition, B-GOS consists of monosaccharide and disaccharide such as glucose,

galactose, and lactose which do not possess prebiotic activity. As such, purification of B-GOS is achieved by fermenting the monosaccharides in the product with *S. cerevisiae* in which monosaccharides are removed by S. *cerevisiae* through anaerobic glycolysis, converting monosaccharides into ethanol and CO_2 (Goulas et al, 2005). This process removes 92% of glucose and ~4% galactose (Goulas et al, 2005). GOS is therefore the active and desired ingredient in B-GOS. In mice models of asthma supplementation with GOS significantly alter the gut microbiota and attenuates HDM-induced AHR in addition to attenuation airway eosinophilia and upregulating T regulatory cells (Verheijden et al, 2015a; Verheijden et al, 2015b; Verheijden et al, 2015c; Verheijden et al, 2016; Verheijden et al, 2018). Interestingly, 5g/d and 10g/d of GOS supplementation for 3-weeks significantly increases faecal bifidobacteria populations in healthy adults while 2.5g/d shows no significant effect (Davis, Martinez, Walter, & Hutkins, 2010). This suggests that the GOS dosage in the present study (2.9g/d) may not have been adequate to induce change in gut bifidobacteria populations. The gut-lung hypothesis suggests that increases in gut bifidobacteria and lactobacilli species and the production of SCFAs from prebiotic supplementation can alter immune homeostasis to enhance immune regulation and attenuate inflammation which can manifest in the airways to alleviate conditions such as asthma and HIB. Without sufficient changes in gut bacterial composition and activity there would be no influence on immune cell maturation and polarisation and subsequently no change in airway inflammation and HIB severity.

Williams et al (2016) also instructed participants to ingest supplements in the mornings before trials which was not allowed in the present study. There is suggestion that acute supplementation (4 hours) may have a protective effect on lung function in asthma patients. Halnes et al (2017) assessed the effect of 3.5g inulin in combination with a probiotic yoghurt (*Lactobacillus acidophilis* LA5, *Lactobacillus rhamnosus* GG, and *Bifidobacterium*

lactis Bb12) on lung function and airway inflammation pre and 4-hours post ingestion. In the symbiotic group absolute FEV_1 (Δ 0.1 L), percentage predicted FEV_1 (Δ 4%), and percentage predicted FEV_1/FVC ratio (Δ 3.8%) increased from baseline. Additionally, sputum lymphocyte, neutrophil, macrophage, total sputum cell counts, IL-8 concentrations and fraction of exhaled nitric oxide were reduced from baseline. These effects were accompanied by increased gene expression of G-couple protein receptor-41 (GPR41) and GPR43 in sputum cells. These receptors are activated by SCFAs, acetate, propionate, and butyrate which inhibits nuclear factors-kappa B (NF-κB), a transcription factor involved in upregulating airway inflammation (Williams et al, 2019). Inulin and soluble fibre ingestion increase serum concentration of SCFAs between 4–6-hour post ingestion and may therefore have an acute effect in attenuating airway inflammation. While the study design of Halnes et al (2017) cannot allude to the individual or combine contribution of inulin or probiotics to the improvement in lung function and airway inflammation it suggests that prebiotic ingestion could have an acute effect. If so, an additional acute prebiotic effect in the Williams et al (2016) study may also partly explain the between-study differences.

We saw no change in baseline pulmonary function measures (FEV_1, FVC, FEV_1/FVC ratio, PEF, $FEF_{25-75\%}$) following B-GOS supplementation compared to placebo or baseline. This is in keeping with the finding by Williams et al (2016) who saw no change in baseline FEV_1, FVC, and PEF from pre to post B-GOS supplementation. Similarly, no changes in FEV_1, FVC, and FEV_1/FVC ratio have been reported following 12g/d supplementation of the prebiotic inulin for 1-week in asthma patients (McLoughlin et al, 2019). Improvements in PEF have however been seen in allergic asthma patients following supplementation with a synbiotic (7.2g/d GOS; 0.8g/d FOS; *Bifidobacterium Breve:* 10^{10} CFU). Van De Pol et al (2011) found that 4-weeks symbiotic supplementation improved PEF compared to placebo, increasing evening PEF by ~10 L/min and morning PEF by ~30 L/min across the 4-weeks.

The improvements in PEF may be influenced by the high dose of GOS used by Van De Pol et al (2011) however, the 2-way cross over design of the study means that the individual contribution of the prebiotics (GOS and FOS) and probiotic (*Bifidobacterium Breve*) to the improvement in PEF is not known.

7.4.3. Asthma control response to B-GOS

The ACQ7 score did not significantly change in the B-GOS or placebo group. McLoughlin et al (2019) found a significant reduction in ACQ-6 scores in asthma patients following 1-week supplementation with inulin at a dose of 12g/d. ACQ6 scores reduced from 0.8 at baseline to 0.5 at the end of supplementation. Additionally, 63% of participants with uncontrolled asthma (ACQ6 $\geq$ 0.75) reached the minimum clinically important difference of 0.5. While we saw no improvement in ACQ7 scores in the present study with B-GOS supplementation only two participants had asthma classified as not well controlled (ACQ7 $\geq$ 1.5) at baseline. In addition two participants had well controlled asthma (ACQ7 $\leq$ 0.75). With the minimum clinically important difference in ACQ7 scores being 0.5, it is likely that baseline asthma was sufficiently controlled, thereby mitigating a significant improvement with B-GOS supplementation. Future research should look to include a larger samples size to increase the heterogeneity of the cohort which may allow for sub analysis in both well and not well controlled asthma patients when assessing ACQ7 scores.

7.4.4. Differential white blood cell counts response to B-GOS

In the present study we saw no change in all white blood cells parameters (eosinophils, neutrophils, lymphocytes, monocytes, basophils) for counts or percentage after B-GOS supplementation. Previously, inulin supplementation (12g/d for 1-week) in asthma patients

significantly reduced sputum percentage eosinophils from baseline (Δ -1%) but had no effect on sputum percentage neutrophils, macrophages, and lymphocytes (McLoughlin et al, 2017). Furthermore, acute supplementation (4 hours) with a symbiotic (3.5g inulin; *Lactobacillus acidophilis* LA5, *Lactobacillus rhamnosus* GG, and *Bifidobacterium lactis* Bb12) significantly reduced sputum total cell counts (Δ -2.0 * 10^6/mL), sputum neutrophils (Δ -67.5 * 10^4/mL), sputum macrophages (Δ -99.1 * 10^4/mL), and lymphocytes (Δ - 1.4 * 10^4/mL) from pre to post supplementation, whereas sputum eosinophil counts were unchanged. Additionally, sputum total cell counts, and sputum lymphocytes were significantly reduced compare to placebo treatment (Halnes et al, 2017). In a murine model of HDM induced asthma Verheijden et al (2015) showed that a 1% GOS supplementation throughout sensitization and HDM challenge significantly reduced HDM-induced airway eosinophilia by 21.2% compared to a control diet. In a separate murine study GOS was shown to reduce HDM-induced increases in bronchi alveolar lavage fluid total cell counts and eosinophil counts by greater than 57% (Verheijden et al, 2015). Concomitantly, HDM challenge in mice induces increase in percentage blood eosinophils which prebiotic and resistant starch feeding attenuates by approximately 2% compared to a control diet (Thorburn et al, 2015). In mice both GOS and resistant starch therefore attenuate airway challenge induced eosinophilia, however, in the present study EVH did not induce increases in blood eosinophil counts or percentage and therefore the effect of B-GOS on challenge induced eosinophilia could not be determined. Monocytes, neutrophils, and basophils did increase following the EVH test, which is discussed in more detail in section 7.4.6., however no day*time or day*time*group effect was seen for these measures. Blood eosinophil counts and percentages are good predictors of airway eosinophilia (≥3%) in asthma patients (Zhang et al, 2014). Both blood percentage eosinophils (r = 0.691; AUC = 0.907) and blood eosinophil counts (r = 0.683; AUC = 0.898) positively correlate with and predict sputum eosinophil counts. The optimal

cut-off for blood percentage eosinophils is 2.7% (sensitivity = 92.2%; specificity = 75.8%), and for blood eosinophil counts is 0.26 x 10^6/L. In our cohort only 3/7 (43%) of the B-GOS group had blood percentage eosinophils and eosinophil counts above these cut-offs. The lack of participants with airway eosinophilia in the present cohort may have ameliorated the effect of B-GOS on baseline blood eosinophil counts and percentages. Future work should look to include a larger sample size so that sub analysis on the effect of B-GOS on blood eosinophils can be conducted in patients with eosinophilic and non-eosinophilic asthma.

7.4.5. Serum cytokines and chemokines, and EBC pH response to B-GOS

Baseline serum concentrations of eotaxin, CCL5, MCP-1, SCF, IL-13 and TSLP did not change in response to B-GOS treatment. Additionally, only eotaxin showed a significant change from pre to post EVH test which is discussed in greater detail in section 7.4.6. Eotaxin is elevated in sputum and blood serum in asthmatics compared to healthy controls (Williams et al, 2016; Yamada et al, 2000) and is involved in the recruitment of eosinophils to the airways (Rankin, Conroy, & Williams, 2000). As such, airway eotaxin concentrations positively correlate with the percentage of airway eosinophils, the chemotactic activity of eosinophils and the severity of bronchial hyperactivity (Yamada et al, 2008; Dent et al, 2004; Ying et al, 1997). Baseline blood serum concentrations of eotaxin in the present study (~44 pg/mL) were substantially lower than that previously reported in stable asthmatics (232 pg/mL) and in mild to moderate asthma patients with HIB (~200 pg/mL) (Tateno et al, 2004; Williams et al, 2016). The reason for such a difference is unclear given the similarity in cohorts but differences in analysis platforms (ELISA versus Multiplex assays) or asthma phenotypes (eosinophilic versus non-eosinophilic) may have contributed. Baseline eotaxin concentrations was unresponsive to B-GOS supplementation which agrees with the finding of

William et al (2016) who found no change in baseline eotaxin concentrations following 3-week supplementation with 5.5.g/d B-GOS (48% GOS content).

CCL5 additionally plays a role in the recruitment of eosinophils and is elevated in bronchoalveolar lavage fluid, EBC, and blood serum of asthma patients compared to healthy controls (Teran et al, 1995; Zietkowski, Tomasiak, Skiepko, & Bodzenta, 2008; Giuffrida et al, 2014). CCL5 is increased in bronchoalveolar lavage fluid following allergen exposure in asthma patients and correlated with the number of eosinophils in the airways (Teran et al, 1996). Additionally, CCL5 concentrations in EBC have been shown to significantly increase from pre (8.76 pg/mL) to 30 minutes post (10.82 pg/mL) an exercise challenge in patients with asthma and exercise-induced bronchoconstriction (EIB) (Zietkowski et al, 2010). Our results however showed no significant increase in blood serum concentrations of CCL5 from pre (10337 pg/mL) to 30 minutes post EVH test (12075 pg/mL). This contrasts the findings of Zietkowski et a. (2009) who found significant increases in blood serum CCL5 concentrations from pre to 30 minutes post an exercise challenge test in participants with asthma and EIB. This discrepancy is likely due to the poor statistical power of the present study as while there was no significant effect a medium effect size was reported (d = 0.65) suggesting a significant increase in blood serum CCL5 concentration may be found from pre to post EVH test in a larger cohort. Baseline blood serum CCL5 concentrations in the present study (10,337 pg/mL) were similar to concentrations reported previously in asthma patients with EIB (7089 pg/mL) (Zietkowski et al, 2009). In the present study baseline CCL5 concentrations were unchanged after B-GOS supplementation. Concomitantly, GOS supplementation has been shown to attenuate CCL5 concentrations in lung homogenates in HDM challenged mice by approximately 50%, in part due to the upregulation of Treg cells (Verheijden et al, 2015; Verheijden et al, 2016). This suggests that GOS can attenuate challenge induced increases in CCL5. Therefore, the lack of an effect of B-GOS

supplementation on CCL5 concentrations may be a type-II error due to the low sample size, and as with all measures B-GOS dose may have been too low to augment an effect.

MCP-1 is unregulated in bronchial tissues of asthmatic subjects and is found in higher blood serum concentrations in asthma patients compared to healthy controls (Sousa, Lane, Nakhosteen, & Yoshimura, 1994; Giuffrida et al, 2014). MCP-1 is involved in the migration and infiltration of monocytes and macrophages and in the polarization of Th2 cells helping to orchestrate type-2 inflammation (Sousa et al, 1994; Deshmane, Kremlev, Amini, & Sawaya, 2009). Baseline concentrations of MCP-1 in the present study (~37pg/mL) are like that reported previous in asthma patients (~40pg/mL) and greater than reported in healthy controls (~15pg/mL) (Giuffrida et al, 2013). To our knowledge this is the first to evaluate the effect of B-GOS supplementation on blood serum MCP-1, which remains unchanged. Like other chemokine concentrations these results need to be interpreted with care due to the low sample size and B-GOS dosage.

SCF is a chemokine involved in the haematopoiesis and migration of mast cells and ILC2s, two inflammatory cells with a large influence in the pathophysiology of HIB and asthma (Virk, Arthur, & Bradding, 2016; Fonseca et al, 2019). SCF mRNA expression is significantly greater in the airway epithelium of asthma patients (70% positive cells) compared to control participants (13% positive cells) (Al-Muhsen, Shablovsky, Olivenstein, Mazer, & Hamid, 2004). Additionally, blood serum concentrations of SCF have been reported to be approximately 21% higher in asthma patients compared to health controls and along with its soluble receptor (c-kit) it positively correlates with asthma severity (Makowska, Cieslak, & Kowalski, 2009). In the present study, baseline SCF concentration unchanged after B-GOS supplementation. Additionally, TSLP concentrations were unaltered with B-GOS supplementations. TSLP is an airway epithelial derived cytokine involved orchestrating type-2 inflammatory airway responses and suppresses the activity of Treg cells

(Ziegler et al, 2013; Nguyen, Vanichsarn, & Nadeau, 2010). TSLP is released from airway epithelial cells upon damage with greater responses seen in asthma patients with EIB compared to asthma patients without EIB and healthy controls (Lai et al, 2014). Serum TSLP concentration in the present study (204pg/mL) was comparable to that previously reported in asthma patients (203pg/mL) and higher than previously shown in healthy controls (44pg/mL) (Skrgat et al, 2015).

Baseline serum IL-13 concentration also did not respond to B-GOS supplementation. IL-13 is released from Th2 cells and type-2 innate lymphoid (ILC2) cells and plays a pivotal role in AHR by initiating airway smooth-muscle contraction and goblet cell hyperplasia (Wills-Karp et al, 1998; Grunig et al, 1998). As such IL-13 is strongly associated with AHR, asthma severity and a rapid decline in lung function with age (Agache et al, 2016). GOS supplementation in a mice models of asthma attenuated HDM-induced increase in lung homogenates IL-13 concentration (Verheijdhen et al, 2015). The baseline concentration of IL-13 in the present study (~12pg/mL) was much lower than reported in allergic asthma patients (119pg/mL) and more in line with measures previously found in healthy control participants (18pg/mL) (Wong et al, 2001). IL-13 is a good predictor of blood eosinophil counts and alongside the blood eosinophil data suggests that the present cohort may lack participants with an eosinophilic/type-2 asthma phenotype (Agache, 2016; Zang, 2014). Mechanistic studies in mice suggest that GOS supplementation attenuates asthma by enhancing the upregulation and activity of Treg cells and attenuating type-2 inflammation leading to reduced airway eosinophilia and AHR (Verheijden et al, 2015a; Verheijden et al, 2015b; Verheijden et al, 2015c; Verheijden et al, 2016; Verheijden et al, 2018). These models rely on sensitization and challenge with HDM which induces a type-2 airway response and airway eosinophilia. In addition, the previously reported protective effect of B-GOS in asthma (Williams et al, 2016) was associated with a significant reduction in CCL17, which is

a chemokine involved in the recruitment of Th2 cells. The cohort in Williams et al (2016) also had high baseline levels of CCL5 and CCL17, which are chemokines involved in type-2 airway responses. Collectively this suggests that the mechanisms of GOS supplementation may primarily act on attenuating type-2 airway inflammation an asthma phenotype which may have been lacking in the cohort of the present study. Due to the heterogeneous nature of asthma future work should collect sputum differential cell counts to accurately phenotype participants and use a larger cohort so that sub-analysis can be conducted within different asthma phenotypes and their response to GOS supplementation.

EBC pH did not significantly change from pre to post EVH test although there was a trend ($p = 0.069$) for EBC pH to increase from pre (5.7) to 30 minutes post-test (5.9). This conflicts the findings by Bikov et al (2014) who shows a significant decrease in EBC pH from pre to 30 minutes post an exercise challenge test in participants with asthma and EIB. This difference may in part be due to difference is sample prep with samples not deaerated in the present study. Following an EVH test however EBC pH does not change from pre to 10 minutes post-test (Mickleborough et al, 2013). Changes in EBC pH may therefore be challenge specific. Baseline EBC pH in the present study (5.7) was lower than most commonly reported in a systematic review by Aldakheel et al (2016) (pH: 6-7) but was similar to participants with stable asthma reported by Hunt et al (2000) (5.8). EBC pH is generally more acidic in asthma patients compared to healthy controls and is reduced in patients suffering with acute asthma compared to stable asthma patients and healthy controls (Aldakheel et al, 2016; Hunt et al, 2000). In present study EBC pH was unchanged following B-GOS supplementation, suggesting 3.65g/d of B-GOS for 3-weeks does not affect EBC pH.

7.4.6. Blood monocyte, neutrophil and basophil counts, and serum eotaxin concentrations response to EVH test

Blood basophil counts significantly increased from pre to 30 minutes post EVH test (Δ 50%). Similar finding has been shown in response to exercise challenge tests with blood basophil counts increasing from pre to post challenge in asthmatics with EIB, however, this response was not observed following isocapnic hyperventilation with cold air (Nagakura et al, 1982). EVH in the present study did cause a significant increase in blood basophils, which might be explained by the dry air (<5% humidity) used in EVH challenges since this is a key stimulus required to induce airway dehydration, airway surface osmolality and the subsequent inflammatory cascade. Hyperpnoea without dry air may therefore not have the same effect on blood basophil counts. While significant increases in circulating basophil counts have been shown in asthma patients in the first hour post an exercise challenge, the same response is observed in healthy control participants suggesting increases in blood basophils are induced by the exercise challenge itself rather than disease status or bronchoconstrictive response (Morgan, Phillips, Moodley, Elliot, & Davies, 1982). It is not known if the same occurs following an EVH test since a healthy control group was not used in the present study. Nevertheless, there was no correlation between the change in blood basophils pre to post EVH test and the peak fall in FEV_1 post EVH test. As such, future work should look to repeat these findings with the addition of a healthy control group to assess whether increased blood basophils are part of HIB pathology or a normal response to an EVH test.

Additionally, we saw significant increases in blood neutrophils (Δ 21%) and monocytes (Δ 24%) cell counts in participants with asthma and HIB from pre to 30 minutes post EVH test. To our knowledge this the first time that these changes have been reported in participants with asthma and HIB in response to an EVH challenge. Following allergen but not histamine induced bronchoconstriction there is an increased activity of blood neutrophils and

monocytes (Carroll, Durham, Walsh, & Kay, 1985; Tanizaki et al, 1982). Blood monocytes show a strong relationship with asthma attacks, increasing in the pre-attack stage and peaking during asthma attacks, subsequently attenuating on alleviation of symptoms (Tanizaki et al, 1982). It must be noted that exercise neutrophilia occurs during and immediately after high intensity exercise due to demargination from endothelial cells and bone marrow (Quindry, Stone, King, & Broeder, 2003; Pyne, 1994). Similarly exercise increases circulating monocyte counts (Peake, Neubauer, Walsh, & Simpson, 2017). In the present study participants average V_E across EVH tests was ~74% of MVV. In healthy participants 8 minutes of normocapnic hyperpnoea at 70% MVV significantly reduces twitch transdiaphragmatic pressure by approximately 18% showing evidence of diaphragm fatigue (Renggli, Verges, Notter, & Spengler, 2008). Seated hyperpnoea at around 70% MVV may therefore increase respiratory muscle work to an extent that elicits respiratory muscle fatigue. As such, it cannot be excluded that increases in blood neutrophil and monocytes counts following an EVH test may be a response to respiratory muscle work rather than HIB pathology. Additionally, both blood monocyte and neutrophil counts did not correlate with the peak fall in FEV_1 post EVH test. Our results showed a significant increase in blood monocytes and neutrophil post EVH test which may suggest a potential involvement in the pathology HIB which has not been implicated previously, however, these results need replicating with the addition of a healthy control group to confirm whether these changes are part of HIB pathology or a normal EVH test response.

Blood serum CCL11 concentrations were shown to increase from pre to 30 minutes post EVH test (Δ 18%) in the present study. Previously CCL11 has been measured in EBC pre and post an exercise challenge test in participants with asthma and EIB (Zietkowski, Skiepko, Tomasiak-Lozowska, Zietkoska, & Bodzenta-Lukaszyk, 2011). Zietkowski et al (2011) found CCL11 concentrations in EBC to be significantly increased from 10 minutes to 24

hours post-test compared to pre-test in participants with asthma and EIB, while no change was seen in healthy controls and asthma participants without EIB. Our results support Zietkowski's findings, suggesting that an increase in CCL11 follow exercise/EVH challenge is involved in EIB/HIB. CCL11 is produced by multiple cells including airway epithelial cells, endothelial cells, lymphocytes, macrophages, eosinophils, and airway smooth muscle cells (Luster, 1998). CCL11 is the strongest chemoattractant for eosinophils inducing airway infiltration and activation of eosinophils (Rankin et al, 2000). While we saw increases in serum CCL11 we did not see changes in blood eosinophil counts, this in part may be explained by the fact that CCL11 peaks in EBC samples at 6 hours post challenge (Zietkowski et al, 2011). As such, both CCL11 and eosinophils may play a more prominent role in the late phase response in HIB.

7.4.7. Limitations of the study

A limitation of the current study was the sample size which may have caused a type II error for the peak fall in FEV_1 post EVH test and for markers of systemic inflammation (Jones, 2004). While previous work has suggested a sample size of seven is adequate to detect a minimum perceptible change in the fall in FEV_1 post EVH this was determined using a breath-by-breath EVH test method (Williams et al, 2015; Williams et al, 2016; Santanello et al, 2013). The EVH test in the present study used the dry gas meter method which may have a negative impact on test reproduciblity (Price et al, 2015). The adoption of the breath-by-breath technique, along with a larger sample size may provide further insight into the effects of B-GOS supplementation on HIB severity. The present study did not include measures of faecal bacteria or SCFAs to analyse the effect of B-GOS supplementation on alterations in the gut microbiota. A GOS content below 5g/d for 3-weeks has been shown to have no

bifidogenic effect in healthy adults. The present study adopted supplementation with the commercial B-GOS dose (3.65g/d) which delivers a daily GOS dose of 2.9g which may not have been enough to see changes in faecal bifidobacteria. Additionally, changes in gut bifidobacteria to prebiotic supplementation is confounded by participant's baseline levels (Kolida et al, 2007; De Preter et al, 2008). A larger cohort and faecal bacteria measurements provide further insight into whether the dosage used in the present study induces gut bacterial changes and whether individual bifidobacteria responses to B-GOS supplementation relate to improvements in asthma and HIB severity.

7.4.8. Conclusion

In conclusion, 21 days supplementation with 3.65g/d of B-GOS (80% GOS content) did not alter the severity of HIB, lung function, asthma control, or systemic markers of airway inflammation in participants with mild to moderate asthma and HIB. Additionally, B-GOS supplementation did not alter EBC pH. We did however observe significant increases in blood monocytes (Δ 24%), neutrophil (Δ 20%) and basophil (Δ 50%) counts, and blood serum CCL11 concentrations (18%) from pre to 30 minutes post EVH test in participants with mild to moderate asthma and HIB.

Chapter 8 - General Discussion

8.1. Summary of key findings

Chapter 4 reported a prevalence of HIB in 47 British University field hockey of 19% with a higher prevalence found in males (30%) than females (5%). In addition, data in chapter 4 confirms the underdiagnosis and undertreatment of HIB with 67% on HIB-positive participants not prescribed asthma/EIB medications and 0% with a previous diagnosis of HIB or EIB confirmed by an objective test. Moreover, during HIB screening baseline spirometry identified a participant with baseline airway obstruction (FEV_1 <70%), this participant was excluded from performing an EVH test but was referred to their general practitioner. These findings highlight the importance of screening in athletes to identify HIB/EIB and to support the respiratory health of athletes. Such screening practises for EIB and HIB should implement the ATS criterion as the prevalence of HIB was shown to range from 19-38% depending on the diagnostic FI criteria used. Finally, chapter 4 found that symptoms of dyspnoea namely the "unpleasantness or discomfort of breathing" (A1) and the sum of A1 and scores relating to the intensity of sensory dimensions (IPDS), were higher in HIB-positive than HIB-negative participants. In conjunction, the peak fall in FEV_1 post EVH test correlated negatively with A1 and IPDS scores. An A1 score of three and IPDS score of seven had an exceptional negative prediction value (100%) in excluding a HIB diagnosis following the EVH test and both A1 and IPDS had good sensitivity and specificity in predicting HIB (A1: Sensitivity = 100%; Specificity = 63%; IPDS: Sensitivity = 89%; Specificity = 66%).

Chapter 5 found IL-13 and TSLP were undetectable in EBC samples collected with the RTube device in participants with asthma and HIB at baseline and post EVH test when analysed with ELISAs. This was despite participants having an average drop in FEV_1 of 31%, with four participants presenting with severe HIB defined by a fall in FEV_1 ≥50% in participants not on maintenance ICS therapies or ≥30% in participants on a maintenance dose of ICS (Weiler et al, 2016). Following on from this **Chapter 6** found IL-5 and TNF-α could

not be detected in EBC samples collected with the RTube device from asthma participants and healthy controls when analysed using a bioplex multiplex assay. Additionally, coating the RTube condensing chamber and sample storage equipment with 1% BSA and 0.01% Tween20 alongside concentrating samples 8-fold did not result in detection of IL-5 and TNF-α. It is suspected that the RTube device material and design reduce its ability to detect these biomarkers, all of which have been measured with other devices using the same analysis platforms. From our findings we recommend researchers to use caution in the using the RTube device to collect EBC for the measurement of cytokines. As such, this method could not be used to measure airway inflammation in the assessment of B-GOS supplementation in chapter 7.

Chapter 7 found that 3.65g/d supplementation with B-GOS (80% GOS content) for 21-days did not alter the peak fall in FEV_1 post EVH test or asthma control in participants with mild-to-moderate asthma and HIB from baseline or compared to placebo treatment. In conjunction, there was no change in differential white blood cell counts or percentages (eosinophils, neutrophils, lymphocytes, monocytes, basophils), blood serum cytokine and chemokine concentrations (IL-13, TLSP, CCL11, CCL5, MCP-1 SCF), and EBC pH with B-GOS supplementation. Significant increases in blood neutrophil (Δ 20%), monocytes (Δ 24%), and basophil (Δ 50%) cell counts were reported from pre to 30 minutes post EVH test at baseline. Additionally, significant increases in blood serum CCL11 concentration (18%) was reported from pre to 30 minutes post EVH test at baseline. Increases in neutrophils, monocytes, basophils and CCL11 require replicating with the addition of a healthy control group to confirm whether these changes are part of HIB pathology or a normal EVH test response.

8.2. Assessment of hyperpnoea-induced bronchoconstriction

The diagnosis of EIB and HIB relies upon the data from objective exercise or surrogate challenge tests (Weiler et al, 2016). Spirometry is commonly used in the diagnosis of respiratory disorders and helps to identify obstructive and restrictive respiratory diseases (Miller et al, 2005). The clinical utility of spirometry in the diagnosis of EIB in athletes is limited however as lung function is typically within the normal range (Bonini & Palange, 2015). In Chapter 4 FEV_1, FVC, FEV_1/FVC, PEF, and $FEF_{25-75\%}$ was no different between HIB-positive and negative athletes. In all HIB-positive athletes FEV_1, FVC and PEF was within the normal range (>80%) with only one athlete having a mild reduction in FEV_1/FVC ratio (79% predicted). $FEF_{25-75\%}$ however, was reduced in 44% (4/9) of HIB-positive athletes but only 29% (11/38) of HIB-negative athletes, although there was no significant difference between groups. Similarly, in Chapters 5 and 7 the cohort average for all lung function variables within asthma and HIB cohorts was in the normal range apart from $FEF_{25-75\%}$ in chapter 5 (77%). Individual cases of reduced lung function were more common in HIB participants that additionally suffered from asthma (e.g. Chapter 5 and 7), particularly for FEV_1 which was reduced (<80%) in 22% of participants with asthma and HIB collectively. This however likely reflects the obstructive nature of asthma rather than HIB (James et al, 2005). Future work comparing lung function between asthma patients without HIB, asthma patients with HIB, HIB participants without asthma and healthy controls would help to confirm this. Data from chapter 4, 5 and 7 support the poor clinical utility of spirometry in identifying EIB/HIB, especially in athletes (chapter 7) where lung function is regularly in the normal range (Bonini & Palange, 2015; Bonini, Lapucci, Petrelli, Todaro, Pamich & Rasi, 2007).

Like spirometry, respiratory symptoms have been shown to have poor diagnostic accuracy (~50%) in diagnosing EIB or HIB (Ansley et al, 2012). Previously however, symptoms have

been assessed retrospectively with participants at rest and asymptomatic, rather than shortly after an exercise or an EVH test when bronchoconstriction is present (Dickinson et al, 2011; Parson et al, 2007; Burnett et al, 2016). This is problematic because the severity of EIB may relate poorly to respiratory symptoms that are assessed retrospectively and reliant on memory (Conner and Barret 2012). Conversely, momentary conscious respiratory symptoms are more likely to relate to the severity of bronchoconstriction if assessed *in situ*, i.e. shortly after an EVH test (Conner and Barret 2012). Chapter 4 showed that symptoms of dyspnoea, namely the "unpleasantness or discomfort of breathing" (A1) and the sum of A1 and scores relating to the intensity of sensory dimensions (IPDS), were higher in HIB-positive than HIB-negative participants and correlated with the peak fall in FEV_1 post EVH test. An A1 score of three and IPDS score of seven had an exceptional negative prediction value (100%) in excluding a HIB diagnosis following the EVH test and both A1 and IPDS had good sensitivity and specificity in predicting HIB (A1: Sensitivity = 100%; Specificity = 63%; IPDS: Sensitivity = 89%; Specificity = 66%). These symptoms may offer a useful tool to rule out the presence of HIB/EIB and to support the need for further objective testing. Price et al (2016) found through conducting semi-structured interviews that HIB-positive athletes typically locate symptoms lower down the respiratory tree (chest/lungs vs larynx) and recover rapidly post exercise from presenting respiratory symptoms (2-10 minutes) compared to HIB-negative athletes. While data is sparse, data from chapter 7 complements Price et al (2016) and supports the notion that HIB/EIB positive and negative athletes can be distinguished using certain respiratory symptoms and patterns. While no current validated questionnaire exists for EIB, these findings highlight the potential to develop such a questionnaire in the future.

While the findings of chapter 4 are encouraging the lack of a validated symptoms questionnaire and the poor diagnostic accuracy of spirometry support the need for objective

testing to identify EIB. While more time consuming and requiring greater resources compared to spirometry and symptom history the EVH test is easy to implement and is well tolerated by most participants (Weiler et al, 2016). All participants with asthma and HIB in chapter 5 and 7 tolerated the EVH test and were able to achieve the ventilatory requirements of a valid test (≥60% MVV), even in participants who suffered from severe bronchoconstriction. Within chapter 4, 85% of university field hockey players were able to achieve ≥60% MVV highlighting only a small portion of individuals (15%) unable to achieve such ventilatory demands required for a valid test. Interestingly, only one HIB-positive athlete was unable to achieve the target ventilation, while no other athlete unable to achieve the target had a history of EIB, asthma or another respiratory disorder. The percentage of athletes unable to attain the target ventilation was similar between male (15%; 4/27) and female athletes (15%; 3/20). All athletes unable to achieve the ventilation requirements were unsuccessful at achieving the target ventilation at a second attempt of the EVH test. In these individuals it may be advisable to perform alternative bronchoprovocation tests (e.g. exercise or mannitol challenge test) to confirm the presence or absence of AHR. Regardless this thesis supports the use of the EVH test in identifying EIB/HIB.

The inability to predict EVH test responses and the severity of bronchoconstriction is a significant issue in EVH testing. This is exemplified in chapter 7 where one participant was excluded from the study due to having a large drop in FEV_1 (>60%) from pre to post EVH test at screening. There are currently no tools available to predict EVH test responses, if such tools could be developed this would allow for ventilation targets to be altered to cater for participants suspected of have large drops in lung function. While not able to predict the severity of bronchoconstriction an increased likelihood of a positive EVH test response is greater in participants with underlying asthma (Sano et al, 1998; Cabral et al, 1999). In chapter 5 and 7, 41 participants with a current diagnosis of asthma performed an EVH test

during screening for eligibility. Of these participants 56% had a positive EVH test response. In chapter 4, 75% (3/4) of athletes with asthma had a positive EVH test while only 12% of athletes without asthma had a positive EVH test. These findings are consistent with previous reports highlighting a prevalence of EIB/HIB in approximately 50% of asthma patients (Sano et al, 1998; Cabral et al, 1999) while the prevalence in the general population is estimated much lower (5-20%; Aggarwal, Mulgirigama, & Berend, 2018; Johansson et al, 2014; Aguiar et al, 2018; Molphy et al, 2014).

Data from this thesis supports the poor accuracy of spirometry in identifying EIB/HIB and an increased likelihood of HIB in individuals with an underlying diagnosis of asthma. Furthermore, data from chapter 4 supports an association between symptoms of dyspnoea and HIB, however, with the lack of a validated symptoms questionnaire this thesis supports the need for data from objective exercise or surrogate tests to identify EIB/HIB.

8.3. Unmet need in the treatment of hyperpnoea-induced bronchoconstriction

A further theme that has developed throughout this thesis is the burden of HIB in both athletes and individuals with asthma. This is most obvious in chapter 4 were a HIB prevalence of 19% was found in university field hockey players. Interestingly, 67% on HIB-positive participants were not prescribed asthma/EIB medications and 0% had a previous diagnosis of HIB or EIB confirmed by an objective test. These findings support Dickinson et al (2011) who screened 228 elite British athletes and found a prevalence of EIB at 34% with 73% of EIB-positive athletes having no previous diagnosis of EIB. Alternatively, Ansley et al (2012) found 51% of professional football players regularly using asthma medications do not have a positive bronchodilator or EVH test response. Collectively there is a major issue in the misdiagnosis and treatment of EIB. This highlights the importance of screening athletes with

EIB using objective testing to ensure EIB-positive athletes receive appropriate treatment. In doing so the ATS criterion should be adopted, this is because we reported in chapter 4 that HIB prevalence ranged from 19%-38% depending on which of three commonly used criterion were adopted (Parsons et al, 2013; Weiler et al, 2016; Anderson et al, 2001; Hurwitz et al, 1995). Furthermore, we reported a higher prevalence of HIB in male (30%) compared to female athletes (5%). While not investigated in this thesis, the peak fall in FEV_1 following an exercise challenge test fluctuates across the menstrual cycle while oral contraceptives can alleviate asthma symptoms (Stanford et al, 2006; Jung et al, 2018). Estimations of HIB severity in females may therefore be influenced by menstrual cycle phase and contraceptive therapies. Future work is required in this area with our findings recommending that future studies should document / control for menstrual cycle phase and contraceptive therapies. This is important in female athletes requiring objective testing to confirm AHR for the use of asthma medication in line with WADA guidelines, as tests conducted during a bronchoprotective phase of the menstrual cycle may result in a false-negative result.

EIB/HIB in asthma patients is associated with poor asthma control (Aggarwal, Mulgirigama, & Berend, 2018), however, in chapter 7 baseline ACQ-7 scores were not associated with the peak fall in FEV_1 from pre to post EVH test ($r = 0.18$). This is consistent with reports in children with asthma where asthma control is poorly associated with EIB (Madburn et al, 2011; Rapino et al, 2011). This is important as in chapter 5 and 7 participants had asthma and HIB, seven of which suffered with severe bronchoconstriction. This means that even if an in individual has well controlled asthma, they could be suffering with severely uncontrolled EIB without detection. Therefore, appropriate treatment to control asthma may not be adequate to also control EIB. This is evident in chapter 5 and 7 where all participants were prescribed a short-acting β_2-agonist and all, but one participant were taking a maintenance therapy in the form of an ICS. This shows that the maintenance therapy in participants who developed

severe bronchoconstriction (N=7) was not adequately controlling HIB. These participants are likely to be heavily reliant on their β_2-agonist therapy while exercising. In athletes, who train multiple times a day, this issue could lead to an overuse of β_2-agonist therapy leading to tolerance or a worsening of bronchoconstriction (Swystun et al, 2000). Furthermore, a reliance of β_2-agonist therapy may put an athlete at a higher risk of surpassing urine substance thresholds set by WADA leading to an AAF and anti-doping violation (WADA. Medical Information To Support The Decision of TUECS – ASTHMA. https://www.wada-ama.org/en/resources/therapeutic-use-exemption-tue/medical-information-to-support-the-decisions-of-tuecs-asthma. Access: 03/01/2021). These findings therefore further support the need to investigate alternative therapies to treat EIB/HIB to reduce the reliance on pharmacological therapies.

Supplementation with the prebiotic, B-GOS, has been shown to attenuate HIB by 40% and reduce systemic markers of airway inflammation in participant with asthma and HIB (Williams et al, 2016). However, to date Williams et al (2016) is the only study to assess the effect of B-GOS supplementation in participants with asthma and HIB. B-GOS may have therapeutic potential in participants with asthma and HIB and therefore warranted further investigation. The findings in chapter 7 suggested that 21 days supplementation with 3.65g/d of B-GOS does not affect HIB severity or asthma control in participants with mild-to-moderate asthma and HIB. Additionally, B-GOS supplementation did not affect differential white blood cell counts or percentages (eosinophils, neutrophils, lymphocytes, monocytes, basophils), blood serum cytokine and chemokine concentrations (IL-13, TLSP, CCL11, CCL5, MCP-1 SCF), or EBC pH. This finding contrasted previous work (Williams et al, 2016) and sheds doubt on the efficacy of B-GOS to attenuate HIB, although, GOS dosage was lower (2.92g/day vs. 5.28g/day) than used by Williams et al (2016) with the small sample size additionally likely contributing to lack of an effect seen.

A limitation of the study in chapter 7 is that no measure of the faecal microbiota was taken to assess the effect of the B-GOS dosage on the gut microbial composition. Changes in gut Bifidobacterium to prebiotic supplementation is confounded by participant's baseline levels, with lower baseline levels resulting in large responses (Kolida et al, 2007; De Preter et al, 2008). Measures of faecal bacteria would have allowed us to assess whether individual Bifidobacterium responses to B-GOS supplementation related to improvements in asthma and HIB severity. Furthermore, the study is susceptible to type II errors due to the low sample size (Jones, 2004). While previous work has suggested a sample size of seven is adequate to detect a minimum perceptible change in the fall in FEV_1 post EVH this was determined using a breath-by-breath EVH test method (Williams et al, 2015; Williams et al, 2016; Santanello et al, 1999). The effect of B-GOS supplementation on airway inflammation requires investigating, however, as shown in chapter 5 and 6 this cannot be achieved by collecting EBC with the RTube device as it lacks the efficacy to collect EBC with measurable IL-13 and TSLP concentrations in participants with asthma and HIB at rest and while suffering with bronchoconstriction. Additionally, the RTube lacks the efficacy to collect IL-5 and TNF-α in participants with asthma at rest.

8.4. General limitations

The specific limitations of each study are discussed in greater detail within the corresponding experimental chapter. A universal limitation was the inclusion criteria for asthma participants which involved a doctor's diagnosis of asthma requiring the treatment with up-to-date asthma medication. Asthma is commonly misdiagnosed as the symptoms of asthma mimic many alternative respiratory disorders, as such, asthma is over diagnosed and over treated (Heffler, Pizzimenti, Guida, Bucca, & Rolla, 2015). The reliance on a doctor's diagnosis of asthma

could have contributed to the inclusion of participants without asthma. The inclusion of reversibility testing would have helped to confirm a diagnosis of asthma and is recommended for future research. The study participants in chapter 5 and 7 did however have confirmed HIB showing evidence of airway hyper-responsiveness, a key feature of asthma. In addition, approximately 50% of asthma patients suffer from EIB and HIB (Sano, 1998; Lucia, 1999) and therefore the documentation of HIB in these participants would further support a diagnosis of asthma on top of doctor's diagnosis of asthma.

Additionally, asthma is a heterogeneous disease that is classified across a spectrum of severities and encompasses numerous phenotypes which possess differences in the underlying molecular pathology (Wenzel et al, 2013). Asthma phenotypes are commonly characterised by measuring airway gene signatures or sputum differential cell counts (Kuo et al, 2017), neither of which was conducted on asthma participants included in experimental chapters. Alternatively, chapter 5 and 7 included differential white blood cell counts which can be used to predict sputum eosinophilia and therefore can be informative on predicting asthma phenotypes (Zhang et al, 2014). Furthermore, in asthma patients with EIB/HIB airway eosinophilia is a distinct feature of this phenotype and is associated with the severity of EIB (Hallstrand et al, 2005; Yoshikawa et al, 1998). This phenotype also presents with high intraepithelial mast cells and these mast cells present with a distinct tryptase and CPA3 high and chymase low characterisation (Hallstrand et al, 2011; Lai et al, 2014). This suggests that in chapter 5 and chapter 7 in which participants had asthma and HIB these participants most likely had an asthma phenotype that was eosinophil/type-2-high in nature. Finally, in chapter 7 there was a large number of measurements which were not normally distributed. The combination of low participant numbers alongside the heterogeneity of asthma likely contributed to this. Future research should look to replicate these findings in larger participant

numbers to allow for the heterogeneity of asthma and to reduce the risk of type II analysis errors.

8.5. Significance of findings and future research directions

In chapter 4 we assessed the largest cohort of field hockey players to date for the prevalence of HIB. In doing so we found a high prevalence of HIB (19%) which was greater in males (30%) than females (5%). The diagnostic criteria used drastically influenced prevalence rates (19-38%). Furthermore, we found for the first time that symptoms of dyspnoea assessed *in situ* were associated and predictive of the presence of HIB following an EVH test. Future research studies should look to focus on:

- Establishing the prevalence of HIB in large cohorts of other sports to help further understand the burden of HIB within individual sports. Research should continue to collect data on the prevalence of HIB in field hockey players so subsequent meta-analysis can be conducted in very large participant numbers. Furthermore, future research should adopt the ATS criterion when estimating the prevalence of HIB to ensure continuity between studies.

- Sex differences should be more widely reported and needs investigating in other sports. In doing so future research needs to take into account the influence of menstrual cycle phase and contraceptive therapies on HIB and try to document or control for these factors. In addition, future research should try to establish the effect of the menstrual cycle phase and the accompanying fluctuation in sex hormones on the EVH test response in both healthy controls and participants with documented HIB. The effect of contraceptive therapies on the EVH test response also warrants investigating.

- Future research should further investigate other symptom questionnaires or respiratory symptoms assessed *in situ* with HIB and EIB to identify symptoms predictive of HIB/EIB. The identification of further predictive questions may allow for the development and validation of an HIB/EIB diagnostics questionnaire.

- Furthermore, there is potential to conduct semi structured interviews post exercise and EVH challenges to identify symptom and symptoms patterns which may distinguish positive and negative responders. Such research would help to further develop questions that could form part of a diagnostic questionnaire.

- Greater field-based testing needs to be conducted on EIB-positive individuals to assess whether they frequently suffer with bronchoconstriction following their respective sports. If certain EIB-positive athletes rarely suffer with bronchoconstriction following their respective sport, then EIB-specific symptoms will not develop and will not be detected by such a questionnaire. Future work should additionally look to assess the impact of appropriate therapy on EIB severity and exercise performance in newly identified EIB cases.

The findings in chapter 5 and 6 expand the current knowledge of the use of EBC to measure airway inflammation. The findings of chapter 5 and 6 found the following significant findings and provide direction for future research:

- IL-13 and TSLP were undetectable in EBC samples collected with the RTube device in participants with asthma and HIB at baseline and post EVH test. Furthermore IL-5 and TNF-α could not be detected in EBC samples collected with the RTube device from asthma participants and healthy controls.

- Coating the RTube condensing chamber and sample storage equipment with 1% BSA and 0.01% Tween20 alongside concentrating samples 8-fold did not result in detection of IL-5 and TNF-α.
- Future research should assess the effect of coating the RTubes polypropylene condensing chamber with surfactants. Surfactant's coating may result in a greater recovery of biomarkers with the RTube device as surfactant-polypropylene interactions are greater than protein-polypropylene interactions (Duncan et al, 1995).

In chapter 7 we found that 3.65g/d of B-GOS for 21-days did not affect HIB severity, asthma control, or systemic markers of airway inflammation. In chapter 7 we additionally showed for the first time that blood neutrophil (Δ 20%), monocytes (Δ 24%), and basophil (Δ 50%) cell counts significantly increased from pre to post EVH in participants with asthma and HIB. Future research studies should look to focus on:

- Confirming the optimal dose of the current commercially available B-GOS mixture (80% GOS content) required to induce increases in faecal Bifidobacterium numbers and increases in faecal and blood SCFAs in healthy adults.
- Future research should collect faecal samples from asthma and HIB participants receiving B-GOS to assess whether individual Bifidobacterium responses to B-GOS supplementation relate to improvements in asthma and HIB severity.
- Mice models of asthma have shown significant reductions in airway inflammation with GOS supplementation with mechanisms alluding to the attenuation of type-2 inflammation. The collection of sputum samples for the assessment of asthma phenotypes would be useful in understanding whether the effect of B-GOS supplementation is specific to certain asthma phenotypes. Additionally, it would allow the effect of B-GOS supplementation on airway inflammation to be assessed.

- To confirm some of the future research questions outlined above and the effect of B-GOS supplementation in asthmatics with HIB studies will need conducting on a larger scale with a larger sample size.
- Blood monocyte, neutrophil, and basophil counts should be assessed pre and post EVH test in participants with asthma and HIB, asthma without HIB, HIB without asthma, and healthy controls. Comparisons between these groups will help to establish whether these cells are implicated in HIB and/or asthma pathophysiology or whether increases are a normal response to an EVH test. Furthermore, the dynamics of these cell count changes should be documented across to early and late phase response.

8.6. Conclusion

In conclusion, this thesis found the prevalence of HIB in field hockey players to be high at 19%. The thesis findings support previous work (Dickinson et al, 2011) highlighting a high prevalence of HIB in athletes and an underdiagnosis and treatment. In participants with HIB and asthma we found B-GOS supplementation did not affect HIB severity, asthma control or systemic markers of airway inflammation. These findings contrasted previous work (Williams et al, 2016) and sheds doubt on the efficacy of B-GOS to attenuate HIB, although, GOS dosage and low sample size was noted as potentially influencing factors. Future work should look to confirm the efficacy of B-GOS using a B-GOS dosage providing ≥5.28g/d of GOS in a larger sample size and taking direct measures of airway inflammation. The measurement of airway cytokine concentrations (IL-5, IL-13, TSLP, TNF-α) could not be obtained in EBC samples using the RTube device, as such, future work should use alternative

measures and methods to assess the effect of B-GOS supplementation on airway inflammation in participants with asthma and HIB.

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Appendix 1 – Multi-dimensional dyspnoea profile questionnaire

Use this scale to rate the **intensity** or **strength** of your breathing sensations, how **much** sensation you have now.
Please focus on how your breathing feels now

0	1	2	3	4	5	6	7	8	9	10
NO SENSATION	SLIGHT SENSATION			MODERATE SENSATION						MAXIMUM SENSATION

Use this scale to rate the **unpleasantness** of your breathing sensations, how **good** or **bad** your breathing feels
Please focus on how your breathing feels now

←	←	0	1	2	3	4	5	6	7	8	9	10
PLEASANT	NEUTRAL		SLIGHT SENSATION			ANNOYING			DISTRESSING			UNBEARABLE

SQ -- Sensory Qualities -Rate the intensity of the breathing sensations you feel (like the loudness of sound, regardless of whether the sensation is pleasant or unpleasant; for example a sensation could be intense without being unpleasant.)

SQ1-My breathing requires muscle work or effort .
SQ2-I am not getting enough air, I feel hunger for air, or I am smothering.
SQ3-My breathing requires mental effort or concentration.
SQ4-My chest and lungs feel tight or constricted.
SQ5-I am breathing a lot. (breathing rapidly, deeply or heavily)

E – Emotional Response-Please tell us about how your breathing sensations made you feel – rate zero for any emotion you did not feel.

E1-Depression
E2-Anxiety
E3-Frustration
E4-Anger
E5-Fear

Appendix 2 – Asthma control questionnaire 7-item

The Asthma Control Questionnaire ©

PARTICIPANT CODE	SUPPLEMENT LETTER	EXPERIMENTAL TRIAL/WEEK NUMBER	DATE

Circle the number of the response that best describes how you have been during the past week:

1. On average, during the past week, how often were you woken by your asthma during the night?
   - 0 Never
   - 1 Hardly Ever
   - 2 A Few Minutes
   - 3 Several Times
   - 4 Many Times
   - 5 A Great Many Times
   - 6 Unable to Sleep Because of Asthma

2. On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?
   - 0 No Symptoms
   - 1 Very Mild Symptoms
   - 2 Mild Symptoms
   - 3 Moderate Symptoms
   - 4 Quite Severe Symptoms
   - 5 Severe Symptoms
   - 6 Very Severe Symptoms

3. In general, during the past week, how limited were you in your activities because of your asthma?
   - 0 Not Limited at All
   - 1 Very Slightly Limited
   - 2 Slightly Limited
   - 3 Moderately Limited
   - 4 Very Limited
   - 5 Extremely Limited
   - 6 Totally Limited

4. In general, during the past week, how much shortness of breath did you experience because of your asthma?
   - 0 None
   - 1 A Very Little
   - 2 A Little
   - 3 A Moderate Amount
   - 4 Quite a Lot
   - 5 A Great Deal
   - 6 A Very Great Deal

5. In general, during the past week, how much of the time did you wheeze?
   - 0 Not at All
   - 1 Hardly Any of the Time
   - 2 A Little of the Time
   - 3 A Moderate Amount of Time
   - 4 A Lot of the Time
   - 5 Most of the Time
   - 6 All of the Time

6. On average, during the past week, how many puffs of Short-Acting Bronchodilator (e.g. Ventolin) have you used on each day?
   - 0 None
   - 1 1-2 Puffs Most Days
   - 2 3-4 Puffs Most Days
   - 3 5-8 Puffs Most Days
   - 4 9-12 Puffs Most Days

		5	13-16 Puffs Most Days
		6	> 16 Puffs Most Days
7.	FEV_1 Pre-Bronchodilator:	0	> 95% Predicted
	FEV_1 Predicted:	1	95%-90%
	FEV_1 % Predicted:	2	89%-80%
		3	79%-70%
		4	69%-60%
		5	59%-50%
		6	< 50% Predicted