Dietary supplementation for the treatment of hyperpnoea induced bronchoconstriction in physically active asthmatics

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List of Abbreviations

5-LOX	5 lipoxygenase
9α, 11β-PGF_2	9α, 11β-prostaglandin F_2
AA	Arachidonic Acid
ANOVA	Analysis of variance
ASL	Airway surface liquid
ASM	Airway smooth muscle
B-GOS	trans-galactooligosaccharide
CCL5	chemokine (c-c motif) ligand 5
CCL11	chemokine (c-c motif) ligand 11
CCL17	chemokine (c-c motif) ligand 17
$CD4^+$CD25 Tcells	T regulatory cells with high expression of surface markers CD4 and CD25
CO_2	Carbon dioxide
COX	Cyclooxygenase
CRP	C reactive protein
CV	Coefficient of variation
DCs	Dendritic cells
DHA	Docosahexanoic acid
EIA	Exercise induced asthma
EIB	Exercise induced bronchoconstriction
ELISA	Enzyme linked immunosorbant assay
EPA	Eicosapentanoic acid
EVH	Eucapnic voluntary hyperpnoea
FENO	Fraction of exhaled nitric oxide
FEV_1	Forced expiratory volume in 1 s
$\%\Delta FEV_1$	Percent change in forced expiratory volume in 1 s
$FICO_2$	Fraction of inspired carbon dioxide
FoxP3	Forkhead box P3 transcription factor
FVC	Forced vital capacity

GALT	Gut associated lymphoid tissue
GI	gastrointestinal
HIB	Hyperpnoea induced bronchoconstriction
IECs	Intestinal epithelial cells
IFN-γ	Interferon-γ
IGE	Immunoglobulin E
IL	Interleukin
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-13	Interleukin-13
IL-33	Interleukin-33
IMT	Inspiratory muscle training
LT	Leukotriene
LTB_4	Leukotriene B_4
LTB_5	Leukotriene B_5
LTC_4	Leukotriene C_4
LTC_5	Leukotriene C_5
LTD_4	Leukotriene D_4
LTD_5	Leukotriene D_5
MALT	Mucosal associated lymphoid tissue
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKT	Natural killer T cell
NLRs	Nucleotide-binding oligomerization-domain protein-like receptors
NO	Nitric oxide
NOD	nucleotide-binding oligomerization-domain protein
O_2	Oxygen

ω3-PUFA	Omega-3 polyunsaturated fatty acids
PBMCs	Peripheral blood mononuclear cells
PCO_2	Partial pressure carbon dioxide
PEF	Peak expiratory flow
$PETCO_2$	Partial pressure of end tidal carbon dioxide
$PETO_2$	Partial pressure of end tidal oxygen
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGD_3	Prostaglandin D_3
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
SD	Standard deviation
SPSS	Statistical package for social sciences
TGF-β	Transforming growth factor beta
Th Cell	T helper cell
Th1	T helper 1 cell
Th2	T helper 2 cell
TLRs	Toll like receptors
TNF-α	Tumour necrosis factor-α
TNFR2	TNF-α receptor 2
Treg	T regulatory cell
TXA	Thromboxane
TXA2	Thromboxane A2
TXA3	Thromboxane A3
$\dot{V}_E$	Minute ventilation
V̇O2max	Maximal oxygen uptake
WADA	World anti-doping agency

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Abstract

Asthma is a significant public health burden with 5.4 million people in the UK currently receiving treatment for the condition. Exercise induced bronchoconstriction (EIB) is a term used to describe an acute asthmatic episode following exercise and is highly prevalent amongst asthma sufferers. Traditional treatment involves the use of pharmacological interventions, the long-term use of which can have significant side effects and reduced efficacy in athletic populations. The purpose of this thesis was to initially establish a reproducible means of measuring bronchoconstriction in asthmatics through an experimental model of EIB termed hyperpnoea induced bronchoconstriction (HIB), and then investigate the use of dietary supplements to treat HIB and suppress markers of airway inflammation.

Accordingly this thesis investigated: (i) the test-retest reproducibility of the bronchoconstrictive response to eucapnic voluntary hyperpnoea in physically active asthmatics and non-asthmatics, (ii) the effects of omega 3 polyunsaturated fatty acid supplementation dose level (6.2 $g \cdot d^{-1}$ vs 3.1 $g \cdot d^{-1}$) on the severity of hyperpnoea induced bronchoconstriction, and (iii) the effects of prebiotic trans-galactooligosaccharide (B-GOS) supplementation on severity of hyperpnoea induced bronchoconstriction and inflammatory markers in physically active asthmatics.

It was found that eucapnic voluntary hyperpnoea incorporating real-time visual feedback of minute ventilation ($\dot{V}_E$) and end-tidal PCO_2 yielded a smallest meaningful change of 88 mL highlighting a highly reproducible bronchoconstrictive response over both short- term (21 days) and long-term (70 days) periods, in physically active asthmatics with HIB. No between-day differences in the bronchoconstrictive response

as measured by both absolute drop in forced expiratory volume in one second (FEV_1, mL) and the percentage change in FEV_1 ($\%\Delta FEV_1$) following the eucapnic voluntary hyperpnoea protocol in the asthmatic participants were evident. Subsequently EVH was deemed a suitable technique for monitoring the effectiveness of treatment interventions.

This thesis shows for the first time that lower doses of ω3-PUFA (3.1 $g \cdot d^{-1}$) are equally as effective as higher doses (6.2 $g \cdot d^{-1}$) in reducing the severity of hyperpnoea induced bronchoconstriction in recreational athletes. Maximum reductions in FEV_1 at day 0 of 28 ± 87%, and -27 ± 17%, had improved to -19 ± 15%; and -18 ± 14% for the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions respectively. Both ω3-PUFA interventions resulted in equal reductions of up to 30% in resting levels of exhaled nitric oxide, and equal suppression up to 44% in inflammatory maker urinary 9α, 11β-PGF_2 concentration following the eucapnic voluntary hyperpnoea challenge. These data highlight that lower ω3-PUFA doses represent a potentially beneficial treatment for physically active asthmatics suffering with HIB whilst reducing the burden of cost, compliance and potential for gastrointestinal distress.

A highly novel finding of this thesis was that a 21 day intervention of a prebiotic galactooligosaccharide (B-GOS) markedly improved the severity of bronchoconstriction in HIB participants, with $\%\Delta FEV_1$ improving by up to ~40%. This occurred in conjunction with reduced markers of airway inflammation including serum concentrations of TNF-α (reduced by 36%), CCL17 (reduced by 22%), and C-reactive protein (reduced by 11%) in the HIB participants. For the first time this suggests that favourable manipulation of the commensal bacteria within the gut by B-GOS will influence immune regulation and inflammatory responses and could prove to be a novel therapeutic target for asthma and HIB.

It is attractive to speculate that combinations of ω3-PUFA, prebiotics, and probiotics could prove to be novel therapies for a range of asthma phenotypes and other respiratory diseases. Furthermore there is scope to develop a greater understanding into the mechanisms behind the effectiveness of the gut microflora as a therapeutic treatment option for inflammatory airway diseases.

1. Chapter 1 – General Introduction

1.1. Introduction and Rationale

Asthma is a highly prevalent chronic inflammatory disorder of the airways that affects both adults and children of all ages. It is the most common chronic disease among children and young adults, with one in four developing asthma before the age of 40 (To, et al. 2012, To, et al. 2010). The condition results in variable airflow obstruction in susceptible individuals but can be reversed with appropriate therapy or spontaneously. Individuals will suffer from symptoms that include, wheezing, breathlessness, coughing, and tightness in the chest (Sears 2008). There is a high heterogeneity in asthma symptoms and severity, and individuals will respond differently to the variety of possible triggers such as dry cold air, exercise, pollen, upper respiratory tract infections, pollution, cigarette smoke, and respiratory allergens (Douglas, et al. 2008). Asthma has a considerable health cost associated with it and, in the UK, the NHS spends £1 billion each year on asthma treatment for 1.1 million children (1 in 11) and 4.3 million adults (1 in 12) (Asthma UK Accessed on 12.06.2013). The prevalence of asthma has increased considerably over recent decades; the World Health Organisation suggests that there are 300 million patients with asthma globally, and this is expected to increase to 400 million by 2025 (Pawankar, et al. 2011). The rise in prevalence has been suggested to be in line with changes in environment and lifestyle associated with western living rather than genetic alterations (Beasley, et al. 2000, Platts-Mills, et al. 2005, Daley 2014).

There is high heterogeneity in asthma severity which has led to the recent concept that there are multiple phenotypes of asthma or groupings of characterisation (Wenzel 2013). Due to these varying phenotypes, difficulty remains in defining the

pathophysiology of asthma. It is commonly thought that a combination of airway smooth muscle (ASM) contraction, excess mucous production, and inflammation are the main symptoms associated with exacerbations (Anderson and Daviskas 2000, Anderson and Kippelen 2005b, Anderson and Kippelen 2005a). Airway inflammation is associated with a variety of leukocytes including eosinophils, mast cells, macrophages, neutrophils, and lymphocytes including B cells, T cells and natural killer cells. These inflammatory cells produce a number of inflammatory mediators that are thought to be key contributors to asthma. These include prostaglandins, leukotrienes, cytokines, histamine, and eicosanoids (Anderson and Kippelen 2005a).

Exercise induced bronchoconstriction (EIB) is a phenotype of asthma that is characterized by a transient narrowing of the airways during or on the cessation of exercise (McFadden Jr and Gilbert 1994, Anderson and Holzer 2000, Suman, et al. 1999). It is estimated that up to 90% of individuals with asthma and 10 to 50% of a given elite athletic population can experience EIB (Naguwa, Afrasiabi and Chang 2012). With such a high prevalence in the asthmatic population there is a clinical, quality of life and economic need to understand the best methods for managing EIB. Standard management techniques for EIB are pharmacological with the use of short acting and long acting bronchodilators (β2-agonists), inhaled corticosteroids and leukotriene modifiers. For the elite athlete, there are number of restrictions imposed by the World Anti-Doping Agency (WADA). The 2012 prohibited list states that all β2-agonists (including both optical isomers where relevant) are prohibited except salbutamol, a short-acting β2-adrenergic receptor agonist (limited to maximum of 1600 micrograms over 24 hr), and formoterol a long-acting β2 agonist (maximum 36 micrograms over 24 hr). All oral or intravenous glucocorticosteroids are prohibited and

a therapeutic use exemption certificate is required for these. In addition to the WADA restrictions, another limitation is that the chronic use of these drugs can cause systemic side effects (reduced bone mineral density, increased airway hyper responsiveness) along with degenerative changes to the respiratory system (Dahl 2006, Anderson, et al. 2006). Research has also shown that EIB in some athletes may not respond well to pharmacologic prophylaxis (Sue-Chu, et al. 2000) and over 50% of asthmatics are poorly controlled due to poor adherence (Barnes 2010). Furthermore, traditional pharmacological treatment such as β2-agonists only target symptoms rather than treatment of the underlying disease and inflammation. The limitations of the current treatments for asthma highlight the importance of novel therapeutic strategies to target underlying asthmatic and immune responses to help improve disease control and efficacy.

Over the past 15 years research has been reported to support the concept that diet is a contributory environmental factor to the increase in asthma prevalence (Allan and Devereux 2011). Adverse changes in diet have been shown to contribute to both an increase in prevalence and severity of asthma (Smit, Grievink and Tabak 1999, Shaheen, et al. 2001, McKeever and Britton 2004, Devereux and Seaton 2005). It has been hypothesised, for example, that the rise in asthma prevalence over the latter half of the 20th century could be related to decreasing omega-3 polyunsaturated fatty acid (ω3-PUFA) intake, and increased consumption of dietary omega-6 PUFA (ω6-PUFA) (Devereux and Seaton 2005). Consequently it seems plausible that favourable dietary manipulation may influence asthma and EIB severity.

Further to these dietary influences, a reduction in the microbial diversity of the human microbiome in western societies as a result of changes in diet, reduced exposure

to infections and pathogens, and increased antibiotic use may also be involved (Wills-Karp, Santeliz and Karp 2001). Both the human gastrointestinal tract and airways are home to unique colonies of commensal bacteria, the composition of which can influence inflammation and immune homeostasis. Murine models and human studies of airway diseases such as asthma and cystic fibrosis show sufferers of these diseases have altered airway and gut microflora when compared to healthy controls (Madan, et al. 2012, Sagar, et al. 2014). Evidence is now accumulating that favourable manipulation of the gut microflora through dietary interventions of pre- and probiotics can influence immune function (Lomax and Calder 2009, Roberfroid, et al. 2010), and consequently affect airway inflammation associated with asthma (Sagar, et al. 2014). Furthermore, manipulation of the gut microflora through a combination of dietary pre- and probiotics has been shown to alter the commensal bacteria of the airways in cystic fibrosis (Madan, et al. 2012).

In relation to asthmatics suffering with EIB, a number of studies have shown that manipulation of diet through supplementation with ω3-PUFA can attenuate the characteristic reduction in pulmonary function, and increase in airway inflammation following bronchial provocation challenges (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010). In addition, a number of research studies suggest that favourable manipulation of the gastrointestinal microbiota through pre- and probiotics can improve lung function and reduce asthmatic episodes during the course of supplementation (Gutkowski, et al. 2010). These research findings highlight the need to gain a greater understanding into the use of dietary supplements for managing EIB in active asthmatic individuals.

1.2. Research aims

The purpose of the series of studies was to investigate the following research questions

I. ***What is the reproducibility of the bronchoconstrictive response to eucapnic voluntary hyperpnoea (EVH) in physically active asthmatics and non-asthmatic controls?***

The research aims were to:

- Determine the test-retest reproducibility of the bronchoconstrictive response to EVH in physically active asthmatics.

  This will allow for greater confidence when tracking changes in the management of hyperpnoea induced bronchoconstriction in response to treatment interventions.

II. ***Previous research has demonstrated the effects of very high ω3-PUFA doses on EIB; it is of interest therefore to understand how effective a lower dose may be in managing EIB. A randomised, double blind, placebo controlled cross-over study was conducted to compare the effectiveness of two doses (6.2 $g \cdot d^{-1}$ vs 3.1 $g \cdot d^{-1}$) of omega-3 polyunsaturated fatty acid (ω3-PUFA) on hyperpnoea induced bronchoconstriction and markers of airway inflammation in physically active asthmatics.***

  The research aims were to evaluate:

- The effectiveness of a lower ω3-PUFA dose on hyperpnoea induced bronchoconstriction in physically active asthmatics when compared to a previously documented higher dose.

- Establish the immunomodulatory effects of 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$doses of ω3-PUFA by assessing markers of airway inflammation in physically active asthmatics.

III. A novel management approach for influencing airway inflammation associated with hyperpnoea induced bronchoconstriction in asthmatics may be through favourable manipulation of the gut microflora. A randomised, double blind, placebo controlled cross-over study to establish the effectiveness of prebiotic trans-galactooligosaccharide (B-GOS) on HIB and markers of airway inflammation was conducted.

The research aims were to evaluate:

- The use of B-GOS in managing the severity of hyperpnoea induced bronchoconstriction in physically active asthmatics.

- If B-GOS can favourably alter the levels of inflammatory markers associated with airway inflammation in physically active asthmatics.

2. Chapter 2 – Review of Literature

2.1. Exercise Induced Asthma

The first record of exercise induced asthma was reported in ~120-200 AD by Aretaeus, who noted that physical exertion could provoke airway obstruction (Adams 1856). Exercise induced asthma (EIA) refers to the transient narrowing of the airways that occurs after vigorous exercise in up to 80% of asthmatics (Anderson and Daviskas 1997) and is characterised by symptoms of coughing, wheezing, shortness of breath and chest tightness primarily on the cessation of exercise. It is associated with airway obstruction and reduced pulmonary function. There are two common terms used to describe airway narrowing associated with exercise: EIA and exercise induced bronchoconstriction (EIB). EIA is used to describe the exacerbation of an individual's asthma when they exercise (Storms 2003) whereas the term exercise induced bronchoconstriction is used to describe the airway narrowing occurring after exercise in individuals with and without a clinical diagnosis of asthma (Anderson and Henriksen 1999). It has been stated that the term EIA should no longer be used because exercise does not induce asthma, but rather it is a trigger of bronchoconstriction (Weiler, et al. 2007). For the purpose of this thesis the term EIB will encompass both of these definitions.

2.1.1. Prevalence of Exercise Induced Bronchoconstriction

Exercise induced bronchoconstriction is a condition that can affect people at any level of exercise, from children to Olympic athletes (Mahler 1993). Bronchoconstriction following exercise has been documented since the second century (Rundell and Jenkinson 2002) and exercise will trigger asthma in most individuals with chronic asthma and in some

individuals who do not have asthma (Weiler 1996). Asthma has been an increasing public health issue over the past 40 years and affects a significant proportion of the global population. The World Health Survey states that on a global scale the prevalence rate of doctor diagnosed asthma is 4.3% (To, et al. 2012). In 2004, the Global Initiative for Asthma (GIBA) combined data from the Phase 1 International Study of Asthma and Allergies (ISAAC) collected from 1992-1996 and the European Community Respiratory Health Survey (ECRHS) from 1988-1994 to generate a global estimate of asthma burden. The report estimated that 300 million people worldwide have asthma, and projected that this number would increase to 400 million by 2025, as countries develop. In the UK, 5.4 million people are currently receiving treatment for asthma this amounts to 1.1 million children (1 in 11) and 4.3 million adults (1 in 12) (Asthma UK Accessed on 12.06.2013). The data highlight the global burden of asthma and the increase in prevalence in western societies. Thus if the occurrence of asthma is increasing, there will be an associated rise in the prevalence of EIB.

Asthma and airway hyperreposnsiveness are amongst the most common chronic medical conditions reported by Olympic athletes with a prevalence of 7-8% (Kippelen, et al. 2012) although large variations exist between sports (Fitch 2012). In the general population up to 80% of clinically recognised asthmatics can experience EIB (Anderson and Holzer 2000). The prevalence of EIB has been reported in a non-elite population of 230 middle and high school athletes (11 – 18 years old) from a range of sports (Rupp, Guill and Brudno 1992). Following the completion of an exercise challenge, 29% of the athletes were identified as EIB positive based on a post-exercise drop in FEV_1 of greater than 15%;

all had been previously undiagnosed (Rupp, Guill and Brudno 1992). This study documents the prevalence in sub-elite athletes however with greater training loads associated with elite level sport it may predispose a greater risk of EIB in this population. Elite athletes have been shown to have a greater prevalence of airway hyper-responsiveness compared to a control population (Langdeau, et al. 2000). The athletes had a 49% prevalence of airway hyperresponsiveness compared with 28% of the sedentary participants ($P = 0.009$).

Within elite sportsmen and women there has been a steady increase in the number of athletes reporting symptoms associated with EIB. Reports from a screening programme organised by the US Olympic Committee found that 57 out of 597 (9.5%) American Olympic Athletes in the 1984 summer games reported suffering from EIB or asthma (Voy 1986). A high prevalence of asthma was also reported at the 1996 Olympic Games. Six hundred and ninety nine athletes completed a medical history questionnaire, 107 reported (15.3%) a previous diagnosis of asthma, 97 (13.9%) recorded the use of asthma medication at some time in the past, and 73 (10.4%) of the athletes were currently taking medication. One hundred and seventeen (16.7%) reported the use of asthma medication, a diagnosis of asthma, or both (Weiler, Layton and Hunt 1998). These studies show both prevalence and medication usage amongst elite athletes is high. Critically these two studies are solely questionnaire based, and no attempts were made to verify or assess EIB by any other forms other than symptoms alone. A diagnosis of EIB through symptoms alone has been shown to be unreliable (Rundell, et al. 2001a, Holzer, Anderson and Douglass 2002) and likely result in both false-positive and false-negative results. Many elite athletes have high thresholds for pain, and often view pain and discomfort as a normal part of training.

Therefore many may neither consider EIB as abnormal nor realise it can be detrimental to performance (Rundell, Wilber and Lemanske Jr 2002). This suggests that elite athletes should be routinely screened for EIB using a suitable bronchial provocation challenge (See section 2.4).

Using the bronchial provocation challenge of methacholine, 49% of 100 competitive athletes exhibited airway hyper-responsiveness compared to 28% of sedentary subjects (Langdeau, et al. 2000), highlighting the greater risk of EIB within an athletic population. The bronchial provocation challenge of eucapnic voluntary hyperventilation (EVH) has been shown to diagnose previously undiagnosed athletes (Dickinson, McConnell and Whyte 2011). With high prevalence rates amongst elite athletes this has led to a concomitant rise in the use of β_2-agonist medication. Of all the athletes competing in the 2002 winter Olympic Games, 5.2% used inhaled β_2-agonists and 4.2% in the 2004 Olympic Games (Anderson, et al. 2006, Anderson, et al. 2003, Fitch 2006). See section 2.5 for more details on pharmacological therapy.

High prevalence's of EIB have been reported in the Great Britain Olympic Teams at the 2000 and 2004 Olympic Games. Prior to the IOC requirement for bronchial provocation testing as evidence for asthma and EIB medical forms of the 2000 Team GB squad (152 Male, 120 Female) assessed prevalence. 21.2% of the squad reported as suffering from asthma (Dickinson, et al. 2005). Prior to the 2004 Olympic Games, British athletes selected to compete in Team GB (165 men, and 106 women) were recruited for bronchial provocation testing. Athletes were only tested for asthma if they had a previous diagnosis of EIB, reported symptoms of EIB or were referred for testing by a team medical officer.

Diagnosis was made in accordance with the International Olympic Committee – Medical Commission requirements of a positive bronchodilator (increase in FEV_1 of $\geq$ 15% following 200 µg of short-acting β_2-agonist) or bronchoprovocation test (decrease in FEV_1 $\geq$ 10% from the pre challenge value). Fifty six of the 271 (20.7%) athlete's tested from the 2004 Team GB received a positive diagnosis. Sixty two athletes had previously been diagnosed with asthma and were prescribed asthma medication; 13 of the 62 (21%) then failed to produce a positive test for asthma. The results show prevalence in elite-athletes is likely to be sport and environment dependent (Table 2. 1) with the highest rates reported amongst swimmers and cyclists, these studies highlight the need for continued monitoring and accurate diagnosis so correct support and medication can be offered to our elite athletes.

Table 2. 1 Prevalence of asthma in the British squads at the 2000 and 2004 Olympic Games. Reproduced with permission from Dickinson, et al. (2005).

	2000		2004	
	N	No (%) as asthmatic	N	No (%) as asthmatic
Athletics	28	7 (25)	58	9 (16)
Badminton	13	2 (15)	11	1 (9)
Canoe/Kayak	12	1 (8)	9	1 (11)
Cycling	27	12 (44)	23	9 (39)
Diving	7	3 (43)	7	1 (14)
Gymnastics	14	0	9	0
Hockey	31	3 (10)	16	5 (31)
Judo	10	2 (20)	8	1 (13)
Rowing	41	8 (20)	36	7 (19)
Sailing	17	0	18	0
Shooting	6	0	6	1 (17)
Swimming	41	17 (41)	36	16 (44)
Triathlon	8	0	6	0
Other	19	3 (16)	28	5 (18)
Overall				
Men	152	29 (19.1)	165	34 (20.6)
Women	122	29 (23.8)	106	22 (20.8)
Total	274	58 (21.2)	271	56 (20.7)

Some of the highest rates of EIB are found among winter sports athletes who may be chronically exposed to dry, cold air. Following assessment of medical history data and a methacholine challenge, plus two symptoms identified via medical history, asthma was found to be prevalent in 33 of 47 (70%) Swedish cross country skiers, compared to just 1 of 29 non-skiing controls (Larsson, et al. 1993). Sue-Chu et al. (1996) provided further evidence of the high prevalence of EIB in cross country skiers. Self-reported symptoms of asthma were prevalent in 46% of 118 Norwegian skiers, and 51% of 53 Swedish skiers. Following methacholine testing, 14% of the Norwegian skiers, and 43% of Swedish skiers suffered with airway hyperresponsiveness (Sue-Chu, Larsson and Bjermer 1996). The differences in bronchial hyperresponsiveness between the Norwegian and Swedish skiers

were attributed to the differing geographical locations and subsequently greater exposure to cold air in the Swedish skiers. It has been suggested that the combination of demanding training at low temperatures and repeated inhalation of cold air may be a pathogenic factor in asthma in this population of athletes (Larsson, et al. 1993); it has been termed "ski asthma" and could be a normal physiological response to extreme environmental stimuli (Bjermer and Larsson 1996, Sue-Chu, et al. 1999). It has been suggested that cross country skiers are an extreme subtype of exercise induced asthmatics, and chronic exposure to cold dry air at high ventilation rates can lead to significant thickening of the bronchial sub-epithelial basement membrane, which is similar to that seen in chronic asthmatics (Karjalainen, et al. 2000)

Indoor winter sports competitors also experience a high prevalence of EIB and asthma like symptoms, and have been shown to have a great degree of small airway dysfunction (Rundell, et al. 2001a, Rundell, et al. 2001b). This high prevalence has been attributed to the inhalation of cold dry air and the high-emissions of pollutants from the ice resurfacing machines.

Studies of endurance based summer sports also show a high prevalence of EIB. A prevalence of 15-23% has been reported in endurance and distance runners and has been associated with atopy, allergy, and asthma (Tikkanen and Helenius 1994, Helenius, et al. 1998). Elite male and female distance runners who were initially classified as atopic (n=39) or non-atopic (n=19) based on skin prick tests of 10 airborne allergens were assessed for EIB. Lung function was assessed following an outdoor 2000 m run at 85% of their individual maximum heart rate during the winter season (mean temperature −6.6°C) and

during the summer pollen season (Helenius, Tikkanen and Haahtela 1998). FEV_1 was measured immediately post, and at 4, 10, and 20 minutes post exercise. EIB (defined as a post exercise drop in FEV_1 of 10%) was observed in 9% of the runners in either summer or winter. When the group's mean change in FEV_1 minus 2 standard deviations was taken as lower limit (a reduction of 6.5% or more in FEV_1) 26% of runners had probable EIB in either the winter or pollen season. A high proportion of long distance runners have been shown to suffer from asthma; 17% of long distance runners reported physician diagnosed asthma compared to 8% of speed and power athletes, and 3% of controls (Helenius, Tikkanen and Haahtela 1997). For athletes competing and training in indoor swimming pools, the prevalence of EIB is also high, attributed to the chlorine compounds in swimming pools (Helenius and Haahtela 2000). In a sample of 738 swimmers, overall prevalence of EIB was 13.4%, in 165 competing at international level, 21% had EIB. The prevalence was less amongst the 537 lower level swimmers at 11.2% (Potts 1996), and therefore could be dependent upon training and competition intensity.

It is clear that exercise induced asthma is highly prevalent amongst asthmatics and elite athletes. The increase in asthma prevalence over the last 30 years has been linked to environmental changes and improved hygiene with fewer children experiencing childhood infections (Umetsu, et al. 2002). However the specific early life infections that limit T-helper type 2 (Th-2)-biased inflammation and asthma are not fully understood. Asthma can result from aeroallergen induced inflammation driven by Th-2 response and mediated by the cytokines IL-4, IL-5, and IL-13, as opposed to T-helper 1 cells which secrete IL-2 and

interferon-γ (Woodruff, et al. 2009, Barnes 2001). The airway cells and their mediators are discussed in section 2.2.2.

Advances in optimal diagnosis and novel treatment methods aimed at athletic populations may help to reduce the burden of the condition and improve the management for these individuals.

2.1.2. Theories for the pathogenesis of EIB

There is still active debate on the acute mechanisms and pathogenesis of EIB. EIB has been described as an exaggerated airway response to airway dehydration in the presence of inflammatory cells and mediators. However, since the late 1970's there has been considerable debate about the mechanisms involved and how exercise provokes airway narrowing in both asthmatic and non-atopic individuals. Two main hypotheses have been proposed; the '*thermal hypothesis*' and the '*osmotic hypothesis*'.

2.1.2.1. The Thermal Hypothesis as a mechanism for EIB

The *Thermal Hypothesis* was a development of the earlier A*irway Cooling Theory* (Deal Jr, et al. 1979). The authors of the *Airway Cooling Theory* concluded that the magnitude of EIB was directly proportional to the thermal load placed on the airways, and it was the level of heat loss that determined the severity of bronchoconstriction. *The Airway Cooling Theory* was revised to the *Thermal Hypothesis* that developed over two main studies (McFadden Jr 1990, Gilbert and McFadden Jr 1992). The revision of the cooling

theory to thermal hypothesis considers that airway cooling alone is insufficient to produce bronchial narrowing and that rapid rewarming is essential for obstruction to develop. It suggests that a thermal gradient must be present at the end of exercise. According to the thermal hypothesis, EIB is a vascular event involving vasoconstriction resulting from airway cooling during exercise, followed by a reactive hyperaemia when the airways re-warm on the cessation of exercise resulting in airway wall oedema (McFadden Jr 1990, McFadden Jr, Lenner and Strohl 1986). The subsequent narrowing of the airways is a direct response to these vascular events. The evidence came from a study that showed by preventing the airways from re-warming following exercise (breathing in cold air) the reduction in FEV_1 was reduced from 25% to less than 10% in asthmatic participants (McFadden Jr, Lenner and Strohl 1986). McFadden and colleagues looked to strengthen the thermal theory in 1999 by comparing FEV_1 before and after hyperventilation with either a warm or cold dry air to determine whether mucosal dehydration causes thermally induced asthma. Eight atopic-asthmatics performed isocapnic hyperventilation while breathing either dry cold (12.5 ± 2.7°C) or dry warm air (24.3 ± 0.7°C). FEV_1 was measured pre and post each challenge, expired temperatures were continuously measured and water loss from the intrathoracic airways was calculated (Equation 1).

$$W_L = ([Wci - Wce] \cdot V_E \cdot time) \cdot 0.5$$

Equation 1. Water loss from the intrathoracic airways calculation Where W_L is the volume of water lost to the environment in $mg \cdot L^{-1}$ air; Wci is the inspired water content in $mg \cdot L^{-1}$ air; Wce is the expired water content in $mg \cdot L^{-1}$; V_E is minute ventilation in $L \cdot min^{-1}$; Time is the duration of the hyperventilation in minutes; and 0.5 is the percentage of water lost from the intrathoracic airways.

The researchers reported that the decrement in FEV_1 was significantly greater when breathing cold air compared to warm air (%Δ FEV_1 cold at 8 min post = 30.0 ± 4.7%, warm = 16.0 ± 4.7%; p = 0.01). Despite greater reductions in FEV_1 during the cold condition, water loss was significantly less when compared to the warm condition (W_L cold at 8 min = 4.8 ± 0.4 g, warm at 8 min = 7.1 ± g; p = 0.001), suggesting the decrement in FEV_1 was temperature dependent. The authors stated that during the cold condition FEV_1 decreased as water losses rose. However, the largest intrathoracic losses were associated with the smallest obstructive response (%Δ FEV_1 cold at 8 min post = 30%, water loss = 4.7 mg; %Δ FEV_1 warm at 8 min post = 16%, water loss = 7.1 mg; p = 0.002). The authors concluded that the water loss associated with hyperpnoea may promote EIB through an effect on the cooling-rewarming gradient rather than through airway dehydration. These conclusions supported previous work into the thermal hypothesis (McFadden Jr, Lenner and Strohl 1986, Gilbert, Fouke and McFadden Jr 1987). Early work by Gilbert and colleagues in 1987, found that after participants had cycled for 4 minutes breathing cold air (−16 ± 2°C), on cessation the airstream temperature increased rapidly, rising twice as fast in asthmatics when compared to non-asthmatic controls. They concluded that a reaction sequence consisting of vasoconstriction and airway cooling during exercise followed by a rapid re-supply of heat when exercise ceases was occurring, suggesting that a rebound hyperaemia may lead to airway oedema and EIB.

The thermal hypothesis assumes that an increase in the intra-airway temperature reflects an increase in the blood flow of the airways and that this provides a significant source of heat to the airways in rewarming them on cessation of exercise (Gilbert, Fouke

and McFadden Jr 1987). This theory has been challenged, with reports that changes in airway temperature are the result of a prolonged warming of the air in the alveoli as a consequence of flow limitations in asthmatics (Anderson and Daviskas 2000). Further, bronchial circulation is a very limited source of heat replenishment for the airways (Solway 1990), and represents only 1% of total cardiac output. Fundamental to the thermal hypothesis is the concept of vasoconstriction causing a reduction in bronchial blood flow. Vasoconstriction is an unlikely response to inhaling hot dry air, and data from animal studies (Baile, et al. 1987) and humans (Agostoni, et al. 1990) demonstrate that there is an increase in blood flow, rather than a reduction when dry air is inspired. Those that developed the initial cooling and thermal theories (McFadden Jr, Lenner and Strohl 1986, McFadden Jr, et al. 1999, McFadden 1983) have acknowledged that bronchial blood flow increases with dry air hyperpnoea and have formally revised the thermal hypothesis. The theory now recognises that airway cooling associated with hyperpnoea will provoke an increase in bronchial blood flow in humans and is thought to regulate thermal losses and prevent tissue damage (McFadden Jr, et al. 1999, Kim, et al. 1996).

Bronchial circulation cannot be ruled out, and it has the potential to contribute to the pathogenesis of EIB. Bronchial circulation is an important source of water for the airways, and increases in osmolality of the airways causes an increase in circulation (Zimmerman and Pisarri 2000). The increase in bronchial circulation would not just increase the delivery of water to dehydrated airways in response to an osmotic stimulus, but would also aid in the clearance of bronchoconstrictive mediators (See section 2.2.1).

Previous research has produced findings that may not be explained by the *Thermal Hypothesis*. Severe EIB has still occurred whilst individuals have been inspiring hot dry air (Aitken and Marini 1985, Anderson, et al. 1985) for example. Twenty out of twenty two asthmatic children recorded EIB after cycling for 8 minutes whilst inspiring hot (32-40°C) and dry (3-10 mg $H_20 \cdot L\text{-}^{1}$) air (Anderson, et al. 1985). Ten asthmatic adults exercised and recovered whilst breathing air at 36°C in an environmental chamber, yet they still had a mean fall in peak expiratory flow of 43% ± 18% (Hahn, et al. 1984). In another study, ten asthmatics and 10 control participants were exposed to 7 air conditions varying in temperature (−2 to 49°C) and relative humidity (10 to 100%). The authors concluded that dehydration and changing osmolality of the airways determined the level of bronchoconstriction as opposed to the thermal exchange (Aitken and Marini 1985).

Inhalation of hot dry gases has been shown to facilitate airway cooling too. It is thought that heat loss of the airways will always occur as a normal part of respiration and develops whenever the inhaled air requires transfer of heat and moisture to condition it (Zawadski, Lenner and McFadden Jr 1988). The *Thermal Hypothesis* cannot explain early findings that EIB severity was greatest when cold air (-10°C) was inspired both during and importantly for 4 minutes after exercise (Deal Jr, et al. 1979). In addition the *Thermal Hypothesis* does not implicate any involvement of the release of pro-inflammatory mediators in EIB. If pro-inflammatory mediators were not involved then pharmacological treatments (see section 2.5) that reduce inflammation would be of no benefit. These findings are not compatible with the concept that the development of obstruction occurs due to cooling and rapid rewarming as proposed by the *Thermal Hypothesis*. In summary,

the weaknesses in the *Thermal Hypothesis* are that it does not include a role for bronchial smooth muscle, or inflammatory mediators in the mechanism of EIB.

In reviewing the *Thermal Hypothesis* literature there may be a number of errors in the research. The McFadden studies may have underestimated the level of water loss occurring within the airway so misjudged the importance of airway dehydration in EIB. Moreover, the authors assumed that the expired air was fully saturated at its exiting temperature. Such assumptions have been questioned. Eschenbacher and Sheppard (1985) undertook studies of asthmatic subjects performing hyperpnoea of dry air at -20°C and +39°C. They found difficulties when measuring the expired air temperature when it was inspired at -20°C, as at -20°C, expirate would freeze and then condense on the thermocouple dampening the response time of the thermocouple. To overcome this, the authors completely separated the inspired and expired airstreams and measured both water and temperature during cold air hyperpnoea. The technical difficulties associated with measuring expired air temperature and water loss are likely to result in the very low expired air temperatures reported in the McFadden et al (1999) paper (Freed, et al. 2000). The alternative hypothesis is the *Osmotic Hypothesis*. This was proposed as a result of the failures of the thermal hypothesis to fully elucidate the mechanisms behind EIB.

2.1.2.2. The Osmotic Hypothesis as a mechanism for EIB

The *Osmotic Hypothesis* was developed after the finding that subjects with EIB were sensitive to inhalation of hypertonic saline solutions (Schoeffel, Anderson and Altounyan 1981). A fall in FEV_1 of 20% or more in response to inhalation of 3.6% saline

and distilled water was observed in patients with asthma, but no response observed in non-asthmatics (Schoeffel, Anderson and Altounyan 1981). Importantly it was found that varying the temperature of the inhaled solution from 22°C to 30°C did not change the response (Schoeffel, Anderson and Altounyan 1981). More recently, it has been supported by the findings that cold air inhalation does not influence the severity of EIB after exercise or eucapnic voluntary hyperpnoea (Evans, et al. 2005). The authors suggested that cold air inhalation or rapid rewarming was not necessary to elicit bronchoconstriction. The similarities in the response to these challenges are consistent with the hypothesis that hyperventilating dry isocapnic/eucapnic air induces asthma via alterations in the osmolality of the airway surface liquid. Further development arose after marked similarities were reported in the airway response to both hyperpnoea of dry air and hypertonic aerosols (Smith and Anderson 1986, Smith and Anderson 1989). The lowest FEV_1 as a percentage of predicted after isocapnic hyperventilation was 45% ± 16% and after inhalation of hypertonic saline was 51% ± 18% ($r = 0.93$) (Smith and Anderson 1989). More recently, hyperosmolarity has been shown to release inflammatory mediators from eosinophils and mast cells contributing to the development of EIB (Broide, et al. 1992, O'Sullivan, et al. 1998, Moloney, et al. 2003).

The osmotic hypothesis first proposed in 1984 (Anderson 1984) has changed little since, with the exception of the addition of the involvement of the epithelial cells in 1989 (Anderson, Daviskas and Smith 1989), and the inclusion of the submucosa in signalling bronchial blood flow (Anderson and Daviskas 1992). Initial evidence showed that the severity of EIB was directly proportional to the water content of the inspired air (Strauss,

et al. 1978) and that when water loss is prevented by inhalation of humid air at body conditions, even severe EIB could be prevented (Strauss, et al. 1978, Anderson, et al. 1982). Anderson et al (1982) suggested that bronchoconstriction induced by water loss from the airways during exercise may be due to changes in the osmolality of the surface liquid in the respiratory tract. With the calculation of the airway surface liquid (ASL) volume to be less than 1 mL in the first 10 generations of airways, it gave further support and focus for the *Osmotic Hypothesis* (Anderson 1984) (Figure 2. 1). This very low volume causes the osmolality of the ASL to change rapidly in response to water loss and dehydration through exercise hyperpnoea and evaporation or deposition of hypertonic aerosol particles.

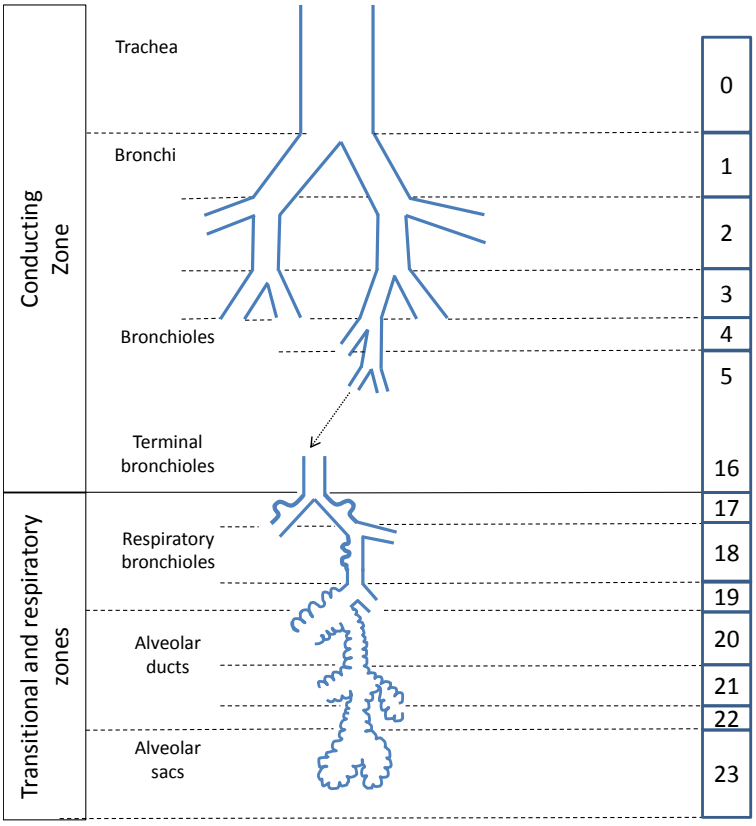

Figure 2. 1 Generation of airways adapted from (Anderson 1984)

Following this research, additional evidence from canine models showed that vasodilation of the tracheal circulation occurred as a result of hypertonic solutions perfusing into the trachea (Deffebach, et al. 1989). This indicates that vasodilation and an increase in blood flow can occur independently of thermal events, but as a direct response to increased osmolality of the ASL. Data from human studies highlight the inability of the human airways to maintain a fluid balance during the breathing of warm air (Tabka, et al. 1988). Tabka et al (1988) showed that whilst exercising and breathing warm dry air, the expired air de-saturates to 80% and the water content falls to 29 $mg \cdot L^{-1}$ when measured by mass spectrometry. They suggested that this shows both dehydration of the airway mucosa and importantly an increase in osmolality of the ASL. Accurate collection and measurement of the ASL in terms of both volume and osmolality is difficult and has not been achieved. The development of the hypothesis would serve to explain several observations about EIB, namely why both intensity and duration of hyperpnoea are important determinants of the response.

Measurements of the intra-airway temperature have been reported (Gilbert, Fouke and McFadden Jr 1987) and were then subsequently used to analyse the magnitude of water lost from the trachea down to the fifth generation of airways (Anderson, Daviskas and Smith 1989). This analysis showed that the airway surface could become dehydrated within a few minutes even if the water loss is low (1 $mg \cdot L^{-1}$). Dehydration of the trachea could occur very rapidly as it has been shown that there is less than 68 mg of ASL to cover it, causing the epithelial cells to become dehydrated within five minutes (Anderson and

Daviskas 1992). Mathematical models highlight the possibility there may be significant dehydration of the airway epithelial cells due to water moving from them to restore the ASL in response to changes in the osmotic gradient (Daviskas, Gonda and Anderson 1990, Daviskas, Gonda and Anderson 1991). This has been supported in human epithelial cells *in vitro* in response to hyperosmotic stimulus (Matsui, et al. 2000). The authors state that normal epithelial cells have relatively high water permeability and thus have a capacity to restore water on the airway surface that may be lost from dehydration.

The osmotic hypothesis is often favoured because it enables an explanation for many of the established facts about EIB, including contributions from inflammatory mediators and airway smooth muscle contraction. Data has accumulated in support of the osmotic hypothesis and it is of interest to note that only one study to date has refuted the osmotic hypothesis (McFadden Jr, et al. 1999) (See section 2.1.2.1).

2.1.2.3. Reconciling the hypotheses for mechanism for EIB

An attempt was made to reconcile these two theories in 2000 by Anderson and Daviskas. They concluded that inspiration of cold and dry air during intense exercise causes not only cooling of the airway, but also increases the hyperosmolar area and dehydrates the airway surface (Anderson and Daviskas 2000). It is clear from the above research that there is a high prevalence of EIB respiratory symptoms in elite athletes that can be explained by the dehydrating effects of intense exercise on the small airways. During moderate exercise only 10 to 12 generations of airways are needed to condition the inspired air. However, during intense exercise or when breathing cold air, the smaller

airways (< 1.0 mm in diameter) are recruited in an attempt to condition the air (Anderson and Daviskas 2000). The recruitment of these small airways may be the critical determinant for the vasculature to contribute to airway narrowing. The involvement of the small airways may be necessary for some subjects to achieve a clinically positive airway response to exercise (>10% fall in FEV_1) (Anderson and Kippelen 2005a); for others it will just enhance their response.

These small airways may actually undergo injury due to the significant dehydration and increases in osmolality that can occur in subjects who exercise intensely for prolonged periods breathing dry and/or cold air. In addition, airway injury may also result from repeated and prolonged exposure to pharmacologic and physical agents and irritants (Anderson and Holzer 2000). As a result of the airways being exposed to these conditions, they may become inflamed, and airway smooth muscle can become more sensitive, also leading to airway injury and remodelling; these events are likely to lead to an exaggerated response to dehydration in asthmatic athletes. There is a wealth of research to suggest that a cooling of the airways followed by a rapid re-warming may not be essential for the development of EIB (Deal Jr, et al. 1979, Aitken and Marini 1985, Anderson, et al. 1985, Hahn, et al. 1984, Evans, et al. 2005, Sheppard and Eschenbacher 1984). It seems essential that sufficient water loss must occur to cause the airway surface to become dehydrated. The thermal theory and osmolality theory merge with the proposal that cold air breathing may enhance the airway response to exercise because the surface area involved in the air conditioning process increases and as a results, very small airways could be subjected to injury from dehydration (Anderson and Kippelen 2008).

In the past 10 years, the focus of the literature has moved away from understanding the acute effects of exercise on the mechanisms of EIB and shifted to gaining a greater understanding of the overall pathogenesis of EIB and airway injury. The shift in focus is concurrent with an increase in the number of individuals reporting EIB, who are otherwise healthy, and exercise regularly both competitively and non-competitively (Anderson and Kippelen 2005a). This greater frequency is even more evident in athletes performing endurance sports especially in challenging environmental conditions (Rundell, et al. 2001a, Langdeau and Boulet 2001, Fitch, et al. 2008). This increased prevalence concurs with a notable rise (320%) in the notification of use of inhaled β2-adrenoceptor agonists before exercise in athletes between the 1984 and 2000 summer Olympic Games (Anderson, et al. 2003).

The acute events that lead to EIB, in both classic asthma and the athlete are depicted below (Figure 2. 2). EIB is most commonly reported in people with clinically recognized asthma shown on the left in yellow. In clinically diagnosed asthmatics EIB stems predominantly from airway dehydration and increases in osmolality associated with the *Osmotic Hypothesis*. The right of the diagram (green area) highlights the multifactorial events that can lead to EIB in athletes, including non-atopic athletes. The severity of EIB in the non-atopic athletes on the right is determined by the ventilation reached and sustained during exercise, and the water content of the air inspired during exercise. The diagram reconciles the *Osmotic and Thermal Hypotheses* with the presence of the airway dehydration (red), and cooling (blue), with the addition of airway injury in the athlete (orange).

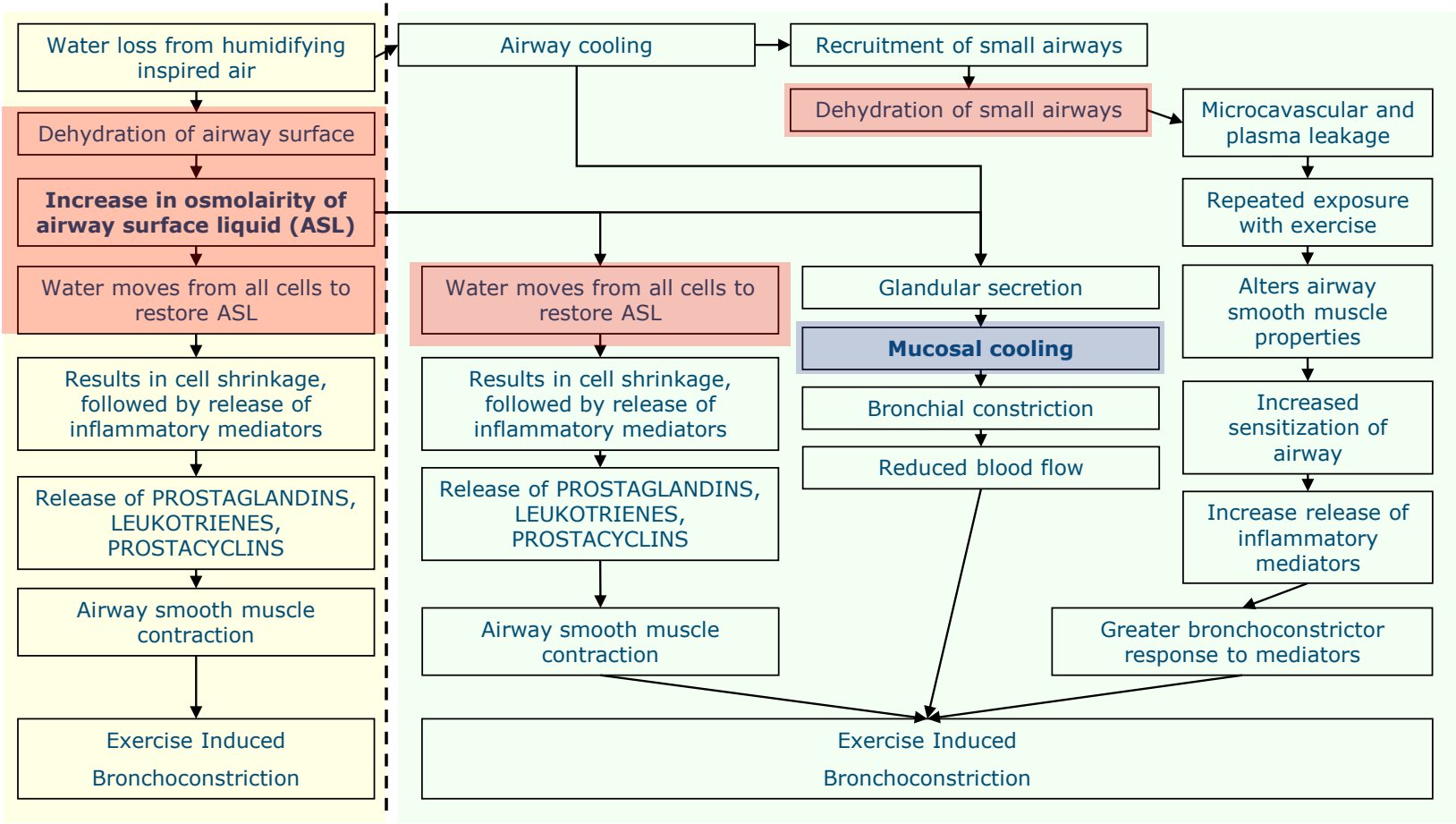

Figure 2. 2 Flow chart depicting acute events leading to EIB, in classic asthmatic (yellow) (left) and progression in the athlete (green) (right). Orange shows the role of the osmotic hypothesis, and blue the role of the thermal hypothesis. Adapted from (Anderson and Kippelen 2005a).

2.2. Pathophysiology of Asthma and EIB

Understanding of the pathogenesis of asthma has changed dramatically with time. Records dating back to 1500BC, indicate that asthma was considered to be a disease caused by "spirits" (Salvi, Suresh Babu and Holgate 2001). It wasn't until 1678 that asthma was first described as a disease rather than purely a symptom, when Thomas Willis (17th century English Physician) described asthma to be due to "obstruction of bronchi by thick humors, swelling of their walls and obstruction." The role of contraction and spasm of the bronchial smooth muscle was first suggested by Sir John Floyer in 1698 (Sakula 1984). With the advent of fiberoptic bronchoscopy in the 1970s and the use of modern molecular biology, it became clear that asthma was a chronic inflammatory disorder mediated by a multitude of cell types and inflammatory mediators (Salvi, Suresh Babu and Holgate 2001). Figure 2. 3 depict the structural changes occurring in the airways during an asthma exacerbation. It is characterised by airway narrowing with constriction of the bronchial smooth muscle, increased mucus production, and inflammation resulting in decreased lumen diameter.

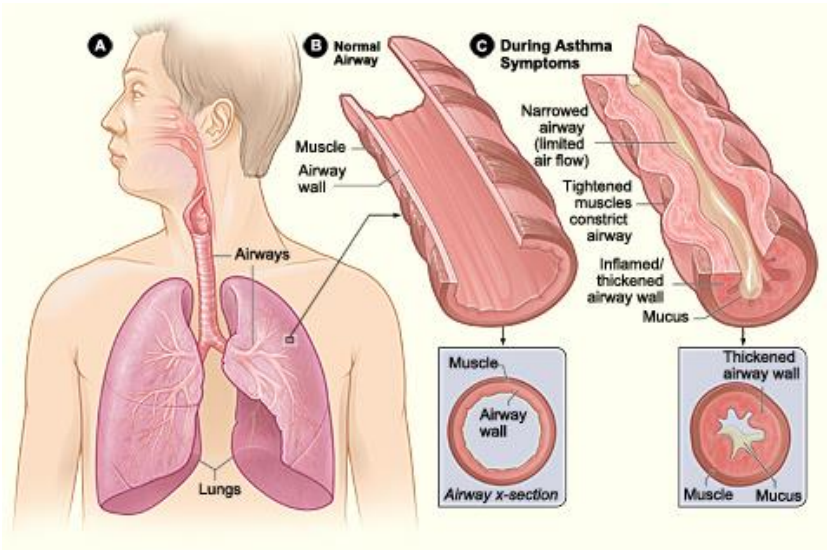

Figure 2. 3 Structure and physiological changes characteristic of an asthma exacerbation (NHLBI)

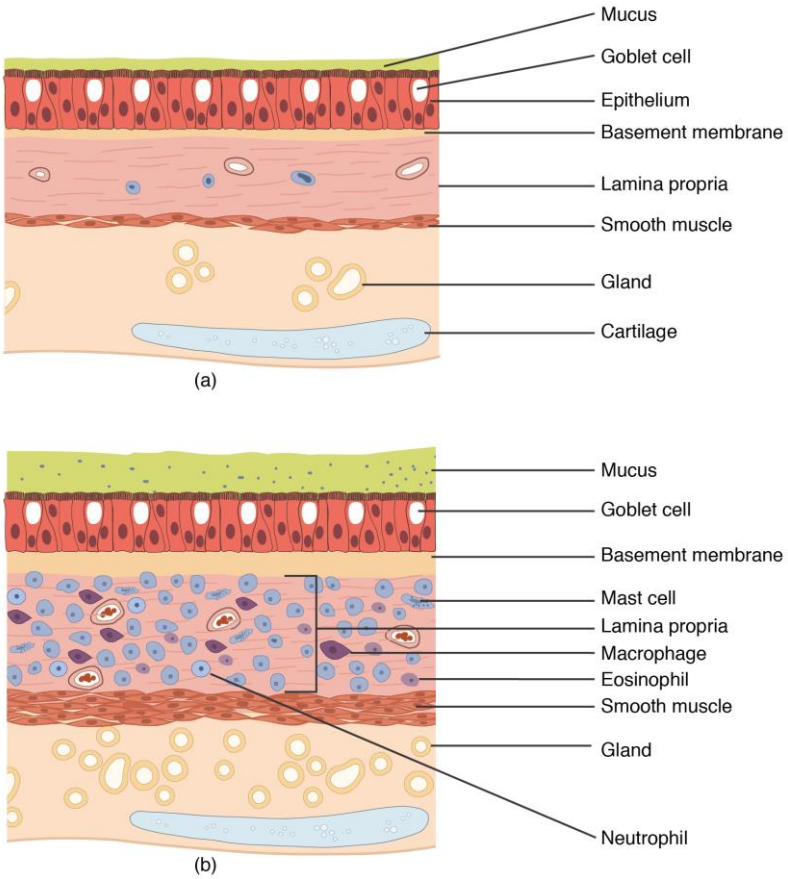

Figure 2. 4 a) normal lung tissue; b) lung tissue with characteristics during an asthma exacerbation, showing increased mucus production from goblet cells, infiltration of mast cells, macrophages, eosinophils, and neutrophils into the lamina propria, and increased thickness of the basement membrane and smooth muscle. Adapted from OpenStax College (07/08/2013).

Asthma and EIB are now well recognised as inflammatory borne conditions, with significant contributions from inflammatory cells of the immune system that infiltrate the walls of the bronchi and bronchioles during an asthma exacerbation (Figure 2. 4) these include eosinophils, mast cells, and macrophages. As described by Rundell and Jenkinson (2002) the symptoms of EIB are merely the 'tip of the iceberg' and reflect remodelling and inflammation of the airways (Figure 2. 5).

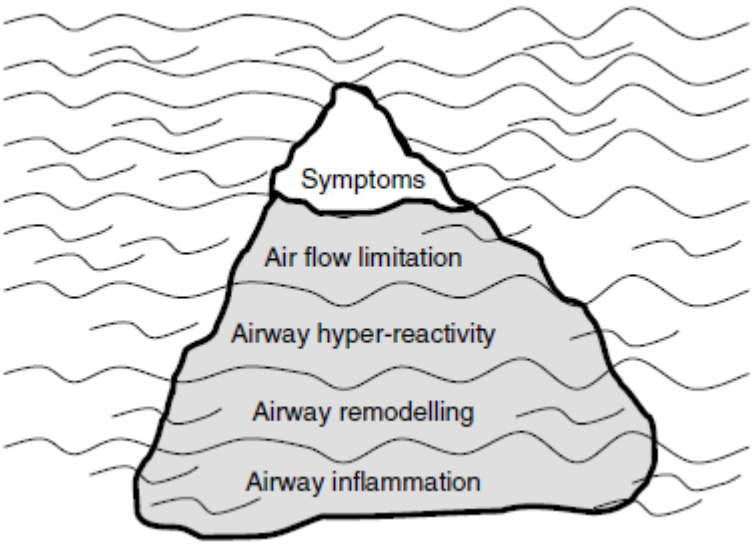

Figure 2. 5 The 'asthma iceberg' schematically illustrates airway inflammation and remodelling as underlying cause of AHR, airflow limitation and symptoms presented by EIB (Rundell and Jenkinson 2002).

Exercising individuals have a need to humidify and warm a high volume of inspired air. The dehydrating and osmotic effects of this respiratory water loss are thought to stimulate the release of a number of pro-inflammatory mediators. It has been proposed that it is the regulatory volume increase, after cell shrinkage that is the key event leading to the release of pro-inflammatory mediators (Anderson and Daviskas 2000). There is potential that all cells could be subjected to cell volume loss including the epithelial cells, mast cells, eosinophils, macrophages, and sensory nerve cells. The airways are narrowed by contraction of bronchial smooth muscle in response to the mediators; the effect will be amplified if airway oedema is present (Anderson and Daviskas 2000). The influx of mediators leads to airway inflammation, with EIB regarded as an inappropriate inflammatory response.

2.2.1. The immune system and inflammation

The immune system is comprised of a network of cells, proteins, tissues, and organs that coordinate to defend the body against attacks from foreign elements. The immune system can be viewed as a series of organs that are distributed throughout the body to provide the human with defence against pathogens wherever these may enter or spread (Janeway, et al. 2001). An immune response is generally divided into innate and adaptive immunity. Innate immunity occurs immediately, when circulating innate cells recognize a problem. Adaptive immunity occurs later, as it relies on the coordination and expansion of specific adaptive immune cells. Immune memory follows the adaptive response, when mature adaptive cells, highly specific to the original pathogen, are retained for later use. An important part of the adaptive immune system aside from the lymphatic system and spleen is the mucosal immune system, termed the mucosal associated lymphoid tissue (MALT) (Janeway, et al. 2001). The mucosal surfaces of the body are located at sites that make them particularly vulnerable to infection. They are thin permeable barriers between the exterior and interior of the body, and include the lungs, the gut, eyes, nose, mouth and throat, and uterus and vagina. The primary function of the immune system is to maintain homeostasis (see section 2.3 for specific details on the intestinal immune system).

Homeostasis is achieved through the recognition and then elimination of harmful stimuli such as pathogens followed by the repair and removal of damaged or abnormal tissue. When the cellular elements of the immune system are challenged by harmful stimuli there is the local response of inflammation.

Inflammation is generally manifested by the accumulation of fluids and blood cells at the point of tissue damage, and is largely a reaction of blood vessels (Horwitz

and Busse 1995). Acute inflammation is a short-term response that usually results in the infiltration of leukocytes to the damaged region, removal of the stimulus, and repairing of the damaged tissue. In contrast, chronic inflammation is a prolonged, deregulated and defective response that involves active inflammation, tissue destruction and attempts at tissue repair (Weiss 2008). Persistent inflammation is associated with chronic conditions and diseases such as allergy and asthma.

While a controlled inflammatory response is thought to be beneficial in providing protection against infection for example, it can become detrimental if dysregulated (e.g. septic shock). At a basic level, the acute inflammatory response is coordinated by the delivery of blood components (plasma and leukocytes) to the site of injury and inflammation (Robbins, Kumar and Cotran 2003). This response is triggered by receptors of the innate immune system, such as Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) (Barton 2008). This initial recognition is mediated by tissue-resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators (Medzhitov 2008), including cytokines (see section 2.2.3), eicosanoids (see section 2.2.5) and chemokines (see section 2.2.4), all of which have been implicated in asthma and EIB. The main and most immediate effect of these mediators is to elicit a local inflammatory exudate of plasma proteins and leukocytes (primarily neutrophils). These are normally restricted to the blood vessels but the exudates components now gain access to the extravascular tissues at the site of infection or injury through the post-capillary venules. The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes (Medzhitov 2008). This selectivity is afforded in part by chemokine receptors on leukocytes, which occurs at the

endothelial surface, as well as in the extravascular spaces (Pober and Sessa 2007). When the leukocytes reach the afflicted site, the neutrophils become activated either by direct contact with pathogens, or through the actions of cytokines secreted by tissue-resident cells.

A successful acute inflammatory response results in the elimination of the infectious agent, or successful repair of the physical damage. The switch in lipid mediators from pro-inflammatory prostaglandins (section 2.2.5.2) to lipoxins which are anti-inflammatory is crucial for the transition from inflammation to resolution (Medzhitov 2008). Lipoxins inhibit the recruitment of neutrophils, and instead, promote the recruitment of monocytes which remove dead cells and initiate tissue remodelling (Serhan and Savill 2005). Resolvins and protectins (see section 2.2.5.3), which constitute another class of lipid mediator, transform growth factors produced by macrophages, and play a crucial role in the resolution of inflammation, including the initiation of tissue repair (Serhan and Savill 2005, Serhan, Yacoubian and Yang 2008).

If the acute inflammatory response fails to eliminate the pathogen or repair the damaged tissue, the inflammatory process persists. Neutrophil infiltration is replaced with macrophages, and in some instances T cells. If the combined effect of these cells is still insufficient, a chronic inflammatory state can occur (Medzhitov 2008). Persistent asthma is characterised by chronic inflammation of the bronchial mucosa, with high levels of circulating T cells and eosinophils (Corrigan and Kay 1990).

The view of asthma has changed from one primarily involving smooth muscle contraction to one of complex interactions between inflammatory mediators and effector cells (Horwitz and Busse 1995). Pathologic evidence for inflammation in

asthma was observed in early autopsy specimens of patients who died from ‘status astmaticus’ (a prolonged and severe asthma attack that does not respond to treatment) (Ellis 1908, Huber and Koessler 1922, Messer, Peters and Bennett 1960). These included hypertrophy of the airway smooth muscle, thickening of basement membrane, mucous deposition in the terminal bronchioles with glandular hyperplasia, and the presence of inflammatory cells. The inflammatory infiltrate was shown to compose of mainly eosinophils as well as neutrophils, lymphocytes, and mononuclear cells (Messer, Peters and Bennett 1960). These early autopsy studies led to the belief that inflammation represented a terminal event in severe asthma, however more recent understanding is that inflammation is not restricted to severe asthma, but rather is a general characteristic of asthma (Horwitz and Busse 1995). Bronchoalveolar lavage (BAL) collections give further insights into the inflammatory basis of asthma, with increased percentages of eosinophils, mast cells, and epithelial cells in asthmatics compare to non-atopic non-asthmatic controls. In addition increases in activated T-cell populations have been consistently noted in asthma (Salvi, Suresh Babu and Holgate 2001).

T helper cells (Th cells) are critical to coordinating the activity of the immune and inflammatory response; they orchestrate the immune response by controlling T-cell subsets, B-cells and innate immune responses. The T helper cell response is defined by two distinct pathways involving two subtypes of Th cells; Th-1 and Th-2 cells. It is thought that immune regulation involves a homeostasis between Th-1 and Th-2 cells. Th-1 cells drive cellular immunity to fight viruses and other intracellular pathogens, eliminate cancerous cells, and stimulate delayed hypersensitivity skin reactions.

It is known that a deregulated Th-2 response overwhelms the protective mechanisms that normally develop to prevent asthma, with an increased production of pro-inflammatory cytokines (see section 2.2.3). This inappropriate Th-2 response causes pulmonary inflammation, airway eosinophilia, mucus hypersecretion and airway hyperreactivity to a variety of stimuli (including allergens and airway dehydration) (Umetsu, et al. 2002). Cytokines play an integral role in the coordination of the inflammatory processes associated with asthma. Solely Th-2 mediated cytokines is an oversimplification of the pathogenesis of asthma and EIB. There are additional mediators in the form of lipid derived eicosanoids, including prostaglandins (PGs), leukotrienes (LTs), and lipoxins (LXs), which have important roles in the pathophysiology of asthma (Drazen 1998) (see section 2.2.5).

Th-2 cells drive humoral immunity and up-regulate antibody production (Kidd 2003). Over-expression of either can cause disease and either pathway can down-regulate the other. The Th-2 pathway is often thought to underlie allergy, atopy and immunoglobulin-E based diseases (Kidd 2003). However, the specific conditions and changing environments that are responsible for driving the development of Th-2 immune responses are not clear.

One of the main key effector cells in asthma are eosniophils, but their role in EIB remains less clear. Eosinophilic granulocytes are attracted and activated at the site of inflammation by chemokines which are proteins of the cytokine family, and these play another mediating role in asthma (Lukacs, Oliveira and Hogaboam 1999, Lukacs 2001) (see section 2.2.4). Synthesis of leukotrienes occurs in mast cells, eosinophils, basophils, monocytes, macrophages, and other airway cells. There is strong evidence that mast cells release the mediators histamine, cysteinyl leukotrienes and

prostaglandins following exercise and are released into the airways during EIB (Rundell and Jenkinson 2002, Mickleborough, Lindley and Ray 2005, Hallstrand, et al. 2005).

2.2.2. Airway Cells and Inflammation in EIB

Many different inflammatory cells are involved in asthma, although the precise role of each cell type is not certain (Barnes 1996), and a number are associated with EIB. Mast cells are purported to be the most important in regards to mediator release within the airways; in addition, elevated cell counts of T-lymphocytes, macrophages, neutrophils and eosinophils have been made and thus increase eicosanoid and cytokine release into the airways during EIB of asthmatic individuals (Rundell and Jenkinson 2002, Hallstrand, et al. 2005). The issue is whether these cells are merely markers of chronic airway inflammation, or if they have a relationship with the acute response associated with EIB. The significance of mast cells is that they are likely to be important in the pathogenesis of EIB in both asthmatic and non-asthmatic EIB individuals. Mast cell mediator release can occur by immuno-globulin (Ig) E-allergen-induced activation as well as by non-IgE-dependent stimuli such as dry cold air (Togias, et al. 1985, Togias, et al. 1988). This is an important point if mast cells are involved in airway hyperresponsivenes (AHR) in both asthmatics and non-atopic EIB sufferers.

Induced sputum collected from EIB positive asthmatics and EIB negative asthmatics has been compared for its cellular constituents (Hallstrand, et al. 2005). The total volume of the induced sputum was higher for the EIB positive group, compared to the EIB negative group (7.7 vs 3.4 mL, $p = 0.003$). The concentrations of eosinophils, and columnar epithelial cells were found to be greater in the EIB positive group

compared to the EIB negative group (Table 2. 2). Alterations in epithelial permeability may be responsible for the higher volume of induced sputum in positive EIB asthmatics. Disruption of the airway epithelium also increases the transit of granulocytes into the airways (Erjefält, et al. 2004). Significantly higher percentages of eosinophils and higher levels of serum eosinophilic cationic protein have been found in EIB-positive asthmatics than in non-EIB controls (Yoshikawa, et al. 1998).

Table 2. 2 Cellular findings in induced sputum from EIB positive and EIB negative asthmatics data expressed as median (interquartile ranges). ¥ Mann-Whitney *U* Test; ‡Inflammatory cells and columnar epithelial cells expressed as % of nonsquamous epithelial cells; § squamous epithelial cells expressed as percentage of total cells; ¤ unclassified cells Adapted from Hallstrand, et al. (2005)

	Asthma		***P* Value ¥**
	EIB Positive (n=10)	**EIB Negative (n=10)**	
Percentage			
Eosinophils ‡	2.07 (0.58-3.49)	0.44 (0.00-1.49)	0.123
Lymphocytes ‡	1.50 (0.78-4.36)	1.20 (0.88-1.74)	0.353
Macrophages ‡	36.89 (26.02-51.44)	43.80 (16.14-52.22)	1.000
Neutrophils ‡	36.20 (24.40-56.41)	35.29 (28.50-64.86)	0.579
Columnar epithelial cells ‡	7.07(3.65-14.35)	2.43 (1.40-10.16)	0.143
Squamous epithelial cells **§**	18.51 (10.41-25.70)	34.99 (14.46-46.49)	0.19
Other cells ¤	4.77 (3.15-8.42)	7.226 (3.56-10.14)	0.393
Concentration (X 10^4)			
Eosinophils ‡	3.58 (12.06-68.71)	0.49 (0.00-1.10)	0.043
Lymphocytes‡	2.73 (0.90-7.38)	0.99 (0.63-2.27)	0.105
Macrophages ‡	49.63 (23.18-119.01)	22.59 (15.05-65.88)	0.123
Neutrophils ‡	45.33 (23.60-127.58)	44.03 (14.35-69.73)	0.247
Columnar epithelial cells ‡	13.70 (5.91-20.13)	2.91 (1.78-7.65)	0.011
Squamous epithelial cells	32.42 (20.43-62.47)	38.62 (15.15-70.03)	0.912
Other cells ¤	6.68 (3.52-17.62)	5.60 (3.67-10.05)	0.579

The bronchial epithelium is favourably situated where gene-environment and environment-environment interactions can occur. The airway epithelium acts as the barrier between the external environment and the internal body and airways. It is continuously exposed to gases and particulates from the external environment and as

such is involved in many of the reactions that lead to airway inflammation and smooth muscle contraction. When the airways are exposed to infectious agents, inhaled pollutants, and particulates the epithelium acts as the protective barrier. During exercise, in an attempt to equilibrate the inspired air to a humidity and temperature of the lower airways, heat and water are transferred out of the airways. At the airway epithelium there is a transfer of water from osmotically sensitive epithelial cells; these osmotic stimuli are known to directly activate inflammatory mast cells (Gulliksson, et al. 2006). In addition, the stimulus of exercise or hyperpnoea is also sensed by the airway epithelium leading to the activation of inflammatory mediator release from leukocytes in close contact to the epithelium (Hallstrand 2012). Induced sputum suggests that asthmatics who suffer from EIB have a disrupted epithelium with increased shredding of epithelial cells into the airway lumen (Hallstrand, et al. 2005, Hallstrand and Henderson 2009). In addition, the intensity of cellular airway inflammation and the generation of the inflammatory eicosanoids and cytokines have been associated with susceptibility to EIB. Increased urinary eicosanoid and serum cytokine concentrations are associated with a more severe EIB response to an exercise challenge in asthmatics (Mickleborough, et al. 2003, Mickleborough, et al. 2006).

Airway inflammation associated with asthma, and allergic asthma is multifaceted but Th-2 cells are important in driving the processes and are mediated by the inflammatory cytokines IL-4, IL-5, and IL-13. The "Th2 hypothesis for asthma" was first suggested by Mosmann in 1989 (Mosmann and Coffman 1989), who earlier had discovered the presence of two distinct subtypes of helper T cells called Th-1 and Th-2 (Mosmann, et al. 1986). The two subclasses of helper T cells differ in their production of cytokines and are reciprocally inhibitory. Th-2 lymphocytes produce IL-

4, IL-5, IL-9, and IL-13, which are active in allergic airway inflammation whereas Th-1 produce interferon γ (IFN- γ) and IL-2 which activate mechanisms important in defence against viruses and bacteria (Mosmann and Coffman 1989). The Th-2 hypothesis suggests that asthma is caused by a relative increase in Th-2 cellular response in combination with a decrease in Th-1 response. The subsequent alterations in cytokine milieu within the lung with excess Th-2 products in conjunction with decreased Th-1 products are predicted to drive the asthma phenotype (von Hertzen and Haahtela 2000).

Additional cells of both the adaptive and innate immune systems are the dendritic cells (DCs). DCs primarily initiate and maintain adaptive Th-2 cell responses to inhaled allergens in asthma (Lambrecht and Hammad 2009). They are antigen presenting cells as part of the adaptive immune system, and immature dendritic cells, or dendritic cell precursors are key components of the innate immune system. DCs bridge the innate and adaptive immunity (Hammad and Lambrecht 2008) associated with asthma, but physical stimuli (exercise and dry air) can modify and exacerbate the disease. These exacerbating factors interfere with the innate immune system and homeostasis of the lung structural cells. Subsequently it is increasingly appreciated that asthma is more than a disorder of the adaptive immune response, but is influenced by pattern recognition of the innate immune cells such as the bronchial epithelial cells, mast cells, and basophils. The lung possess an elaborate network of DCs found throughout the conducting airways, lung interstitium, vasculature, pleura, and bronchial lymph nodes (GeurtsvanKessel and Lambrecht 2008). DCs perform a unique function in the pulmonary immune response recognizing inhaled antigens through expression of pattern-recognition receptors such as Toll-like receptors and NOD-like receptors. These recognise virtually any inhaled pathogen, allergen, or substance to produce the cascade

of immune response (Barrett, et al. 2009). Additionally lung DCs express numerous receptors for inflammatory mediators that are released upon damage or trauma to the airways to further enhance the immune response. DCs are primarily associated with allergen induced asthma, with little research to suggest a direct link to the onset of EIB following exercise in asthmatics. However, DCs are shown to respond and be recruited into inflamed airways in response to a variety of stimuli (McWilliam, et al. 1996). The drying of the airway surface liquid and changes in osmolality of the airways that is characteristic of EIB (see section 2.1.2.2) results in a cascade of inflammatory mediators that may too be interacting with the DCs.

2.2.3. Cytokines in Asthma and EIB

The word 'cytokine' originates from the Greek word 'cyto' meaning cell and 'kine' meaning movement. They are simple peptides, proteins or glycoproteins (Akira et al. 1993). Cytokines are regarded as a catalyst for cell-cell communication, which can also include growth factors, and cytokines with chemoattractant properties (chemokines) (see section 2.2.4). They act on target cells to cause a wide array of cellular functions including activation, proliferation, chemotaxis, immunomodulation, release of other cytokines or mediators, growth and cell differentiation, and apoptosis (Chung and Barnes 1999).

When cytokines are produced they influence target cells. The type of target cell provoked by a particular cytokine is determined by the type of specific receptor on its surface. The term 'autocrine regulation' is used when the receptor is present on the surface of the same cell that produces the cytokine (Figure 2.6A). Alternatively, the

target receptor may be present only on the surface of another cell. If the target cell resides close to the producer cell this is termed ‘paracrine regulation’ (Figure 2.6B). Also, a producer cell can exert its actions on target cells that reside in distant parts of the body or in other tissues or organs; this is termed ‘endocrine regulations’ (Figure 2.6C).

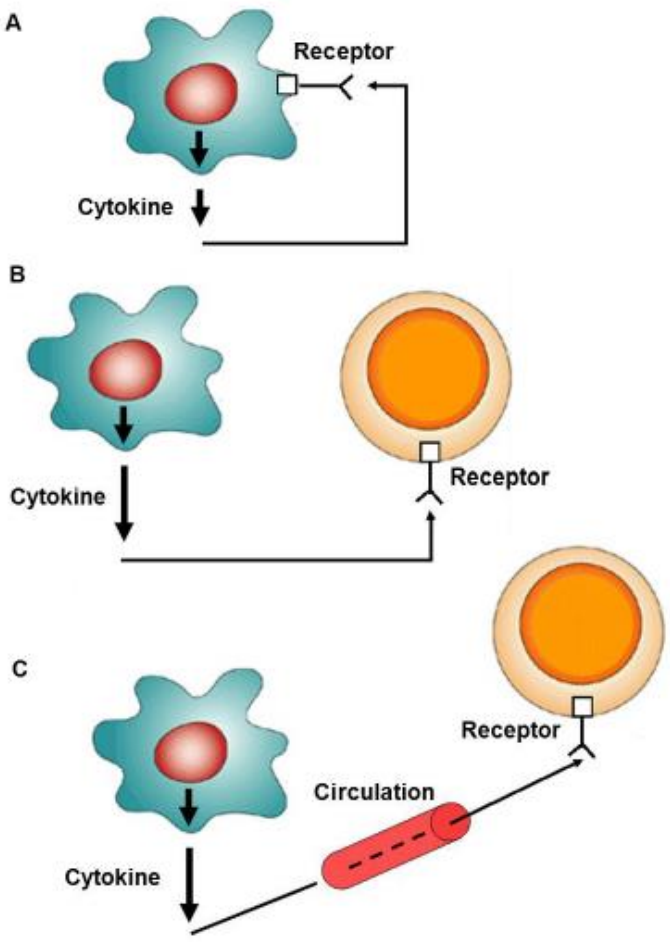

Figure 2. 6 Cytokine regulation, (A) autocrine regulation; (B) paracrine regulation; (C) endocrine regulation. Reproduced with permission from (Mills 2013).

The airway inflammation underlying asthma is regulated in part by a network of mutually interacting cytokines. The exact functional role of each individual cytokine in the pathogenesis of asthma remains to be fully established. The effect of an individual

cytokine in the context of disease may not be easy to predict because it can be influenced by other cytokines released simultaneously by the same cell or from other target cells. Many cytokines play an integral role in the coordination and persistence of the inflammation process within the airways in asthma and they are capable of inducing many pro-inflammatory effects characteristic of the disease (Chung and Barnes 1999). Ultimately they contribute to the release of mediators such as cys-LTs, airway remodelling, bronchoconstriction, and bronchial hyperresponsiveness. Each cytokine will have varying roles in the development of these processes and can be evaluated by studying their properties, presence in the airway walls, and secretions in patients suffering from asthma. Cytokines such as tumour necrosis factor-α (TNF-α) are increased in response to exercise (Mickleborough, et al. 2003), with TNF-α specifically involved in inducing airway hyperresponsiveness (Anderson and Kippelen 2005a). Within the range of cytokines produced as part of airway inflammation associated with EIB, TNF-α has specifically received attention when assessing EIB in athletes in response to dietary interventions. In addition to this, recent evidence also suggests a key component to the initialisation of pro-inflammatory states is interleukin-33 (IL-33).

2.2.2.1. Tumour necrosis factor-alpha (TNF-α)

The cytokine tumour necrosis factor-alpha (TNF-α) has been implicated in both asthma and EIB (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Bradding, et al. 1994). Bronchial mucosal biopsies taken from asthmatic individuals have shown a seven fold increase in the number of mast cells staining for TNF-α compared to non-asthmatic controls, suggesting that TNF-α is up-regulated in asthma (Bradding, et al. 1994). Circulating concentrations of TNF-α were assessed prior to, and following an

exercise challenge test in ten clinically diagnosed EIB participants, and ten controls. Within the EIB group, there was a significant increase in plasma concentrations of TNF-α following the exercise challenge, peaking 60 minutes after the challenge compared to no increases in the control group (Mickleborough, et al. 2003). Importantly, TNF-α concentration was significantly reduced following dietary intervention of high dose omega-3 polyunsaturated fatty acids in these EIB participants, and was associated with improvements in pulmonary function following an exercise challenge compared to placebo (Mickleborough, et al. 2003).

2.2.2.2. Interleukin-33 (IL-33)

Classically, T cell derived Th-2 cytokines such as IL-4 and IL-5 have been implicated in asthma (Bradding, et al. 1994, Doucet, et al. 1998, Shi, et al. 1998); in addition it is now recognised that IL-33 also plays an important contribution to allergic responses and asthma (Prefontaine, et al. 2009). IL-33 is a recently described member of the interleukin-1 family of cytokines, and is expressed by many cells following a pro-inflammatory stimulation. It is widely expressed by T helper 2 cells and mast cells, both of which have been implicated in the pathogenesis of EIB (Wenzel 2013) (Section 2.2.2). It is thought to function as an alarm following cell necrosis to alert the immune system to tissue damage or stress (Miller 2011). It mediates its biological effects via interaction with receptors (ST2 and IL-1RAcP), which are widely expressed by innate immune cells and Th2 cells. Importantly IL-33 strongly induces Th2 cytokine production and can promote the pathogenesis of Th2 diseases such as asthma (Miller 2011). The gut and the lungs appear to be prominent IL-33 expressing organs and IL-33 mRNA expression is found in multiple tissue related cells. Importantly initial gene

expression studies in a range of tissues revealed expression of IL-33 in lung tissue and high expression in bronchial smooth muscle cells (Schmitz, et al. 2005, Smith 2010). Further studies to investigate which cells are the main IL-33 responsive cells in the lung demonstrated that bronchial epithelial and endothelial cells were important (Yagami, et al. 2010). Released IL-33 from these tissue cells could provide a local signal to sustain the accumulation and effector function of innate inflammatory mediators to exacerbate conditions further.

IL-33 exerts its cytokine activity through a signalling receptor known as ST2. ST2 is expressed predominantly on immune cells that are associated with allergic and asthmatic inflammation (Section 2.2.2). The concentration of serum ST2 is significantly increased in asthmatics following an exacerbation (Oshikawa, et al. 2001). Furthermore, the serum ST2 levels during the asthma exacerbations were statistically correlated with the percentage predicted peak expiratory flow. The results highlight that serum ST2 (as a marker of IL-33 activity) may be related to Th2 mediated inflammation during acute exacerbations in atopic asthmatics (Oshikawa, et al. 2001). Research is beginning to show that IL-33 mediates its biological effects via ST2 and subsequently drives the production of Th2 associated cytokines both *in vitro* and *in vivo*, this leads to pathological changes in mucosal organs such as the lungs (Schmitz, et al. 2005).

In a murine model of ovalbumin induced airway inflammation, intranasal administration of IL-33 induces antigen specific IL-5 T cells, furthermore it also increases airway hyper-responsiveness, goblet cell hyperplasia, eosinophilia, and accumulation of IL-4, IL5, and IL-13 in the airways (Kondo, et al. 2008, Stolarski, et al. 2010)

Bronchial biopsies from asthmatics have been reported to have an increased IL-33 expression when compared to controls and further supporting the importance of TNF-α, this cytokine has been shown to increase up-regulation of IL-33 *in vitro* (Prefontaine, et al. 2009). Furthermore, traditional asthma treatment of inhaled corticosteroid has been shown to completely suppress some inflammatory cytokines, but not IL-33 (Yagami, et al. 2010). This suggests that additional treatment methods that influence IL-33 activity are warranted considering the role of IL-33 in asthma.

2.2.4. Chemokines in Asthma and EIB

Chemokines are a large family of peptide chemotactic mediators that control leukocyte migration to the site of inflammation and subsequent activation. As described in section 2.2.2, asthma is characterized by an infiltration of primarily mononuclear cells (lymphocytes and macrophages) and eosinophils. The ability to control leukocyte infiltration into the lungs is viewed as a key regulator of disease severity (Lukacs 2001), and so the expressions of chemokines are a key marker of asthma severity (Lukacs, Oliveira and Hogaboam 1999). The expression of distinct chemokines within the airways raises the prospect that specific profiles of chemokines may mediate various stages or phenotypes of asthma; no specific chemokine profile of EIB is currently known. Chemokines bind to G-protein coupled serpentine receptors and are divided into two main groups on the basis of their sequence homology and the position of the first two cysteine residues, CxC (alpha), and CC (beta) (Rossi and Zlotnik 2000). There are 6 known CXC chemokine receptors and ten known CC chemokine receptors. To date, 16 CxC ligands (CxCL1-16) and 28 CC ligands (CCL1-28) have been identified (Table 2. 3).

Table 2. 3 Chemokines and their receptors – recreated from (Lukacs 2001)

Chemokine Receptor	Ligands (CCL or CxCL)	Cellular expression of receptor
CC Family		
CCR1	CCL3, 7, 9, 15, 16, 23	D, DC, E, M, N, NK, MC, T
CCR2	CCL2, 7, 8, 12, 13	BA, DC, EC, F, M, MC, NK, N, SM, T
CCR3	CCL5, 7, 8, 11, 13, 24, 26	BA, E, EP, T
CCR4	CCL17, 22	BA, DC, M, T
CCR5	CCL3, 4, 5	DC, M , NK, T
CCR6	CCL20	B, DC, N, NK, T
CCR7	CCL19, 21	B, NK, T
CCR8	CCL1, 16, 17	N, NK, T
CCR9	CCL25	T
CCR10	CCL27, 28	B, M, T
CxC Family		
CxCR1	CxCL1, 7, 8	E, EC, MC, N, NK
CxCR2	CxCL1, 2, 3, 5, 6, 7, 8	E, EC, MC, N
CxCR3	CxCL9, 10, 11	E, EC, NK, T
CxCR4	CxCL12	DC, NK, M, T
CxCR5	CxCL13	B, M, T
CxCR6	CxCL16	T

B, B Cell; BA, basophil; DC, dendritic cell; E, eosinophil; EC, endothelial cell; EP epithelial cell; F, fibroblast; M, monocyte/macrophage; MC, mast cell; N, neutrophil; NK, natural killer cell; SM, smooth muscle; T, T cell.

Many of the CC ligands, and CxC ligands had multiple names, but their nomenclature has now been standardised (Table 2. 4) (Rossi and Zlotnik 2000, Zlotnik and Yoshie 2000). As eosinophils are implicated in the pathophysiology of asthma, research has tended to focus primarily on chemokines that have chemotactic activity for eosinophils. Chemokines such as CCL5 (RANTES), CCL7 (MCP-3), and CCL13 (MCP-4), have been identified in the airways of asthmatics. These three chemokines are associated with eosinophil recruitment through the CCR3 receptor which is highly expressed in eosinophils (Powell, et al. 1996, Stellato, et al. 1997). The airway epithelium also produces high levels of chemokines namely CCL5, CCL11, and CCL13 (Stellato, et al. 1997, Wang, et al. 1996, Taha, et al. 1999). This high level of chemokine expression may favourably signal eosinophils to the asthmatic airways. Subsequent degranulation of these cells will lead to the release of mediators at the site of inflammation. These same chemokines can also influence other asthma related leukocyte cells such as basophils and Th2 lymphocytes (Lukacs 2001).

Lymphocytes that are activated in the lymph node are recruited into the asthmatic airways. The T helper cells produce an array of Th2 cytokines (IL-4, IL-5, and Il-13), see section 2.2.3 for further details. The Th2 mediated cell response results in the production of a characteristic subset of chemokines that include CCL11, CCL13, CCL22, CCL1, and CCL17 (Sekiya, et al. 2000, Andrew, et al. 1998) and induces Th2 lymphocyte migration through specific receptors (CCR4, CCR8, and CCR3). The continued activation of these cells induces pathophysiological dysfunctions associated with asthma such as excess mucous production (Lukacs 2001).

Table 2. 4 Standardised nomenclature for chemokines and their receptors. Adapted from (Zlotnik and Yoshie 2000).

New nomenclature	Abbreviation	Common (full) name	Receptors
CCL1	TCA3	T-cell activation-3	CCR8
CCL2	MCP-1	Monocyte chemotactic protein-1	CCR2
CCL3	MIP-1α	Macrophage inflammatory protein-1α	CCR1, CCR5
CCL5	RANTES	Regulated on activation, normal T cell expressed and secreted	CCR1, CCR3, CCR5
CCL7	MCP-3	Monocyte chemotactic protein-3	CCR1, CCR2, CCR3
CCL11		Eotaxin	CCR3
CCL13	MCP-4	Monocyte chemotactic protein-4	CCR2, CCR3
CCL17	TARC	Thymus and activation-regulated chemokine	CCR4, CCR8
CCL22	MDC	Macrophage-derived chemoattractant	CCR4
CCL24		Eotaxin-2	CCR3
CCL26		Eotaxin-3	CCR3
CxCL8	Il-8	Interleukin-8	CxCr1, CxCr2
CxCL12	SDF-1	Stromal-derived factor-1	CxCr4

With evidence of EIB in asthmatic individuals being associated with eosinophilic airway inflammation (Lee, et al. 2006), it highlights the potential role of eosinophil chemo attractant chemokines in the manifestation of the condition. Research suggests that local and systemic expression of the CC ligand chemokines such as CCL11 (eotaxin), CCL5 (RANTES), and CCL17 (TARC), are increased in patients with asthma (Lilly, et al. 1999, Kalayci, et al. 2004). Research also shows a significant rise in chemokine levels following marathon running (Ostrowski, et al. 2001). Plasma concentrations of CCL3 and CxCL8 peaked 0.5 h after the run and where 3.5-fold, and 4.1-fold higher than pre marathon levels. Although the authors did not investigate EIB,

it suggests that following exercise there is an increase in circulating levels of chemokines which could contribute to the exacerbation of EIB post exercise in asthmatics. In support of a potential role of chemokines in EIB; *in vitro* hyperosmolar challenges which cause changes in osmolality of the airways similar to that seen in exercise result in an increased release of CxCL8 from human bronchial epithelial cells (Gon, et al. 1998, Hashimoto, et al. 1999). Therefore this suggests that the osmotic changes that occur in asthmatic airways following exercise can result in increased chemokine release from the bronchial epithelial cells.

The exact role of individual chemokines in EIB has not been fully elucidated. The chemokine response of CCL5, CCL11, and IP-10 to a standardised exercise challenge in asthmatic children (aged 6 – 17 years) have been assessed (Tahan, et al. 2006). The authors found that the exercise challenge did not induce any significant changes in plasma chemokine levels despite a positive reduction in FEV_1 (> 10% reduction) in 50% of the children. Correlation analysis showed that in children with a positive exercise response (> 10% reduction in FEV_1 post exercise) there was a weak inverse correlation between eosinophil counts and CCL11 levels ($r = -0.616$; $P = 0.01$), and a stronger inverse correlation between FEV_1 and CCL17 ($r = -0.865$; $P = 0.001$). This suggests that systemic CCL17 values may be one factor in determining the severity of EIB in children with asthma, with an association to the reduction in FEV_1. It is possible that the level of bronchoconstriction exhibited by the children in Tahan *et al.* (2006) (maximum $\Delta\%FEV_1$ of −17.9%) was too little to see a significant increase in circulating systemic chemokines and may not reflect changes taking place in the local environment of the lung following exercise. Taking measurements directly from the lung in the form of exhaled breath condensate (EBC) has provided more robust

evidence for the role of CCL5 and CCL11 in EIB (Zietkowski, et al. 2009, Zietkowski, et al. 2011).

Asthmatics with EIB had significantly higher CCL5 concentration in EBC collected during the first 24 h after an exercise challenge. Additionally there was a significant correlation between the maximum increase in CCL5 concentration following exercise with baseline FeNO, bronchial hyperreactivity to histamine, and also an increase in serum eosinophil cationic protein or FeNO 24 h after the exercise in the EIB asthmatics (Zietkowski, et al. 2009). The increased expression of CCL5 in EIB asthmatics, signifies greater levels of migration and activation of inflammatory cells including eoisnophils to the inflamed airways in asthmatics with EIB.

Similar results were found when investigating CCL11. Twenty seven asthmatics (17 with EIB, 17 without EIB) cycled for 9 minutes at an intensity of 85% of age predicted heart rate maximum; spirometery, EBC, and FeNO were assessed pre and post the exercise challenge. Bronchial provocation with a histamine challenge was also performed 24 h before and 24 h after the exercise test. In the asthmatics with EIB a statistically significant increase in CCL11 concentrations in EBC collected during the first 24 h after the exercise test with a maximum increase at 6 h was reported (Zietkowski, et al. 2011). Significant correlations between maximum increase in CCL11 concentrations in EBC after exercise, and an increase in both eosinophil cationic protein and FeNO 24 h after exercise in asthmatic EIB group was seen. These results highlight the connection between EIB and increased expression of chemokines associated with eosinophil migration; and as such are a potential marker for EIB severity.

2.2.5. Eicosanoids

The discovery of the eicosanoids dates back to the 1930s, when they were first isolated from human seminal plasma (Goldblatt 1935), and the prostate gland (Von Euler and Bergstrom). Eicosa means twenty in Greek, and the eicosanoids are lipid derived signalling molecules that are generated primarily through an oxidative pathway from arachidonic acid (AA), but also from the pathways originating from eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) (Harizi, Corcuff and Gualde 2008) (Figure 2.7). At sites of inflammation the production of eicosanoids are considerably increased, and their biosynthetic pathways are of particular clinical relevance because their products are involved in the pathology of diseases related to immune function and inflammatory conditions including asthma (Wan and Wu 2007, Wang and DuBois 2010). Eicosanoids generated from AA are generally considered to be pro-inflammatory, where as those generated from EPA and DHA are considered to be less pro-inflammatory, even anti-inflammatory, and possess inflammatory resolving properties (see section 2.2.5.3).

The generation of inflammatory based eicosanoids are dependent upon the availability of free arachidonic which is associated with dietary intake of omega-6 polyunsaturated fatty acids (see section 2.6.1.3). When tissues are exposed to physiological and pathological stimuli such as growth factors and hormones, arachidonic acid is liberated from membrane phospholipids by the action of phospholipase A2 (PLA_2) and can then be converted into different eicosanoids (Harizi, Corcuff and Gualde 2008). Arachidonic acid derived eicosanoids exert complex control over a wide range of physiological processes. Arachidonic derived eicosanoids have been associated with a vast number of inflammatory disorders such as arthritis,

(Sperling 1995) and risk factors associated with atherosclerosis and cancer (Wang and DuBois 2010, Thiemermann, Mitchell and Ferns 1993) and importantly asthma (Boyce 2008).

2.2.5.1. Eicosanoids in Asthma and EIB

Asthma is recognized as a mediator driven inflammatory process within the lung. The arachadonic acid derived eicosanoids cysteinyl leukotrienes and prostaglandins have been implicated in the inflammatory cascade that occurs in asthmatic airways. The cyclooxygenase pathway that results in the generation of prostaglandins is involved in maintaining a balance in the airways with both PGD_2 and thromboxane A_2 causing bronchoconstriction, whereas PGE_2 is bronchoprotective (Wenzel 1997). The leukotrienes are exclusively proinflammatory in nature and are potent bronchoconstrictors. LTB_4 may play an important role in attracting neutrophils and eosinophils into the airways whereas the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) are active in the development of airway inflammation and lead directly to bronchoconstriction through effects on vascular permeability, mucus secretion, and smooth muscle constriction (Hallstrand and Henderson 2009). LTD_4 is the most potent, and LTE_4 the least potent (Hallstrand and Henderson 2009) (figure 2.5). A direct association between an increase in urinary excretion of cysteinyl leukotrienes and EIB is suggested by the findings of Mickleborough et al. (2003). They report significant increases in urinary excretion of 9α-11β, PGF_2 and LTE_4, and circulating cytokines TNF-α and interleukin 1-β as an acute response to exercise to the volitional point of exhaustion in subjects with mild EIB (Mickleborough, et al. 2003).

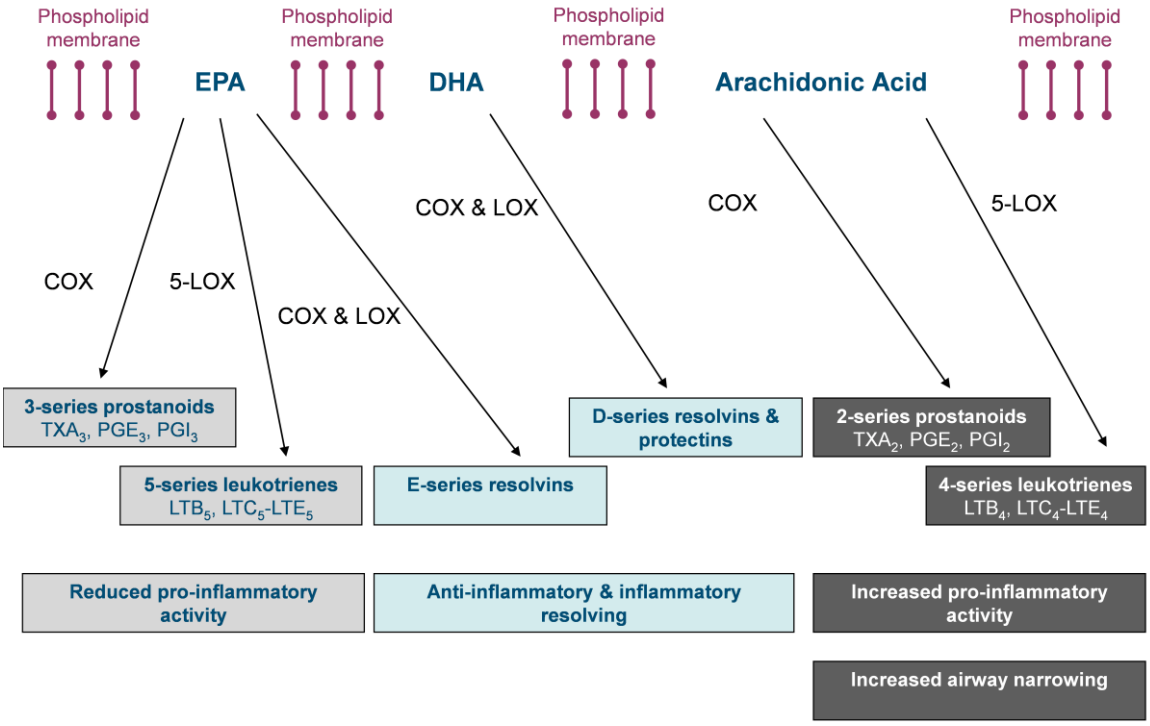

Figure 2. 7 Generation of eicosanoids. DHA, docosahexanoic acid; EPA, eicosapentanoic acid; AA, arachadonic acid; COX, cyclooxygenase enzymatic pathway; 5-LOX, 5 lipoxygenase enzymatic pathway; TXA, thromboxane; PG, prostaglandin, LT, leukotriene

2.2.5.2. Leukotrienes and Prostaglandins in Asthma and EIB

The leukotrienes are pro-inflammatory lipid mediators that were first discovered after challenging animal lungs with snake venom (Feldberg and Kellaway 1938). The authors found that cobra venom caused the release of histamine from perfused lungs and this observation has since been explained by leukotriene synthesis induced by the enzyme phospholipase A_2 which is a major component of snake venom. The leukotrienes are formed from AA by the 5-lipoxygenase (5-LO) pathway in the presence of 5-LO-activating protein (FLAP). The intermediate LTA_4 is then converted to LTB_4 via LTA_4 hydrolase. The formation of the cysteinyl LTs (LTs C_4, D_4, and E_4) is initiated by the action of LTC_4 synthase (Figure 2.8). The cysLTs play an important role in propagating airway inflammation, causing mucus production, smooth muscle

contraction, increased vascular permeability, and have been shown to contribute to airway remodelling in murine models of asthma (Hallstrand and Henderson 2009, Henderson Jr, et al. 2002). The inflammatory cells responsible for the synthesis of LTs are eosinophils, mast cells, basophils, monocytes, and macrophages.

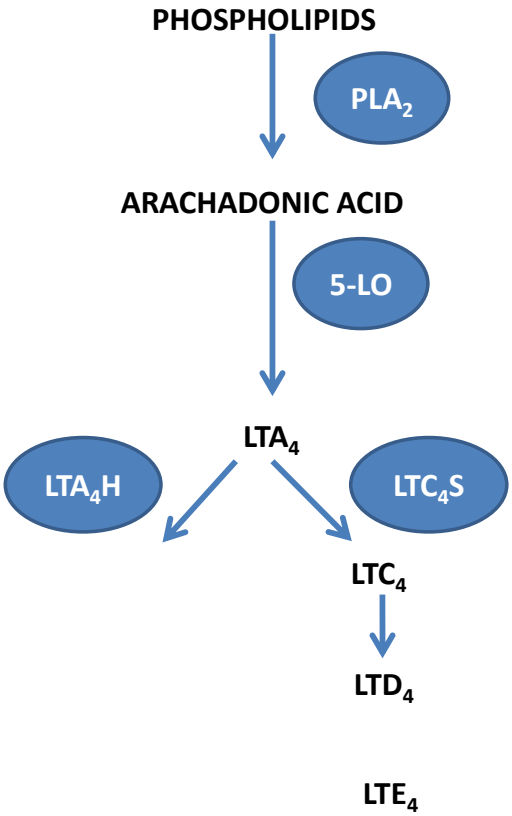

Figure 2. 8 The formation of leukotrienes (LTs) from arachadonic acid metabolism through the 5-lipoxygenase pathway. Ovals indicate the enzymes involved in leukotriene biosynthesis; phospholipase A_2 (PLA_2), 5-lipoxygenase (5-LO) with its activating protein (FLAP), LTA_4 hydrolase (LTA_4H), and LTC_4synthase (LTC_4S).

Hallstrand et al (2005) studied two asthmatic groups, one group with moderate to severe EIB (≥ 30% fall in FEV_1 after exercise challenge) and one without EIB, and showed that both the concentration of cysLTs (727.7 vs 151.9 pg/mL, $P = 0.01$) and the ratio of cysLTs to PGE_2 (1.85 vs 1.04, $P = 0.002$) within the airways were increased in the asthmatics with EIB. PGE_2 is a bronchoprotective eicosanoid produced in large amounts from the epithelium, whereas the cysLTs are the products of inflammatory cells. The altered ratio of these in the EIB asthmatic group and higher concentrations of cycLTs suggest that airway epithelium injury and influx of inflammatory cells may be an underling basis of EIB (Hallstrand and Henderson 2009). Pharmacologic inhibition of the $cysLT_1$ receptor through cysteinyl leukotriene antagonist (montelukast) along with antihistamines (loratadine) can reduce both EIB severity and release of inflammatory mediators (Hallstrand, et al. 2005). However, just inhibiting the release of cysLTs alone does not completely reduce EIB highlighting that there is further eicosanoid action in bronchoconstriction in the form of the prostaglandins.

The production of inflammatory prostaglandins begins with the liberation of AA from the membrane phospholipids by phospholipase A_2 in response to inflammatory stimuli. AA is converted to PGH_2 by the cyclooxygenase enzymes (COX-1, COX-2); COX-1 is expressed in most tissues of the body and acts to maintain homeostasis of processes such as mucus secretion. COX-2 is mainly an inducible enzyme primarily involved in the regulation of inflammation (Harris, et al. 2002). Cell specific prostaglandin-synthases convert PGH_2 into a series of prostaglandins (PGI_2, $PGF_{2\alpha}$, PGD_2, and PGE_2) (Figure 2. 9). Prostaglandins have been shown to have both bronchoprotective and bronchoconstrictive mechanisms. PGE_2 is a bronchoprotective eicosanoid produced in large amounts from the epithelium, whereas $PGF_{2\alpha}$ and PGD_2

mediate bronchoconstriction in asthmatics (Pang, et al. 1998). PGD_2 is the major eicosanoid released from stimulated mast cells. PGD_2 is unstable and rapidly metabolised and eliminated from circulation, as such quantification of PGD_2 is an unreliable indicator of *in vivo* production. 9α-11β-$PGF_{2\alpha}$ is the primary metabolite of PGD_2 *in vivo* and can be reliably quantified in human urine, the concentration of which is increased nearly three-fold upon exposure to allergens in asthmatics (O'Sullivan, et al. 1996).

Increased urinary concentrations of 9α-11β-$PGF_{2\alpha}$ have been found in response to acute episodes of asthma and EIB (Mickleborough, et al. 2003, Kumlin, et al. 1992, Nagakura, et al. 1998). Furthermore the *In vivo* production of TXA_2 and cys-LTs responses to specific aeroallergens have been studied (Kumlin, et al. 1992). TXA_2 is a 2-series prostanoid generated from AA via the cyclooxygenase enzymatic pathway, and the cys-LTS are generated from AA via the 5-lipoxygenase pathway and both are pro-inflammatory eicosanoids (Figure 2. 9). Bronchoprovocation with aeroallergen in the atopic asthmatics caused significant increases in urinary concentrations of cys-LT LTE_4 (34 ± 6 before, versus 56 ± 7 ng·mmol^{-1} creatinine after allergen challenge; n=5) and the TXA_2 urinary metabolite 11-dehydro-TXB_2 (164 ± 29 versus 238 ± 25 ng·mmol^{-1} creatinine) ($p < 0.05$). Following administration of a leukotriene-antagonist the provocative dose to cause a 20% reduction in FEV_1 (PD_{20}) was significantly increased ($p < 0.05$), suggesting a direct role for AA derived leukotrienes in airway narrowing. These findings support that notation that the cys-LTs from the 5 lipoxygenase pathway are mediators for allergen induced asthma and the release of LTE_4 into the urine appears to be direct and dose dependent (Kumlin, et al. 1992).

In addition to the 5-lipoxygenase derived eicosanoids, those derived from cyclooxygenase (Figure 2. 9) are also raised in asthmatics. Urinary levels of the PGD_2 metabolite 9α, 11β-PGF_2 were measured in 30 asthmatic children admitted to hospital, 14 children with EIB, and 14 without EIB following an exercise challenge (Nagakura, et al. 1998). Increased urinary levels of 9α, 11β-PGF_2 observed on the first day of hospital treatment, gradually decreased on the third ($p < 0.05$) and on the sixth day ($p < 0.01$). In EIB, there was a significant increase in urinary levels at 1 h ($p < 0.01$) and at 5 h ($p < 0.01$) after the exercise challenge, but no change in children without EIB (Nagakura, et al. 1998). This further highlights the role of AA derived eicosanoids not only in asthma but specifically in EIB. The pro-inflammatory eicosanoids of the COX and 5-LOX pathways are inextricably associated with the pathogenesis of both asthma and EIB. Symptoms of asthma and allergies can be significantly improved with long term treatment of leukotriene-antagonists (Wood, et al. 1999, Horwitz, McGILL and Busse 1998) further supporting the role of leukotrienes in pathogenesis of asthma. These AA derived eicosanoids are lipid derived mediators from ω6-PUFA. It supports the notion that modulation could occur through dietary supplementation with higher levels of EPA and DHA competitively inhibiting the formation of arachidonic acid derived eicosanoids and having a therapeutic effect (see section 2.6.1.4).

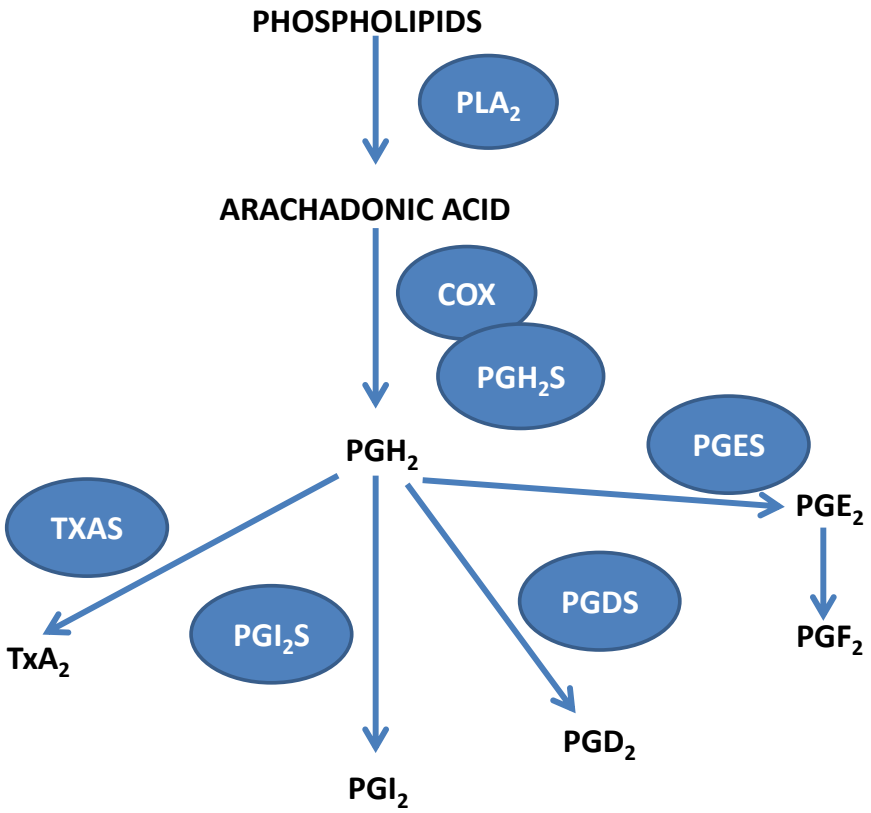

Figure 2. 9 The formation of prostanoids from arachadonic acid metabolism through the cyclooxygenase pathway. Ovals indicate the enzymes involved in prostanoid biosynthesis; Phospholipase A_2 (PLA_2), cyclooxygenase (COX), Prostaglandin H_2 Synthase (PGH_2S), Thromboxane A Synthase (TXAS), Prostacyclin I_2 Synthase (PGI_2S), Prostaglandin D Synthase (PGDS), Prostaglandin E Synthase (PGES).

2.2.5.3. Resolvins and Protectins in Asthma and EIB

As highlighted in section 2.2.1, acute inflammation is essential in the response to dangerous stimuli such as potential infection, or tissue injury. The magnitude and duration of inflammation is dependent upon mechanisms that amplify inflammation, and endogenous braking programs that control the resolution of inflammation (Serhan 2007). Resolution of inflammation is an active coordinated process controlled by endogenously generated autacoids at sites of inflammation (Serhan and Savill 2005, Serhan 2007, Serhan, et al. 2000). During inflammation, it is the enzymatic transformation of polyunsaturated fatty acids (PUFAs) that lead to the generation of the

recently discovered inflammation-dampening and resolution promoting ω-3 PUFA-derived lipid mediators called resolvins and protectins (Weylandt, et al. 2012). Chemically, these compounds are hydroxylated derivatives of the parent ω-3 PUFA eicosapentanoic acid (EPA) for the E-series resolvins, and docosahexanoic acid (DHA) for the D-series resolvins and protectin D1 (Figure 2. 10). These pro-resolving mediators that are enzymatically generated from ω3-PUFA act as agonists to specific receptors for chemokines, cytokines and eicosanoids. Resolvins exert counter-regulatory effects on leukocyte function, preventing uncontrollable neutrophil infiltration, decreasing cytokine, chemokine, and reactive oxygen species generation, and promoting the clearance of apoptotic neutrophils from sites of inflammation (Uddin and Levy 2010). Protectin is generated from DHA and is a protective anti-inflammatory molecule that can counter leukocyte action to promote the resolution of inflammation (Levy, et al. 2007). Levels of protectin D1 (PD1) have been shown to be significantly lower in the exhaled breath of asthmatics (Levy, et al. 2007). In a murine model of asthma, when PD1 was administered before a methacholine challenge, airway eosinophil and T lymphocyte recruitment was decreased, as were levels of proinflammatory mediators, cytokine IL-13, cysteinyl leukotrienes, and PGD_2, and airway hyperresponsiveness and mucous production (Levy, et al. 2007). The formation of resolvins and protectins from EPA and DHA may offer an important new concept in establishing the protective effects of ω-3 PUFA in inflammatory disease.

To date, research has focused primarily on murine models of asthma and the influence of PD1 and resolvin E1 (RvE1) on airway inflammation and allergic asthma (Aoki, et al. 2008, Haworth, et al. 2008). Intraperitonetal administration of RvE1 caused subsequent reductions in eosinophil and lymphocyte recruitment, Th2 cytokine

concentration and airway hyperresponsiveness to methacholine (Aoki, et al. 2008), and reduces the production of IL-23, and IL-6 (Haworth, et al. 2008). Furthermore, RvE1 treated mice have been shown to have lower mucous production from goblet cells compared to non-treated mice (Aoki, et al. 2008). In a murine model of allergic asthma *in vivo*, RvE1 administered intravenously inhibited the development of allergic airway inflammation and when given during the resolution phase of inflammation, RvE1 accelerated the resolution of airway inflammation, (Haworth, et al. 2008). The bronchoprotective effects of RvE1 during resolution were attributed to the inhibition of Th17 effector lymphocytes and increased generation of interferon-gamma (IFN-γ) and lipoxin A_4 (Haworth, et al. 2008). There is potential that RvE1 can prevent the development of bronchial hyperresponsiveness, mucus eosinophil accumulation and Th2 cytokine mediator release (Aoki, et al. 2008). As RvE1 is a ω-3 PUFA derived mediator, it highlights a potential therapeutic use of ω-3 PUFA in EIB (See section 2.6.1.4).

As stated previously, the pathogenesis of asthma is associated with excess generation of pro-inflammatory cytokines, chemokines, and eicosanoids (section 2.2). Due to the multitude of pro-inflammatory mediators and their numerous interactions with inflammatory cell types, an anti-inflammatory therapy designed to target a single pro-inflammatory mediator is unlikely to be successful (Uddin and Levy 2010) . No current pharmacologic asthma therapies are designed to selectively promote resolution of pulmonary inflammation. Also of interest, is that airway mucosal epithelial cells from individuals with asthma or cystic fibrosis have depleted stores of ω-3 DHA compared with healthy controls (Freedman, et al. 2004). This further suggests an

additional role to support the incorporation of ω3-PUFA in diets of asthmatic individuals.

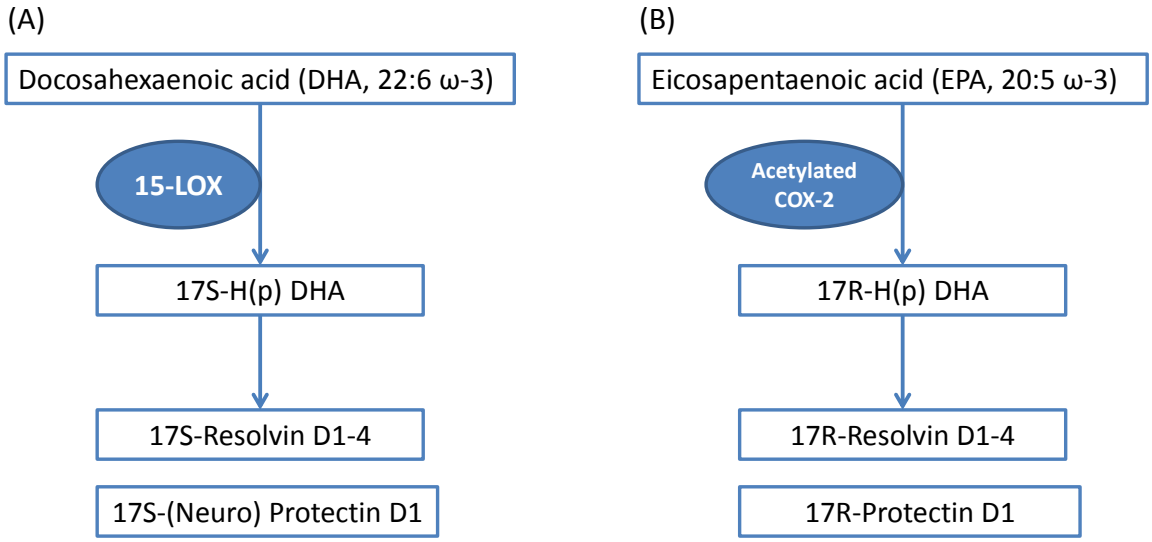

Figure 2. 10 (A) Formation of D-series resolvins and protectins from DHA, through the 15-lipoxygenase (15-LOX), and acetylated cyclooxygenase-2 (COX-2) pathways. Leads to 17S or 17R-H(p)DHA, and subsequent processing through epoxide intermediates and lipoxygenase action. Final products are the aspirin triggered 17R resolvins D1-D4, and the 15-LOX dependent 17S-resolvins D1-D4. 17S and 17R protectin D1's are also formed. (B) Formation of E-series resolvins from EPA, through acetylated cyclooxygenase-2 (COX-2) pathway to form 18S and 18R-H(p) EPE. Subsequently 5-LOX, enzymatic epoxidation or reduction lead to resolving E1 or E2. Each compound can exist as 18S or 18S-epimer which is dependent upon its precursor molecule.

2.3. The gut, the immune system and inflammation

The immune system as described in section 2.2.1 is comprised of a network of cells, proteins, tissues, and organs that coordinate to defend the body against attacks from foreign elements. An important part of the adaptive immune system is the mucosal immune system, termed the mucosal associated lymphoid tissue (MALT), which includes the gut associated lymphoid tissue (GALT). The human intestine represents the largest mass of lymphoid tissue in the body. Figure 2. 11 depicts the anatomy of the intestinal immune system and it's interaction with the commensal bacteria. A single layer of intestinal epithelial cells (IECs) provide a physical barrier that separates the trillions of commensal bacteria in the intestinal lumen from the underlying lamina propria. The IECs were once considered to just provide a simple barrier but are a crucial cell lineage for maintaining intestinal immune homeostasis (Artis 2008). The IECs are continually exposed to substances in the intestinal lumen (nutrients, commensal bacteria, immunoglobulin, and mucous from goblet cells) in response to this exposure a cascade of events occurs associated with immune function. Epithelial stem cells proliferate and give rise to daughter cells with the further potential to proliferate. These IECs differentiate into villous or colonic enterocytes, which are able to absorb nutrients (small intestine) and water (colon) thus influencing systemic metabolism. In addition to differentiated enterocytes and goblet cells, progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Beneath the IECs, the lamina propria is made up of stromal cells (myofibroblasts), B cells (especially IgA-producing plasma cells), T cells, macrophages and dendritic cells which cab regulate humoral and cellular gut immunity through a number of toll like receptors (Denning, et al. 2007, Uematsu, et al. 2008).

Certain subsets of T cells and dendritic cells localize between the IECs (Artis 2008, Abreu 2010). Dendritic cells, with their dendrites are localised between the tight junctions of IECs and are in a unique position to sample and respond to environmental cues in the lumen of the intestine (alterations in commensal bacteria) and select appropriate immune responses (Coombes and Powrie 2008). As such, dendritic cells can have a subsequent influence on tolerance and immunity in the intestine through alterations in inflammatory cell and mediator production (Coombes and Powrie 2008, Varol, et al. 2009). Furthermore, the small intestine has regions of specialized epithelium termed follicle-associated epithelium cells and microfold (M) cells that overlie the Peyer's patches and sample the intestinal lumen (Abreu 2010). These cells secrete a number of chemokines that are important in the recruitment of inflammatory cells and dendritic cells in the Peyer's patches (Mowat 2003, Zhao, et al. 2003)

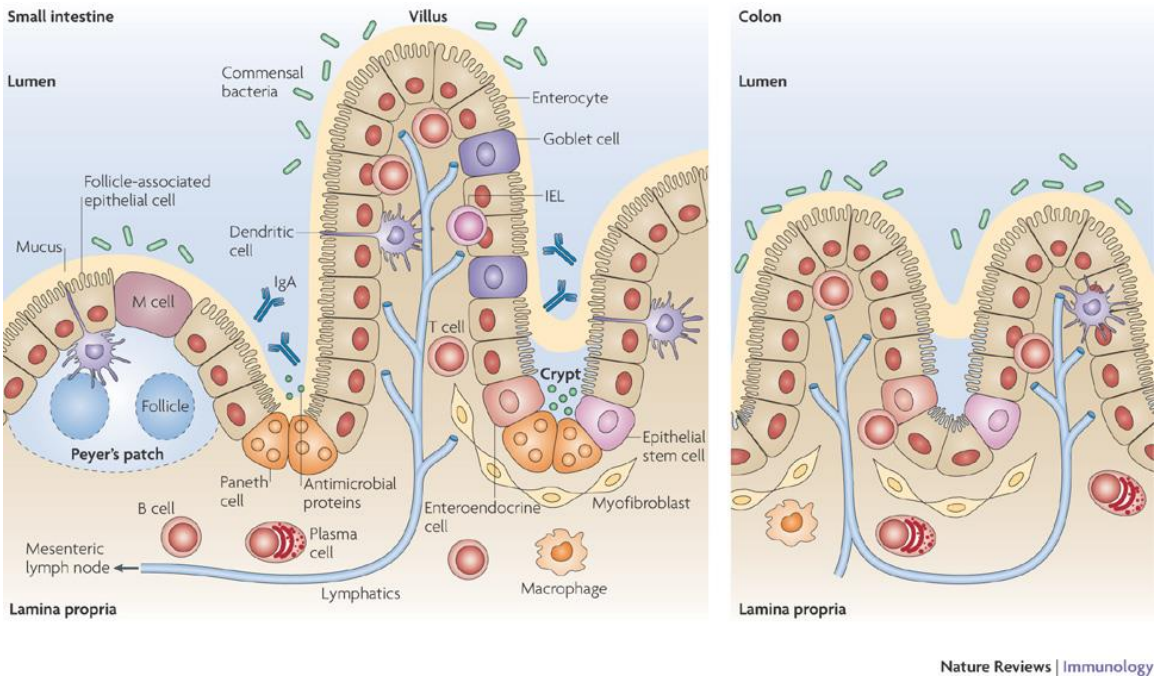

Figure 2. 11. Anatomy of the intestinal immune system (Abreu 2010). IEL, intraepithelial lymphocyte

In combination with the GALT, the intestinal immune system also comprises of resident microbiota with the human gastrointestinal tract (GI tract) colonized by over a 1000 species of microbes known as the commensal bacteria. The GALT and commensal bacteria comprise a complex ecosystem that functions to significantly contribute to systemic metabolism and immune function. As a result they can impact significantly on health and disease (McCracken and Lorenz 2001). Presently a total of 1057 intestinal species of Eukaraya (92), Archaea (8), and Bacteria (957) have been identified (Rajilić-Stojanović and Vos 2014). This microbial colonisation of the GI tract affects the GALT for example; the commensal bacteria in the GI tract cause the intraepithelial lymphocytes to greatly increase in number, potentially impacting on immune and inflammatory function.

The adult gastrointestinal immune system comprises of a stable alliance among the commensal microbiota, immune mediators, and the epithelial barrier (McCracken and Lorenz 2001). All three components are essential for function and maintenance of a stable and mature intestinal immune system. There is a complex relationship between the intestinal immune system and the GI microbiota. The Intestinal epithelial cells are capable of detecting differences in the local environment and will respond to exposure to regulate both innate and adaptive immune responses. Signals from, and alterations in the gut microbiota can be transmitted to adjacent immune cells such as macrophages, dendritic cells, and lymphocytes through molecules expressed on the epithelial cell surface known as toll-like receptors (TLRs). The epithelial cells of the GI tract are in constant contact with the gut microbiota, and the expression of TLRs must be down-regulated in order to avoid over-stimulation. It is important for the intestinal epithelial cells and the mucosal immune system to be able to distinguish between pathogenic and

non-pathogenic agents. Intestinal epithelial cells protect the host by providing a physical barrier and produce a variety of innate antimicrobial substance (McCracken and Lorenz 2001).

2.3.1. The gut microbiota and disease

The intestinal immune system must elicit a robust immune response to harmful pathogens whilst also restraining immune responses to commensal microbes and dietary antigens. There is a delicate balance in this dichotomy and imbalances in either the intestinal immune system or gut microbiota can contribute towards a number of diseases. Dysregulation of the intestinal immune response to normal bacterial flora has been suggested to play a crucial role in several inflammatory and autoimmune diseases including asthma (Elson and Cong 2002).

To support the notion that the gut microbiota plays an integral role in immune and inflammatory responses a number of murine based studies of human diseases such as inflammatory bowel disease have been completed under germ-free conditions. Humans and host animals are highly adapted to have a synbiotic relationship with the commensal intestinal bacteria. Experimental procedures now mean it is possible to keep experimental mice entirely germ-free with no colonization of commensal bacteria in the gut. This is achieved by initially delivering the offspring under sterile caesarean section and hand rearing then aseptically (Macpherson and Harris 2004). Germ-free mice have no bacteria in the intestine or other body surfaces, and comparing these mice to specific-pathogen-free (SPF) mice of the same strain which contain a simple flora produces evidence for the importance of the microflora and the intestinal immune

system in specific diseases. Germ-free mice do not develop a full mucosal intestinal immune system and also exhibit poorly developed spleen and lymph nodes (Macpherson and Harris 2004). Furthermore, the gene expression of the IECs is altered in the absence of commensal bacteria. This under development of the mucosal immune system in germ free mice highlights the importance of commensal bacteria in ensuring an adequate immune response can occur in response to disease.

Germ-free mice are shown to exhibit high numbers of pro inflammatory cytokine secreting cells termed invariant natural killer cells (iNKT) (Olszak, et al. 2012). iNKT cells play an important role in the pathogenesis of ulcerative colitis, irritable bowel disease (IBD), and asthma and are shown to accumulate in high amounts in the lamina propria of the both the colon and airways (Olszak, et al. 2012). This increased accumulation of iNKT cells resulted in increased morbidity in models of IBD and allergic asthma when compared to specific pathogen free mice. It was also associated with increased intestinal and pulmonary chemokine expression (Olszak, et al. 2012). In contrast, colonization of neonatal germ-free mice with a conventional microbiota protected the mice from mucosal iNKT accumulation and related pathology. Interestingly this was not evident when adult germ-free mice were exposed to conventional microbiota suggesting that microbial exposure during early life was important. This had persistent effects on natural killer T cell function which are one component of the innate immune system and have the ability to both lyse target cells and provide an early source of immunoregulatory cytokines, and influence subsequent immune function in later life (Olszak, et al. 2012).

There is growing evidence that children suffering from autoimmune and atopic conditions have reduced diversity of the intestinal microbiota. Reduced diversity of the

microbiota in infants has been shown to increase the risk of allergic disease, atopic eczema, and asthma in later life (Wang, et al. 2008, Sjögren, et al. 2009, Bisgaard, et al. 2011, Hansen, et al. 2012, Abrahamsson, et al. 2014). Epidemiological studies and clinical reports over the last 15 years have revealed dramatic increases in the incidence of several immune disorders including IBD, asthma and allergies, primarily in Western societies (Bach 2002). According to the 'Hygiene Hypothesis' (Strachan 1989), smaller family sizes and increased hygienic living standards have caused a reduction in the incidences of infections in both Western and recently developing countries occurring simultaneously with increased prevalence of autoimmune and allergic diseases (Strachan 1989). The Hygiene Hypothesis was based upon epidemiological data and migration studies highlighting that individuals migrating from a low-incidence country to a high-incidence country become susceptible to immune disorders within the first generation (Okada, et al. 2010). This is too quick for any alterations in genetic makeup and therefore suggests a cause could be due to changes in environment. A weakness of epidemiological studies such as these is that they demonstrate a relationship between disease incidence and socio-economic status but do not provide a cause and effect between infections and immune disorders (Okada, et al. 2010). Animal models, and to a lesser extent some intervention trials in humans, do provide some proof of principle of the Hygiene Hypothesis, but the mechanisms are likely to be multiple and complex.

Western lifestyles can result in disturbances in the gastrointestinal microbiota as a result of reduced early life exposure to microorganisms, increased antibiotic use and dietary changes. This may subsequently disrupt the immunological and inflammatory responses to triggers. The research to support this altered microbiota includes correlations between asthma prevalence and antibiotic use in industrialised countries,

and association between altered faecal microbiota and allergic disease (Wickens, et al. 1999, Kirjavainen, et al. 2001, Kalliomäki, et al. 2001) . Children living on farms have been shown to have a lower prevalence of asthma and atopy, and are exposed to a greater variety of environmental microorganisms compared to a reference population during their childhood (Ege, et al. 2011). Early life (children <1 years of age) exposure to stables and consumption of farm milk has been associated with lower frequencies of asthma, hay fever and atopic sensitisation compared to those aged 1-5 years (Riedler, et al. 2001). In addition to early life microorganism exposure impacting on the GI microbiota and atopic risk, is the influence of early life antibiotic use. Antibiotic treatment has been shown to result in a long term decrease in beneficial anaerobic bacteria (*Bifidobacterium, Lactobacillus, Bacteroides*) and increases in potentially harmful microbes (Clostridium difficile). Antibiotic treatment can also result in reduced levels of short-chain fatty acids and changes in the 16s rRNA microbiota patterns long after antibiotic therapy and this reflects changes in the gut microbiota composition (Nord 1993, Sullivan, Edlund and Nord 2001). The use of antibiotics in the first year of life has been associated with asthma, hay fever, and eczema (Droste, et al. 2000) suggesting early life disturbances in the maturation of the immune system can influence asthma susceptibility. The susceptibility of an individual to suffer with asthma and allergic disease is complex but will involve genetic variants and environmental exposures (bacteria, viruses), alteration of our microbiome and potentially large-scale manipulation of our environment over the past century (Daley 2014).

It is clear that appropriate development of the microbiome during early life is necessary for a strong immune system and reduced risk of autoimmune and inflammatory conditions. These studies support the concept that the gut microflora and

inflammatory conditions are pathogenically closely related, and that the commensal bacteria play an important role in the pathogenesis of certain inflammatory borne diseases. Recent advances in genomic technologies have shown that the human microbiome comprise of 100 trillion bacteria (Human Microbiome Project Consortium 2012). There is evidence that alterations in gut microbiota may influence immune regulation outside of the gut affecting asthma and atopic disease (Noverr and Huffnagle 2004) moreover, dysbiosis of the gut may well be a critical factor in autoimmune diseases such asthma and allergies.

2.3.2. Diet, gut microbiota and asthma

In recent years it has been clearly demonstrated that diet has a considerable effect on the composition of the gut microbiota (Turnbaugh, et al. 2009, De Filippo, et al. 2010). Comparisons of dietary intake and microbiota composition have been made between children from rural Africa with low incidences of asthma and atopy and Europe where there are high incidences (De Filippo, et al. 2010). The diet of children from rural Africa has high fibre content similar to that of early humans at the time of the birth of agriculture. Rural African children showed a significantly richer and more diverse microbiota with greater numbers of beneficial *Bacteroidetes*, and depletion of harmful bacteria *Firmicutes* ($p < 0.001$) compared to European children. In addition rural African children had significantly greater amount of faecal short-chain fatty acids compared to European children ($p < 0.001$). The authors hypothesise from these findings that the diverse gut microbiota in rural African children coevolved with a polysaccharide-rich diet allowing them to maximise energy intake from fibres and benefit inflammatory and immune function (De Filippo, et al. 2010). The study suggests

that development of western diets away from traditional early human diets can have a significant impact on the composition and diversity of the gut microbiota, and a relationship was inferred that it may impact on immune function and susceptibility to atopic diseases.

It is has been frequently proposed in the literature that changes in diet and associated changes in gut microbiota are contributing to increased incidences of inflammatory conditions in western society including asthma (Maslowski and Mackay 2011). Figure 2.12 is a model proposed by Maslowski and Mackay in 2011 in which they suggest that balanced microbial composition results in symbiosis; this provides regulation of immune and inflammatory responses through anti-inflammatory and/or immunomodulatory products such as short chain fatty acids (SCFA), polysaccharide A (PSA) and peptidoglycan (PTGN), which help maintain homeostasis. In contrast, dysbiosis leads to dysregulation of the immune system through lack of beneficial microbial products and an increase in virulence factors, which could leave the host susceptible to inflammation. Dysbiosis may occur through poor dietary intake, as well as through changes induced by factors such as host genetics, maternal transfer and antibiotic use (Maslowski and Mackay 2011).

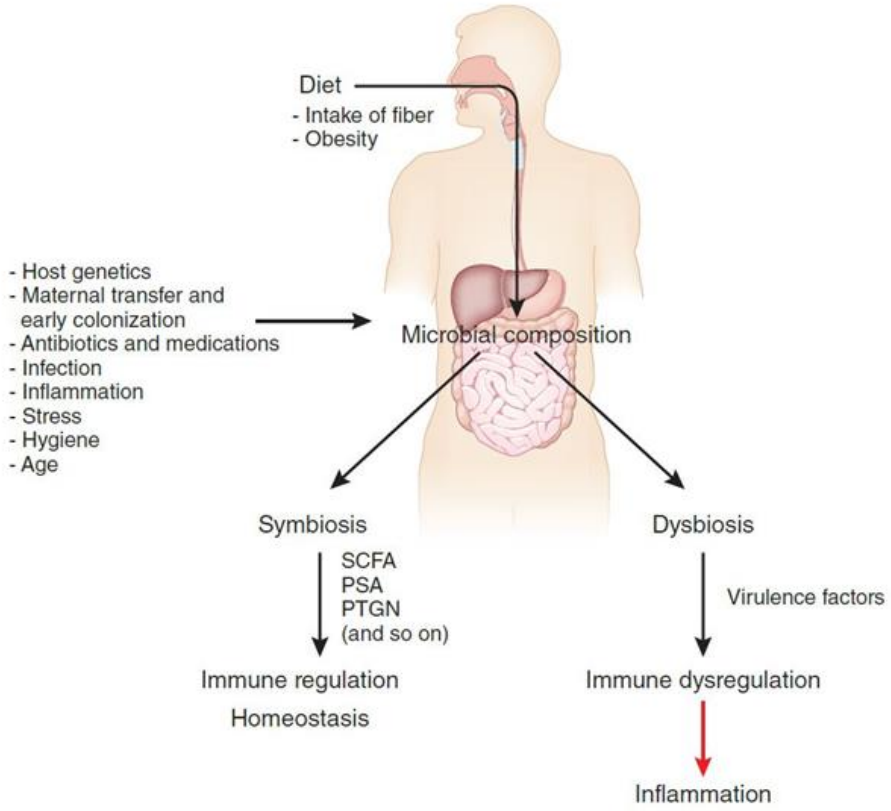

Figure 2.12 Diet, microbial composition and regulation of the immune system. Diet and other environmental and host factors have a major effect on gut microbial composition (Maslowski and Mackay 2011).

Childhood diet has been implicated in the development of asthma (Willers, et al. 2011). Fruit consumption at early age is associated with reduced asthma symptoms, and long-term fruit intake was inversely associated with symptoms and sensitisation to inhaled allergens (Willers, et al. 2011). Studies have also compared children of families with an anthroposophic lifestyle to non-anthroposophic children. Anthroposophic lifestyle means children often abstain from antibiotic use and ingest larger quantities of fermented foods containing probiotic organisms. Rates of allergy and asthma amongst these children were found to be significantly lower than that in control children (Alm, et al. 1999). In addition, the microbiota of anthroposophic children was found to have

greater numbers of beneficial lactic acid bacteria compared to controls correlating to reduced asthma and allergy prevalence (Alm, et al. 2002).

Ingestion of food by the human host will influence the GI microbiota, and the type of breakdown products they generate such as vitamins, and short-chain fatty acids and polysaccharides; thus a poor diet can have a detrimental impact on the gut microbiota. For example in a murine model, feeding mice a high fat/high sugar western diet altered the structure of the microbiota within a single day (Turnbaugh, et al. 2009). This association between disturbances in the gastrointestinal microbiota and susceptibility to inflammatory conditions including asthma raises the possibility that favourable manipulation of the microbiota could be a novel therapeutic target (Chapter 6). Rather than developing new anti-inflammatory drugs, it might be more cost-effective to devote more effort to new approaches, such as monitoring the human intestinal microbiota and manipulating it if required through the use of probiotics and/or prebiotics that stimulate the growth and/or activity of bacteria (Maslowski and Mackay 2011). Recent research has focused on the effect of specific carbohydrates known as prebiotics. These are non-digestible carbohydrates that stimulate the growth of beneficial probiotic bacteria such as bifidobacteria (See section 2.6.2).

The exact mechanisms by which manipulation of the gut microflora can positively impact on inflammatory conditions beyond the gut including airway inflammatory disorders in humans are still poorly understood. In 2004, Noverr and Huffnagle proposed a model for how the gastrointestinal microbiota might regulate immune responses in the airways (Figure 2. 13). The model depicts that antigens are inhaled and are captured by cells of the innate immune system causing stimulation of the adaptive immune responses in the secondary lymphoid organs including the lymph

nodes and spleen. Antigen-specific T cells are then recruited into the lungs upon subsequent antigen exposure. Regulatory T-cell networks are needed to down regulate the development of over stimulated Th2 responses in the airways to alterations in the airway environment bought about from innocuous inhaled and swallowed antigens (i.e. pollen). These swallowed antigens are then acquired by dendritic cells in the GI tract. Under non-inflammatory conditions, these DCs promote the development of a regulatory T-cell response to the antigens. Dietary changes and increased antibiotic use alter the bacterial microbiota, which alter the developmental environment for regulatory T cell responses and impact on immune and inflammatory function. A limitation with this model is it solely looks at pulmonary immune responses to inhaled antigens. As stated in section 2.2.2, EIB develops as a result of an increasing osmotic environment within the airways following exercise causing mucous production and airway smooth muscle contraction, thereby ruling out a role for antigen inhalation and digestion into the GI tract in EIB. However, it may be argued that the airway epithelial cells and dendritic cells respond to the changing osmolality to produce a cascade of inflammatory events. This cascade in turn may be modulated by alterations in inflammatory and immune function from the gut microbiota.

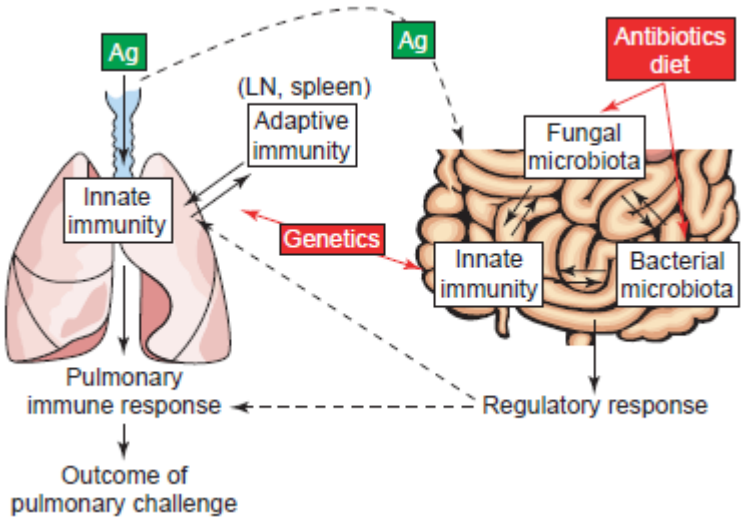

Figure 2. 13 Proposed model for how the gastrointestinal microbiota might be involved in the regulation of pulmonary immune responses (Noverr and Huffnagle 2004)

2.3.3. The concept of the gut microbiota as a therapeutic target for airway inflammation

Beneficially altering the gut microbiota with a pre/probiotic supplement will influence the intestinal epithelial cells and dendritic cells within the gut associated lymphoid tissue (see 2.3 for more details). Figure 2. 14 depicts the interaction of probiotic bacteria with the intestinal epithelial cells (IECs) and intestinal DCs. It shows that probiotic bacteria can encounter DCs through two routes. DCs residing in the lamina propria sample luminal bacteria by passing their dendrites between IECs into the gut lumen (Rescigno, et al. 2001), and DCs can also interact directly with bacteria that have gained access to the dome region of the GALT through specialised epithelial cells termed microfold cells (M cells) (Artis 2008). The interaction of these host cells with microorganism-associated molecular patterns (MAMPs) that are on the surface of the probiotic bacteria will induce a certain molecular response. The host pattern recognition receptors (PRRs) on the dendrites can perceive probiotic signals that include toll-like receptors (TLRs) and DC- specific intracellular adhesion molecule 3-grabbing non-

integrin (DC-SIGN) (Lebeer, Vanderleyden and De Keersmaecker 2010). The molecular response of some IECs will depend upon the subtype of cell, e.g. Paneth cells will produce defensins whereas goblet cells will produce mucous. The important response of DCs to probiotics that may influence systemic inflammation is the production of cytokines, and co-stimulatory molecules to polarize T cells into T helper or T regulatory cells in the mesenteric lymph nodes (MLNs) of the subepithelial dome of the GALT (Lebeer, Vanderleyden and De Keersmaecker 2010). Altering the T helper to T regulatory cell ratio is likely to impact upon systemic immune and inflammatory function which may in turn alter the inflammatory response associated with EIB.

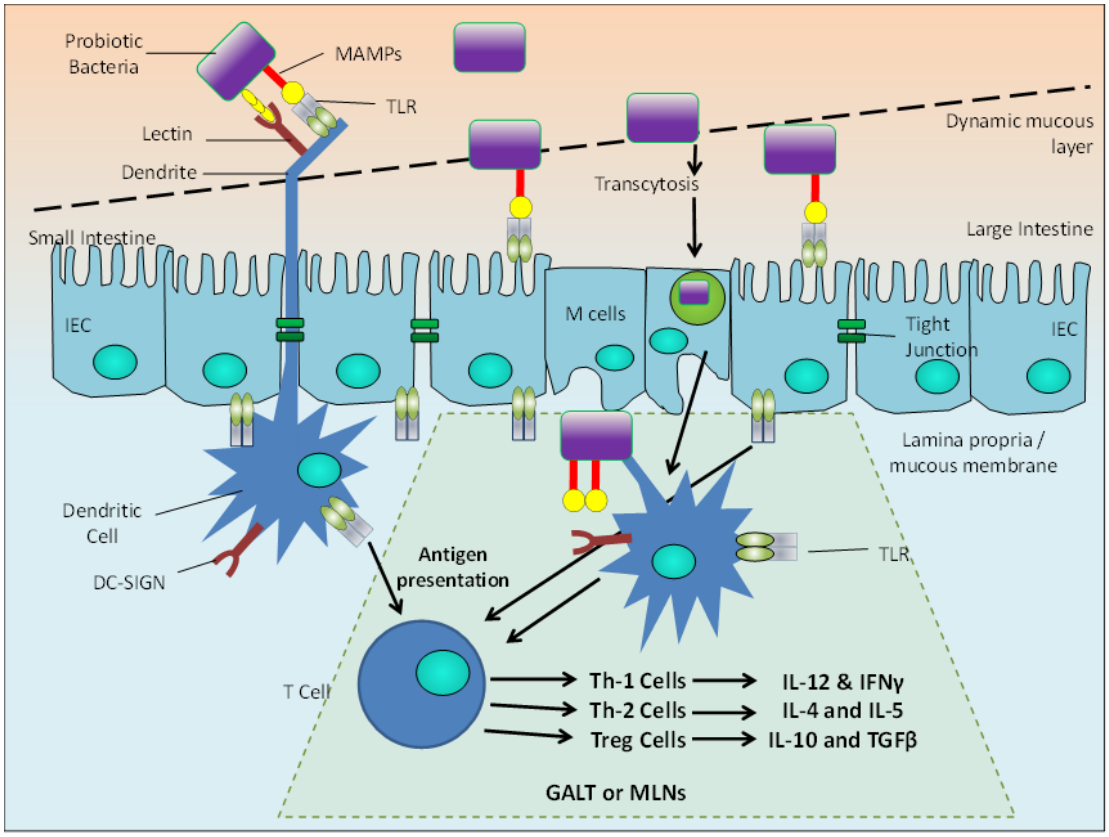

Figure 2. 14 Interaction of probiotic bacteria with intestinal epithelial cells and dendritic cells in the gut associated lymphoid tissue and subsequent influence of T cell expression and cytokine production. MAMPs, microorganism-associated molecular patterns; TLR, toll like receptors; IEC, intestinal epithelial cell; DC, dendritic cell; SIGN, specific intracellular adhesion molecule 3-grabbing non-integrin; IFNγ, interferon-γ; IL, interleukin; TGFβ, transforming growth factor-β; MLNs, mesenteric lymph nodes. Adapted from (Lebeer, Vanderleyden and De Keersmaecker 2010).

2.3.4. The airway microbiota and asthma

The human respiratory tract traditionally viewed as a sterile environment, has now recently shown to actually harbour its own local microbiota (Charlson, et al. 2011) Advances in sequencing technologies have made it possible to identify strains of bacteria that colonise the airways. Contrasting the gut microbiota, the amount of bacteria in healthy lungs is lower, but comprises a relatively diverse number of species (Gollwitzer and Marsland 2014). The most prevalent phyla identified in the airways are *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Studies at the genus level confirmed the predominant presence of Pseudomonas, Streptococcus, Prevotella, Fusobacteria, Veillonella, Haemophilus as well as Neisseria (Hilty, et al. 2010, Erb-Downward, et al. 2011). To date, the exact initial source of the airway microbiota is unknown, but there is evidence to suggest it is determined, not only from the environment but also from the gut microbiota and diet (Madan, et al. 2012, Gollwitzer and Marsland 2014).

A landmark paper in 2010 was the first to describe the existence of a microbiota in the lower airways, and showed clear differences in the composition of healthy airways compared to asthmatics (Hilty, et al. 2010). Asthmatic airways showed significant increased levels of *Proteobacteria*, with potentially pathogenic strains; and reduced levels of beneficial *Bacteroidetes* when compared to healthy controls (Hilty, et al. 2010). This has since been supported by Huang et al. (2011) who showed that total bacteria burden and bacterial diversity where much higher in asthmatic patients with airway hyperresponsiveness compared to healthy controls, although the exact cause and relationship still remains to be established. With evidence to suggest that the gut microbiota and diet could influence the airway microbiota (Madan, et al. 2012), it

highlights a possible novel treatment target and option for airway diseases that may influence the airway microbiome (Hilty, et al. 2010).

2.4. Methods to diagnose exercise induced bronchoconstriction

EIB is usually diagnosed by a ≥10% reduction in forced expiratory volume in 1 second (FEV_1) over the first 20 minutes from the pre-exercise or pre-bronchial challenge value. Exposing exercising asthmatics to allergens may also increase the exacerbation and airway responsiveness (Anderson and Kippelen 2005a). This increased responsiveness is termed "airway hyper-responsiveness (AHR)". The severity of AHR and bronchoconstriction in EIB sufferers is determined by the ventilation reached and sustained during exercise, and the water content of the inspired air during exercise (Anderson and Daviskas 1997).

The need for objective testing to identify EIB is supported by the knowledge that, in athletes, respiratory symptoms alone do not predict EIB(Rundell, et al. 2001a) and may not reliably monitor the condition and responses to interventions and medication. Bronchial provocation challenges have been developed to diagnose and track management strategies for EIB more accurately. There are two distinct types of tests which are termed direct and indirect bronchial provocation challenges. Direct challenges involve the inhalation of methacholine or histamine as a provocative stimulus, and these cause bronchoconstriction predominantly via their direct effect on the airway smooth muscle. In contrast, indirect challenges induce bronchoconstriction by acting on inflammatory cells, epithelial cells and nerves which, upon stimulation, release mediators or neurotransmitters that provoke smooth muscle contraction (Joos and O'Connor 2003).

2.4.1. Indirect Challenges

Indirect challenges have been defined as a challenge that act by causing the release of endogenous mediators that cause the airway smooth muscle to contract, with or without inducing microvascular leakage. Because the responses to these challenges are modified or even completely inhibited by inhaled steroids, the airway response to these challenges may be a close reflection of active airway inflammation (Joos and O'Connor 2003).

Indirect measures include, exercise testing, eucapnic voluntary hyperventilation, inhaled mannitol, and hypertonic saline (Anderson 2011). Indirect measures have been suggested to be a preferred strategy for monitoring the progression of interventions as they provoke bronchoconstriction and airway hyperresponsiveness associated with increased airway inflammation (Rundell and Slee 2008, Brannan, et al. 1998).

2.4.1.1. Exercise Challenges

Recommendations for conducting an exercise challenge test (ECT) have been described by both the European Respiratory Society(Sterk, et al. 1993) and American Thoracic Society(American Thoracic Society 2000). The guidelines state that participants should exercise for six (children under 12 years) to eight minutes (adult) breathing temperate dry air (< 25°C and <50% relative humidity or < 10 mg $H_2O \cdot L^{-1}$) at an intensity to raise minute ventilation ($\dot{V}_E$) up to 21 times the FEV_1 for the last 4 min of exercise (60% maximum voluntary ventilation) (American Thoracic Society 2000). In the absence of a measure of ventilation the heart rate should achieve 90% of age predicted maximum in the last 4 minutes(Sterk, et al. 1993, American Thoracic Society

2000). Pulmonary function testing is performed pre and post the exercise and a reduction in FEV_1 of 10% of the pre-exercise values is widely accepted as the diagnostic criteria (Sterk, et al. 1993, American Thoracic Society 2000).

Exercise challenges have been compared to direct challenges of histamine and methacholine research has shown a weak but statistically significant correlation between ECT and log histamine or methacholine PC_{20} (Anderton, et al. 1979, Eggleston 1979). ECT is less sensitive, but more specific than the direct challenges in differentiating asthmatics from non-asthmatics (Eggleston 1979, Eliasson, et al. 1992). There are many asthmatics with mild bronchial hyperresponsiveness to a direct challenge who are negative to an ECT, but there are individuals who have a positive ECT and a negative direct test (Holzer, Anderson and Douglass 2002). The poor relationship between ECT and direct challenges are indicative of the differing mechanisms involved. In addition to laboratory based protocols on treadmills and cycle ergometers, it is relatively easy to perform a test outside where the participant experiences their symptoms and therefore may be more informative for swimmers and skiers. However, it is usually easier to have the correct inspired air conditions to provoke a response within a laboratory, and the appropriate equipment to measure the intensity of exercise by measuring ventilation rather than heart rate. The severity of the EIB response will be dependent upon the ventilation reached and sustained during the exercise protocol. Severity is related to the rate of water lost from the lower airways which is why ventilation and not heart rate is the primary determinant and why the inspired air needs to be dry. With increasing ventilation rates and dryer air there is a greater recruitment in the number of airways used in an attempt to condition the air this

increases the chances of identifying EIB (Anderson and Daviskas 2000, Anderson and Kippelen 2010).

The limitation with ECTs is that there are many other factors that can affect the response to exercise. Research highlights that prior exercise or intermittent warm up exercise can cause a protective refractory period to develop(Stickland, et al. 2012) increasing the likelihood of a false negative response. In contrast, recent exposure to inhaled or ingested allergens may increase the participant's normal response. In addition studies show there is inherent variability in the EIB response to ECTs with a 25-50% variation in the % fall of FEV_1 so a diagnosis may be missed if only one test is performed (Anderson, et al. 2010). As such, exercise is not the best indirect challenge to identify and monitor EIB.

2.4.1.2. Eucapnic Voluntary Hyperpnoea (EVH)

Eucapnic voluntary hyperpnoea is an alternative to laboratory/field based exercise challenge tests to identify EIB. The EVH test was originally developed, standardised, and validated by members of the US army (Eliasson, et al. 1992, Argyros, et al. 1995, Argyros, et al. 1996) and later adopted to identify EIB in elite athletes (Anderson, et al. 2001). A stepped protocol is used in individuals with severe and/or unstable asthma. In stable asthmatics and athletes, a standard single-step 6-minute uninterrupted EVH challenge is commonly used. This requires participants to hyperventilate a dry gas (<5% rH) at room temperature that contains ~5% carbon dioxide (to maintain eucapnia), 21% oxygen and balance of nitrogen. The target $\dot{V}_E$ is calculated as 30 x baseline forced expiratory volume in 1 second (FEV_1) and equals

approximately 85% of maximal voluntary ventilation (MVV) (Anderson, et al. 2001). This is only a target rate and most high level athletes should achieve at least 25 x FEV_1 with some asthmatics responding to a level of 21 x FEV_1 (Anderson, et al. 2001). Post EVH FEV_1 is then measured in duplicate at set time points (~5, 10, 15 and 20 minutes) to determine the percentage drop in FEV_1. A drop of 10% or more is determined as diagnostic of EIB.

EVH is recommended by the International Olympic Committee-Medical Commission for diagnosing EIB (Argyros, et al. 1996, Rundell, et al. 2004). It has been shown to be a highly diagnostic challenge for provoking bronchoconstriction in clinically recognised asthmatics that are responsive to exercise, and produces symptoms that are comparable to those associated with exercise[229]. The advantage of EVH over an ECT is that participants can often sustain a higher ventilation rate over the 6 minutes. The "square wave" nature of the ventilatory response during EVH allows the participant to rapidly achieve their target $\dot{V}_E$. The total pulmonary ventilation over the 6 min EVH period is therefore likely to be higher than that achieved during an exercise protocol where $\dot{V}_E$ increases progressively over time (Anderson, et al. 2001). EVH has been shown to have greater sensitivity than a methacholine challenge (Holley, et al. 2012), particularly in athletes and military personnel (Dickinson, McConnell and Whyte 2011, Argyros, et al. 1996); results in fewer false-negative EIB diagnoses than exercise challenges (Rundell, et al. 2004, Mannix, Manfredi and Farber 1999), and can diagnose EIB in previously undiagnosed athletes (Dickinson, McConnell and Whyte 2011, Molphy, et al. 2013).

2.4.1.3. Hypertonic saline and inhaled mannitol

In 1981 Schoeffel and colleagues demonstrated that airways of asthmatic individuals respond with bronchoconstriction following the inhalation of nonisotonic aerosols(Schoeffel, Anderson and Altounyan 1981). These findings were later confirmed, highlighting that increases in the osmolality of the airway is responsible for causing the airway narrowing in asthmatic individuals (Sheppard and Eschenbacher 1984, Eschenbacher, Boushey and Sheppard 1984). Subsequently a bronchial provocation test with a hypertonic (4.5%) saline was developed and standardised (Riedler, et al. 1994, Anderson, et al. 1995) followed by the development of Inhaled mannitol (Brannan, et al. 1998). Mannitol inhalation is an osmotic challenge that is used as an alternative surrogate test to identify EIB in clinically recognised asthmatics (Brannan, et al. 1998). The mechanism by which osmotic challenge causes airway narrowing is thought to involve both an increase in osmolality and release of mediators from mast cells and sensory nerves. *In vitro*, human lung mast cells have been shown to release histamine in response to increases in osmolality (Eggleston, Kagey-Sobotka and Lichtenstein 1987). *In vitro*, mannitol was shown to be more potent than sodium chloride at the same level of osmolality in causing a release of mast-cell histamine (Eggleston, Kagey-Sobotka and Lichtenstein 1987).

2.4.2. Direct Challenges

Many earlier studies used a direct airway challenge as a means of diagnosis. The most commonly used direct airway challenges are histamine and methacholine. The histamine challenge directly activates smooth muscle and secretory receptors, whereas the methacholine challenge acts as a non-specific cholinergic agonist to cause

bronchoconstriction. The sensitivity and/or specificity of these methods have been questioned within the literature. Holzer et al. (2002) screened 50 athletes for EIB through both methacholine and EVH challenges. The authors found only 9 (18%) of athletes were positive to methacholine, whereas 25 (50%) athletes, which included the 9 positive methacholine athletes, positive to the EVH challenge. This highlights the increased sensitivity and specificity of EVH over methacholine in diagnosing EIB. This study supports previous research suggesting that indirect challenges such as EVH are the most sensitive and specific bronchial provocation challenges (Haby, et al. 1994, Clough, et al. 1991).

2.5. Pharmacological methods to treat exercise induced bronchoconstriction

At present there is no known cure for asthma, or exercise induced bronchoconstriction, however there are a range of both pharmacological and non-pharmacological methods available to manage symptoms. Athletes commonly use pharmacologic medication to attenuate EIB with asthma medication amongst the most commonly reported medication used by elite level athletes (Helenius and Haahtela 2000). The British Thoracic Society (BTS) has a 5 step guideline (Table 2. 5) for asthma treatment in the general population (BTS 2004). There is a lack of randomised control trials for treatment for EIB however despite this the BTS currently recommends inhaled corticosteroids and inhaled β_2-agonists as the first line of treatment for individuals with EIB. The step guidelines show that, for mild EIB, short acting β_2-agonists should be used in the first instance (step 1). If symptoms persist and are not controlled then inhaled corticosteroids should be added to the treatment (step 2),

followed by long-acting β_2-agonists (step 3) if symptoms remain and are not controlled by the combination of short-acting β_2-agonists and corticosteroids.

Table 2. 5 The British Thoracic Society 5-step asthma medication guidelines (British Thoracic Society Scottish Intercollegiate Guidelines Network 2008)

Step Level	Medication
1 – Mild intermittent asthma	Inhaled short-acting β_2-agonist
2 – Introduction of regular preventative corticosteroid therapy	Corticosteroids
3 – Add on therapy	Increase current medication, Inhaled long acting β_2-agonists, theophylines, leukotriene receptor antagonists, antihistamines
4 – Poor control on moderate dose of corticosteroid and add on therapy	Add forth drug from list above
5 – Continuous or frequent use of oral corticosteroids	Oral corticosteroids

2.5.1. Inhaled β_2-Agonists

Inhaled β_2-agonists are commonly used for an acute asthmatic episode (short acting) often during or after exercise in EIB sufferers, with corticosteroids ineffective in the acute term. In addition to their use following an acute exacerbation, it is recommended that asthmatic athletes inhale short acting β2-agonists 30 minutes before a period of exercise. Ninety percent of those with EIB will have a successful reduction in EIB with the prophylactic use of inhaled β2-agonists (Anderson, et al. 1979). β2-agonist medication act by relaxing the smooth muscle so increasing airflow, decreasing vascular permeability, and moderately inhibiting mediator release (Johnson 2001).

The degree of reduction in EIB that is observed following inhalation of short acting β2-agonists has ranged from 50-100% in clinical trials in both adults and children (Anderson, et al. 1979, Boulet, Turcotte and Tennina 1989, Woolley, Anderson

and Quigley 1990). However, short acting β2-agonists are not recommended as the only source of medication for chronic asthma and EIB if they are inhaled more than 3 times a week (British Thoracic Society Scottish Intercollegiate Guidelines Network 2008).

2.5.2. Inhaled corticosteroids

Chronic asthmatics, and EIB sufferers who exercise regularly can achieve better control using inhaled corticosteroids (ICS). ICS control underlying inflammation, by reducing the number of inflammatory cells including mast cells, eosinophils and lymphocytes. ICS not only reduce bronchial hyper-reactivity, but also improve the diurnal variation that occurs in lung function in asthmatic patients. Asthmatic airways are associated with an increased number of goblet cells producing increased amounts of mucin, and a reduction in the ciliated epithelium. ICS therapy is associated with normalisation of the ciliated epithelium to goblet cell ratio, and reduces inflammatory cell infiltrate. Primarily this is a reduction in eosinophils within the lamina propria of the airways and respiratory epithelium. It is suggested that combination treatment of inhaled β2-agonsits and ICS are used for greatest treatment efficacy (Laitinen and Laitinen 1995).

2.5.3. Leukotriene Modifiers

With evidence that the cysteinyl leukotrienes are important mediators for EIB, there are a number of anti-leukotriene medications. A study in 64 asthmatic children with EIB found that those who received leukotrienes antagonist montelukast (5 $mg \cdot d^{-1}$) for 8 weeks showed significant improvements in symptoms and maximum percent fall

in FEV_1 following exercise when compared to the control group. Short-term acute doses of montelukast have also been used in treating EIB. Six hours prior to either an exercise or EVH challenge participants ingested 10mg of montelukast or placebo (Rundell, et al. 2005). Reductions in FEV_1 after the bronchial provocation challenges where shown to be significantly less following montelukast when compared to placebo ($p < 0.05$), and was effective in up to 59% of the asthmatic participants (Rundell, et al. 2005).

2.5.4. Side effects of pharmacological treatment of exercise induced bronchoconstriction

Inhaled corticosteroids (ICS) are the mainstay of asthma treatment, and have been used in ever increasing dosages. However, there is evidence that long term use of inhaled corticosteroids can result in reduced bone mineral density (BMD). Thirty seven asthmatics who had taken ICS for 18 months where shown to have reduced urinary phosphorus levels and serum osteocalcin, although BMD was unchanged compared to control population (Boulet, et al. 1994). Others have shown significant reductions in BMD, bone turnover markers, and adrenal steroid hormones (Hanania, et al. 1995, Wong, et al. 2000).

Chronic use of β2-agonists can also cause systemic side effects such as increased airway hyper responsiveness along with degenerative changes to the respiratory system (Dahl 2006, Anderson, et al. 2006). Daily use of β2-agonists can increase hyper responsiveness within the airways and contribute to the development of EIB, plus a greater frequency of exacerbations. In cross country skiers, regarded as an

extreme subset of EIB sufferers, research has shown no beneficial effect of inhaled corticosteroid on symptoms, bronchial hyperresponsiveness, or airway inflammation (Sue-Chu, et al. 2000). Further, Anderson and Brannan (2004) suggested three clear adverse effects. 1) Daily use of inhaled β2-agonists can result in the development of tolerance and a reduction in the duration of their protective effect, 2) the severity of EIB can increase when exercise is performed 8-12 hours following the last inhaled dose and 3) prolonged recovery of lung function after an asthma attack. The responses occur due to desensitization of β2-receptors on mast cells making them more sensitive to changes in osmolality increasing pro-inflammatory mediator release (Simons, Gerstner and Cheang 1997). These adverse effects highlight the need and significance for research into alternatively therapeutic strategies.

The NHS currently spends £1 billion each year on asthma treatment (Asthma UK Accessed on 12.06.2013), couple this with potential complications of pharmacological interventions and the continued scrutiny of their use from governing bodies in elite sport, it provides a strong rationale to establish novel ways to treat EIB in both athletes and the general public. One potential area for therapy is the use of dietary manipulation to aid in the management of EIB.

In summary, although the majority of asthma patients' symptoms are well controlled with β_2-agonsits and inhaled corticosteroids, over 50% of asthmatics exhibit poor control largely down to poor adherence (Barnes 2010). These pharmacological interventions, specifically β_2-agonists, are primarily aimed at relieving symptoms rather than treatment of the disease. These limitations of the current treatments for asthma and EIB highlight the need for novel therapeutic interventions that target the underlying

allergy, inflammation, and immune responses to increase the disease control and have greater clinical effect.

2.6. Dietary methods to treat exercise induced bronchoconstriction

There are a number of non-pharmacological methods that could be implemented in the management of EIB; these include altering the training environment, warming up for a refractory period, breathing exercise, and inspiratory muscle training. However, the focus of the present thesis is on the use of nutritional interventions to aid in the management of EIB. Research suggests that increased prevalence of atopy and asthma have been preceded by parallel changes in western diets (Devereux and Seaton 2005). For example it has been suggested that reduced consumption of fruit and vegetables has left the western diet increasingly deficient of antioxidants. A proposed mechanism relates dietary antioxidant intake to reduced lung antioxidant defences so increasing airway susceptibility to oxidant damage resulting in airway inflammation and asthma (Devereux and Seaton 2005). In addition, western diets have seen changes in dietary fat consumption paralleled with increased levels of asthma and atopy. Specifically, there has been a fall in the consumption of saturated fat and an increase in the amount of omega-6 polyunsaturated fatty acids (principally linoleic acid) from vegetable oils and margarine, coupled with a reduction in consumption of omega-3 polyunsaturated fatty acids from fatty fish (Black and Sharpe 1997).

Previous research highlights the effectiveness and possibilities of a number of dietary manipulation strategies. Dietary salt intake has increased in western and developing societies and there is evidence to suggest a causal relationship between salt intake and asthma severity. A review of the available data showed that adopting a low

sodium diet for 2-5 weeks can lead to an improvement in lung function and reduced bronchial reactivity (Mickleborough and Fogarty 2006). In contrast, sodium loading had the opposite effects (Mickleborough, Lindley and Ray 2005, Mickleborough and Fogarty 2006). The precise mechanism by which dietary salt may influence EIB is unknown. In asthma, sodium transport across the smooth muscle cells has been implicated in the regulation of airway smooth muscle tone (Tribe, et al. 1994) furthermore increased dietary salt intake may increase bronchial reactivity thereby increasing the likelihood of exacerbations when asthmatics are exposed to triggers (Burney, et al. 1989). Tribe et al (1994) suggested that a serum-borne factor found in asthmatics caused an increase in cell membrane permeability stimulating sodium influx into cells; consequently, dietary sodium intake would further increase this influx. The increased sodium influx may alter the intracellular calcium concentration in murine mast cells increasing smooth muscle contractility and inflammatory mediator release (Cabado, Vieytes and Botana 1998, Franzius, Hoth and Penner 1994) .

A number of studies suggest a link between increased maternal intakes of vitamin D and reduced risk of childhood asthma (Devereux, et al. 2007, Camargo, et al. 2007, Erkkola, et al. 2009). Children with reduced serum vitamin D in Costa Rica had increased markers of allergy and asthma (Brehm, et al. 2009). As deficiency leads to reduced levels of antimicrobial peptide (Ginde, Mansbach and Camargo 2009), which is important in host defence, this could link vitamin D deficiency to increased risk of respiratory tract infections. Increased respiratory tract infections are associated with wheeze in children and increased asthma exacerbations in adults (Ginde, Mansbach and Camargo 2009).

Furthermore, asthmatic children with a positive response to an exercise challenge (%ΔFEV_1 >10%) presented with lower serum levels of 25-hydroxyvitamin D compared to children who did not respond to exercise (Chinellato, et al. 2011). The precise mechanism linking vitamin D deficiency to increased severity of EIB is unknown. *In vitro* animal studies suggest that vitamin D deficiency is associated with increased mast cells in connective tissue. In addition 1,25-dihydroxyvitamin D3 (an active vitamin D metabolite) promotes apoptosis and inhibits bone marrow derived mast cell precursors and histamine release from mast cells. These *in vitro* studies suggest a role for vitamin D in moderating mast cell derived inflammatory mediators. Recently, 1,25-dihydroxyvitamin D3 has been shown to be a potent regulator of immune response in Th1-cell driven diseases and has a role in controlling transcription factors and co-regulatory molecules to suppress gene transcription in lung inflammation (Sundar and Rahman 2011) potentially enhancing immunoregulation in asthma.

Similarly, vitamin C at a dose of 1500 $mg \cdot day^{-1}$ for two weeks has been shown to significantly reduce maximum fall in post exercise FEV_1 to non-diagnostic levels in eight adult asthmatics, along with improved symptom scores, and reduced inflammatory markers in urine (Tecklenburg, et al. 2007). Reductions in urinary inflammatory prostaglandin and leukotriene levels following the vitamin C intervention again suggested a role for suppressed mast cell activation.

This thesis focuses on two other dietary components which may have therapeutic potential for asthma. A number of studies highlight that modulating the ratio between dietary omega 6 and omega 3 fatty acids can result in an improvement in asthma symptoms (Oddy, et al. 2004) and reduced inflammatory markers (Hodge, et al. 1998). In addition, a novel dietary approach may be to manipulate the gut microflora

though feeding of prebiotic carbohydrate. Prebiotic carbohydrate has the potential to modify the gut microflora which may impact on the systemic inflammation associated with asthma and EIB.

2.6.1. Omega 3 polyunsaturated fatty acids (ω3-PUFA)

Omega 3 polyunsaturated fatty acids (ω3-PUFA) are generally termed essential fatty acids and must be supplied through the diet. The term ω3 is a structural descriptor for a family of PUFA, and it describes the position of the first double bond that is closest to the methyl terminus of the acyl chain. In all ω3-PUFA, this first double bond is on carbon 3, and this differs to ω6-PUFA where the first double bond is on carbon 6 (Figure 2. 15). The simplest ω3-PUFA is alpha-linolenic acid [18:3(ω-3)] and it is synthesized from linoleic acid [18:2(ω-6)] catalysed by Δ15-desaturase. However, humans do not possess the enzyme Δ15-desaturase and so cannot synthesise alpha-linolenic acid, thus it is classed as an essential fatty acid. Humans can however metabolize alpha-linolenic acid into other longer chain, more unsaturated ω3-PUFAs such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:5) (Figure 2. 16). The conversion rate of long chain ω3-PUFA (EPA and DHA) from alpha-linolenic acid is ineffective and therefore intervention studies commonly supplement directly with EPA and DHA (Galli and Calder 2009).

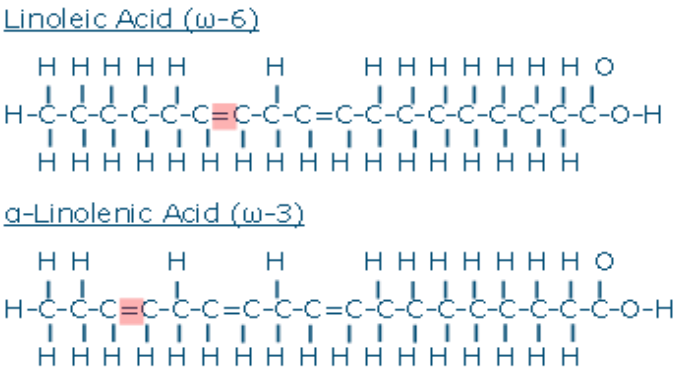

Figure 2. 15 Structure of ω-6 and ω-3 PUFA. Highlight shows location of first double bond

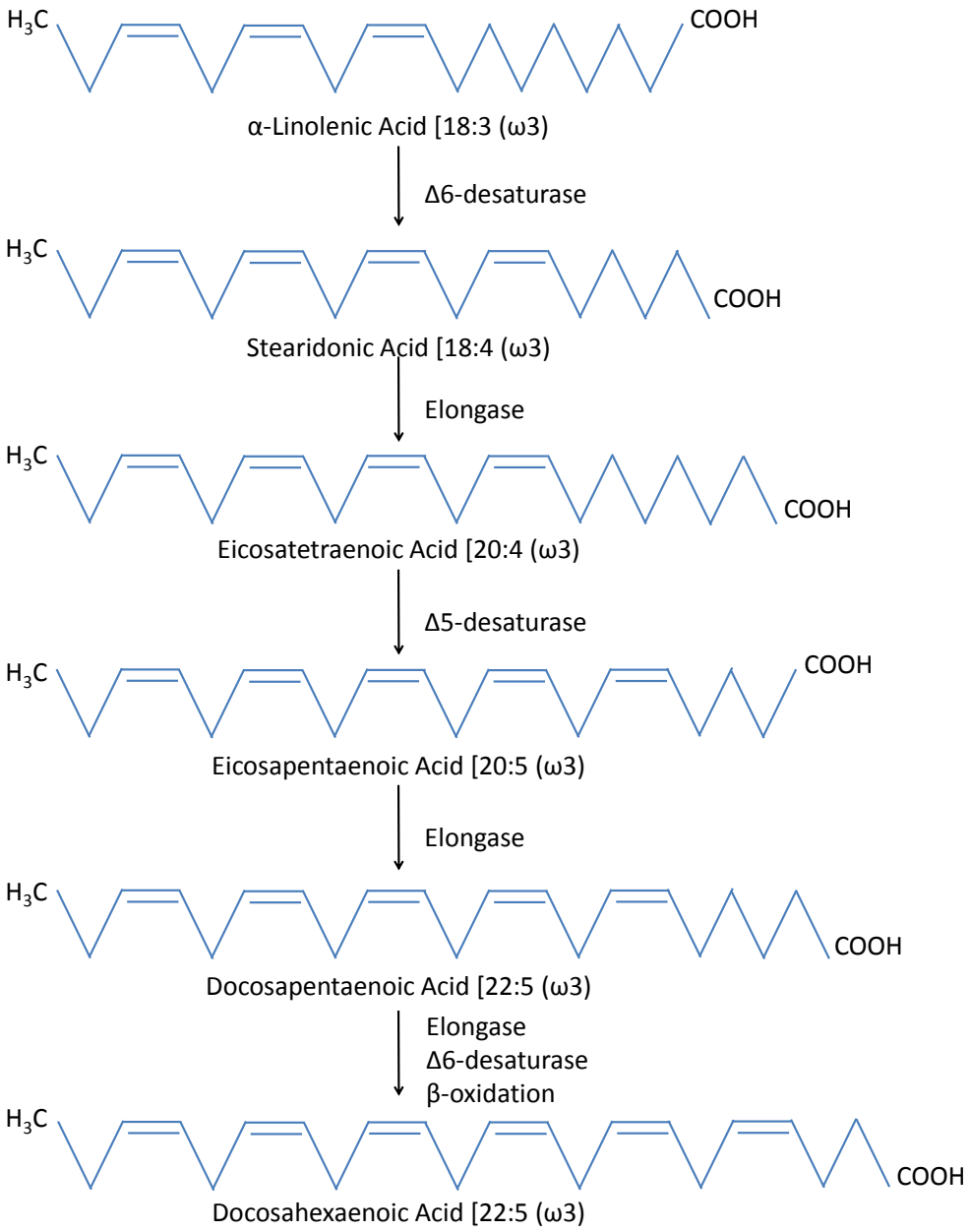

Figure 2. 16 The biosynthesis of the ω3 PUFA family from α-linolenic acid and the formation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

2.6.1.1. Dietary sources and typical intakes of ω3-PUFA

α-Linolenic acid is a common component of green leaves, comprising over 50% of their fatty acids, but as green leaves are low in fat they are not usually a major dietary source of α-linolenic acid (Calder 2012). Linseeds (flaxseeds) and their oil typically contain 45-55% of fatty acid in the form of α-linolenic acid, whereas soybean,

rapeseed, and walnuts typically contain ~10% α-linolenic acid (Simopoulos 2002a). There is very little α-linolenic acid in corn, sunflower, or safflower oil all of which are high in linoleic acid (ω6-PUFA) and are key contributors to the rise of ω6-PUFA intake in western diets (Simopoulos 2002a). ω3-PUFA consumption among Western populations is low, and estimated to be ~0.15-0.25 $g \cdot d^{-1}$ (Calder 2012), whereas ω6-PUFA is the main PUFA typically consumed in 5 to 20 fold greater amounts than α-linolenic acid which can have implications in health and disease (Burdge and Calder 2006, British Nutrition Foundation 2000, Blasbalg, et al. 2011).

Fish and other seafood are much better sources of very long ω3-PUFA than green leafy vegetables (British Nutrition Foundation 2000). Lean fish, like cod or haddock, store lipids in their liver whereas oily fish like salmon, tuna, and sardines store lipids in their flesh/muscle in higher quantities, although it is important to note that different types of fish contain varying amounts of ω3-PUFA with differing ratios of EPA:DHA. This is because the ω3-PUFA content and type is influenced by the metabolic characteristics of the fish, their diet, the temperature of the water in which they live, and the season (Calder 2012). A single serving of a lean fish could typically provide 0.2-0.3 g ω3-PUFA, whereas a single oily fish portion could provide 1.5-3.5 g of ω3-PUFA (Calder 2012). ω-3 PUFA, specifically the biologically active EPA and DHA, possess the most immunomodulatory action, and are more biologically potent that α-linolenic acid (Simopoulos 2002b). The ω6 and ω3 PUFAs are metabolically distinct, and have opposing physiological functions, as such the increased ω6:ω3 ratio in Western diets likely contributes to an increased incidence of disease and inflammatory disorders such as asthma (Simopoulos 2002b).

2.6.1.2. Anti-inflammatory actions of ω3-PUFA

Polyunsaturated fatty acids (PUFAs) are important constituents of the phospholipids of all cell membranes. In animal studies, increasing the dietary intake of ω3 EPA and DHA results in higher concentrations of these fatty acids in inflammatory cells including lymphocytes, macrophages, and neutrophils (Yaqoob, Newsholme and Calder 1995, Brouard and Pascaud 1990, Palombo, et al. 1999). Typically the increase in EPA and DHA in cell membranes is at the expense of ω6 arachidonic acid (Calder 2010). The anti-inflammatory actions of ω3-PUFA are likely to be multifactoral, and due to this complexity, it has made elucidating the exact actions of PUFAs within inflammatory processes difficult (Calder 2010). This difficulty has been further compounded by the variety of experimental approaches that have been used, including the method of presentation of PUFAs of interest to inflammatory cells in order to study their effects. For example, many *in vitro* studies have exposed cells to non-esterified fatty acids, often at concentrations that might not be achieved physiologically (Calder 2010). Despite these difficulties there is a wealth of evidence that the consumption of oily fish or fish oils can have a multitude of anti-inflammatory effects (Simopoulos 2002a) (Table 2. 6). Many experimental studies have provided evidence that incorporating ω3-PUFAs into tissues may modify the inflammatory and immune reactions meaning that they have the potential to be a therapeutic agent for inflammatory diseases.

The most commonly reported anti-inflammatory action of ω3-PUFA within the literature is their influence on the lipid derived mediators eicosanoids (section 2.2.5). Relatively high amounts of ω6 arachidonic acid are present in the phospholipid membranes of inflammatory cells and this fatty acid is the major precursor for

eicosanoids which are produced in greatly increased amounts upon cellular stimulation (Calder 2010). In general, the eicosanoids produced from arachidonic acid act in a pro-inflammatory way, although this is a slight over-simplification as it is now recognised that arachidonic acid derived eicosanoid PGE_2 can be both pro- and anti-inflammatory (Levy, et al. 2007, Vachier, et al. 2002). Increasing dietary intake of EPA and DHA can decrease the production of pro-inflammatory arachidonic acid derived eicosanoids cysteinyl leukotrienes, and prostaglandins. Like arachidonic acid, EPA is a substrate for the cyclooxygenase and lipoxygenase enzymatic pathways; as such, increases in EPA within the phospholipid membranes of inflammatory cells can competitively inhibit the use of arachidonic acid as a substrate for eicosanoid production. The EPA derived mediators are much less biologically active than those produced from arachidonic acid. For example, EPA-derived LTB_5 is 10- to 100-fold less potent as a neutrophil chemoattractant compared with LTB_4 (Lee, et al. 1984). Supplementing the diet with high dosages of ω3-PUFA (3.2 g EPA and 2.2 g DHA, per day) in normal subjects can increase the EPA content in neutrophils and monocytes more than sevenfold (Lee, et al. 1985). The anti-inflammatory effects of fish oils are partly mediated by inhibiting the 5-lipoxygenase pathway in neutrophils and monocytes and inhibiting the leukotriene B_4 (LTB_4)-mediated function of LTB_5 (Lee, et al. 1985). ω3-PUFAs influence cytokine production specifically interleukin metabolism such as TNF-α, IL-6 and IL-1β (Endres, et al. 1989, Khalfoun, et al. 1996, Kremer, Lawrence and Jubiz 1989). With leukotrienes and prostaglandins produced from the metabolism of arachidonic acid, and pro-inflammatory cytokines implicated in the pathogenesis of asthma and EIB, the ratio of ω6:ω3 may be an important determining factor in the severity of symptoms and the disease.

As described in section 2.2.5.3 EPA and DHA also give rise to resolvins and protectin compounds through the cyclooxygenase and lipoxygenase pathways. These mediators have been demonstrated in cell culture and animal feeding studies to be anti-inflammatory and inflammation resolving (Calder 2010). Resolvin E1, D1, and protectin D1 have been shown to prevent neutrophilic infiltration at sites of inflammation, and protectin D1 inhibits TNF-α and IL-1β production. As such, the role of resolvins and protectins from ω3-PUFA may be important in the resolution of inflammation and stopping ongoing inflammatory processes to limit tissue damage (Calder 2012, Calder 2010).

Table 2. 6 Effects of omega-3 PUFAs on factors involved in the pathophysiology of inflammation, recreated from (Simopoulos 2002a, Simopoulos 2002b).

Factor	**Function**	**Effects of ω3-PUFA**
Arachidonic Acid	Eicosanoid precursor, aggregates platelets, stimulates leukocytes	Decreases
Thromboxane	Platelet aggregation, vasoconstriction, increase of intracellular Ca^{++}	Decreases
Prostacyclin ($PGI_{2/2}$)	Prevents platelet aggregation, vasodilation, increase CAMP	Increases
Leukotriene (LTB_4)	Neutrophil chemoattractant, increases intracellular Ca^{++}	Decreases
Fibrinogen	A member of the acute phase response, blood clotting factor	Decreases
Tissue plasminogen factor	Increases endogenous fibrinolysis	Increases
Platelet activating factor (PAF)	Activates platelets and leukocytes	Decreases
PDGF	Chemoattractant	Decreases
Oxygen free radicals	Cellular damage, enhances LDL uptake by macrophages, stimulates arachidonic acid metabolism	Decreases
Lipid hydro peroxides	Stimulates eicosanoid formation	Decreases
IL-1 and TNF	Stimulate neutrophil O_2 free radical formation, stimulate lymphocyte proliferation, express intracellular adhesion molecule-1 on endothelial cells, inhibit plasminogen activator	Decreases
IL-6	Stimulates the synthesis of all acute phase proteins involved in the inflammatory response: C reactive protein, serum amyloid A, fibrinogen, α1-chymotrypsin, and heptoglobin	Decreases

2.6.1.3. Importance of the ω3:ω6-PUFA ratio in health

Mammalian cells lack the enzyme ω3-desaturase so subsequently cannot convert ω6 to ω3 fatty acids. The balance of essential fatty acids, and thus the ratio of ω6:ω3 is important for good health and normal development. When humans consume fish or fish oil, the quantities of EPA and DHA partly replace the ω6-PUFA, primarily arachidonic acid, in the membranes of almost all cells, especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells (Simopoulos 1994). As described in section 2.2.5, arachidonic acid derived from ω6-PUFA, and EPA, DHA derived from ω3-PUFA are the parent compounds for the production of eicosanoids.

With the increased intake of ω6 PUFA in western diets, the eicosanoids derived from arachidonic acid, specifically prostaglandins, thromboxanes, and leukotrienes, are formed in larger quantities than those formed from EPA and DHA. Only small quantities of arachidonic acid are required to produce highly biologically active eicosanoids and if they are formed in large amounts they contribute to the formation of allergic and inflammatory disorders particularly in susceptible people (Simopoulos 2002a, Simopoulos 2002b). Therefore, modern Western diets rich in ω6 PUFA can shift homeostasis to one of a greater pro-inflammatory state.

There is a lack of understanding into the optimal therapeutic dose or ratio of ω6:ω3 with recommendations varying from 1:1, to 4:1 depending on the disease which is expected due to the multifactorial nature of many Western chronic diseases (Simopoulos 2002a). The research highlights that altering the ratio in favour of ω3 can decrease drug dose in chronic inflammation (James and Cleland 1997), and reduce levels of pro-inflammatory mediators associated with airway inflammatory (Broughton, et al. 1997, Mickleborough 2005). The research supports the rationale that the

manipulation of the ω6:ω3 ratio through ω3-PUFA feeding maybe a therapeutic option for asthma, EIB and airway inflammation.

2.6.1.4. ω3-PUFA as a therapeutic intervention in asthma

In western diets 20-25 fold more ω6-PUFA are typically consumed than ω3-PUFA which can result in a greater liberation of pro-inflammatory arachidonic acid metabolites (Simopoulos 2002a). It has been previously hypothesized that the low prevalence of lung disease among Eskimos is a result of their diet high in ω3-PUFA (Horrobin and Manku 1987). This was attributed to the anti-inflammatory role of ω3-PUFA in reducing leukotriene generation. Observational research highlights a relationship between fish intake and decreased risk of asthma (Wong 2005). Despite this, the results from intervention studies have yielded conflicting data.

Significant improvements in daytime wheezing, reductions in concentration of exhaled hydrogen peroxide (marker of airway inflammation), and increase in peak expiratory flow were observed in mild adult asthmatics following 2 months of 200mg·d^{-1} of an EPA/DHA mix when compared to a placebo (150 mg·d^{-1} of olive oil) (Emelyanov, et al. 2002). However no significant changes in FEV_1 were noted which may be attributed to mild level of asthma exhibited in the participants, and the relatively low daily dose of EPA/DHA. Furthermore the improvements that did occur could not be solely attributed to EPA and DHA as the supplements also contained carotenoids, which can act as antioxidants. As asthma is regarded as a long-term irreversible condition a longitudinal ω3-PUFA supplementation study was conducted over a 12 month period by Dry and Vincent (1991). Twelve asthmatics were randomized to

receive 1000 mg·d^{-1} of an EPA/DHA supplement, or placebo under double-blind conditions. In the ω3-PUFA group FEV_1 reportedly increased by 23%, significantly greater than that seen in the placebo group ($p < 0.005$) (Dry and Vincent 1991). This improvement did not however translate to a reduction in medication usage and in addition the authors failed to report the constituents of the placebo. Both of these studies also failed to tightly control for the individuals long-term dietary intake which will likely impact on the habitual ω3:ω6 ratio.

The effects of consuming two different ω3:ω6 ratios (0.1:1 and 0.5:1) each for four weeks in an asthmatic population has been investigated by Broughton et al., (1997). Pulmonary function and urinary 5-series leukotriene excretion were used to assess treatment efficacy following a standard methacholine challenge and baseline saline challenge. The ω3:ω6 ratio of 0.1:1 resulted in a greater bronchoconstrictive response to the methacholine challenges compared to baseline, with a reduction in the provocative dose to induce a fall of ≥ 20% in FEV_1 of 89%. Increasing the ω3 PUFA intake to a ratio of 0.5:1, resulted in a reduction in drop in FEV_1 in response to the methacholine challenge compared to baseline and 9 of the 19 participants actually improved. Those showing no reduction in respiratory measures to the methacholine challenge after the period of increased ω3-PUFA intake where referred to as ω3-PUFA responders (Broughton, et al. 1997).

The lower ratio of ω3:ω6 caused an increase in pro-inflammatory 4-series leukotrienes of 13.3 ± 4.5 ng, ($p<0.05$) in all participants following the methacholine challenge. In contrast, following the higher ω3:ω6 ratio there was no increase in the 4-series leukotrienes. Comparing high and low ω6:ω3 ratios, overall 4-series leukotriene excretion were significantly lower ($p<0.05$) with the high ω3-PUFA intake compared to

the low. Leukotriene E_5 was markedly increased following the high ω3-PUFA diet, compared with baseline in both responders and non-responders although it was 230% greater in responders ($p<0.05$). Higher levels of LTE_5 are associated with reduced levels of airway inflammation. With a urinary ratio of LTE_4 to LTE_5 of <1 following the higher ω3 intake, it may help to elucidate the positive mechanisms for alleviating respiratory symptoms (Broughton, et al. 1997). The research highlights the pro-inflammatory nature of skewed ω3:ω6 PUFA in favour of ω6 PUFA can have a significant impact upon inflammation associated with asthma. As such detailed intervention studies that can increase ω3-PUFA intake are warranted in inflammatory conditions such as asthma. Chapter 5 highlights the use of ω3-PUFA as a treatment intervention in asthmatics with EIB.

2.6.2. Prebiotics

Prior to the 1990's there had been a widespread belief that while food intake may regulate certain metabolic activities associated with micro-organisms, changing the diet had little effect on the overall composition and structure of the microbial communities in the human gut (Macfarlane and Macfarlane 2003, Macfarlane, Steed and Macfarlane 2008). However, it is now recognised that the species composition of the microbiota, as well as many physiological consequences can be modified by small changes in food consumption that includes the introduction of prebiotics into the diet (Macfarlane, Steed and Macfarlane 2008).

The original definition of a prebiotic was proposed by Gibson and Roberfroid (1995) as 'a non-digestible food ingredient that beneficially affects the host by

selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves health'. More recently, this has been amended to 'a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health' (Gibson, et al. 2004).

Prebiotic oligosaccharides can be classified according to their chemical constituents and degree of polymerisation. In addition to be classified as a dietary prebiotic the ingredient must:

- Resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption;
- Be fermented by the intestinal microflora;
- Stimulate selectively the growth and/or activity of the intestinal bacteria associated with health and wellbeing (Gibson, et al. 2004).

Any food ingredient that escapes digestion in the upper gastrointestinal tract has the potential to be prebiotic, but only certain carbohydrates have shown convincing evidence of prebiotic action. A review of the literature (Macfarlane, Macfarlane and Cummings 2006) highlights that the majority of prebiotic research has focused on inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS). FOS and GOS are not sensitive to gastric acid, and do not serve as substrates for hydrolytic enzymes in the upper digestive tract; as such they are capable of stimulating the growth and activity of intestinal bacteria within the lower gut. FOS and GOS have been

assessed and achieve all of the above classification criteria (Gibson, et al. 2004, Bouhnik, et al. 2004). Bouhnik et al (2004) compared the prebiotic characteristics of a number of candidate prebiotics *in vivo*. Following 7 days of consumption, GOS showed the greatest prebiotic effect with a stronger bifidogenic effect than short chain FOS (Bouhnik, et al. 2004). Although this is a rapidly growing research area, the evidence for prebiotic status of other non-digestible carbohydrates (glucooligosaccharides, isomaltooligosaccharides, lactosucrose, polydextrose, soybean oligosaccharides, and xylooligosaccharides) offer preliminary promising data but currently are not sufficient to classify them as prebiotics according to the stated criteria (Bandyopadhyay and Mandal 2014) .

Unlike probiotics where the microbes are introduced to the gut, and actually have to compete against the established colonic communities, an advantage of prebiotics is that they target probiotic bacteria that are already commensal to the large intestine. As such, prebiotics are arguably a more practical and efficient way to manipulate the gut microflora than probiotics. Conversely, if the gut microflora are not present due to disease, ageing, antibiotics, or drug therapy the prebiotic is unlikely to be of benefit and a synbiotic (combined prebiotic and probiotic) approach may be more beneficial (Macfarlane, Steed and Macfarlane 2008).

2.6.2.1. Prebiotic – Trans-galactooligosaccharide (B-GOS)

Galactooligosaccharides are very stable carbohydrates at high temperatures and acidic environments and with a low calorific value of 1.7 $kcal \cdot g^{-1}$ it makes them of particular interest to the food industry. A number of health benefits have been

attributed to the ingestion of prebiotics mainly with the use of FOS, and less so GOS although GOS has the potential to have a greater bifidogenic effect within the gut (Bouhnik, et al. 2004).

GOS is principally formed by enzymatic treatment of lactose by β-galactosidase to produce several oligomers of different chain lengths (Prenosil, Stuker and Bourne 1987). GOS is an inclusive term referring to oligosaccharides with 2 to 6 sugars that have galactose as their main component. It can be produced from lactose in cow's milk, but the main raw material for its production is usually from whey-derived lactose. GOS is then produced by β-galactosidases that have transgalactosylation activities, which results in the formation of 4- or 6-galactosylactose, oligosaccharides, transgalactosylated disaccharides and non-reducing oligosaccharides (Figure 2. 17) (Angus, Smart and Shortt 2005).

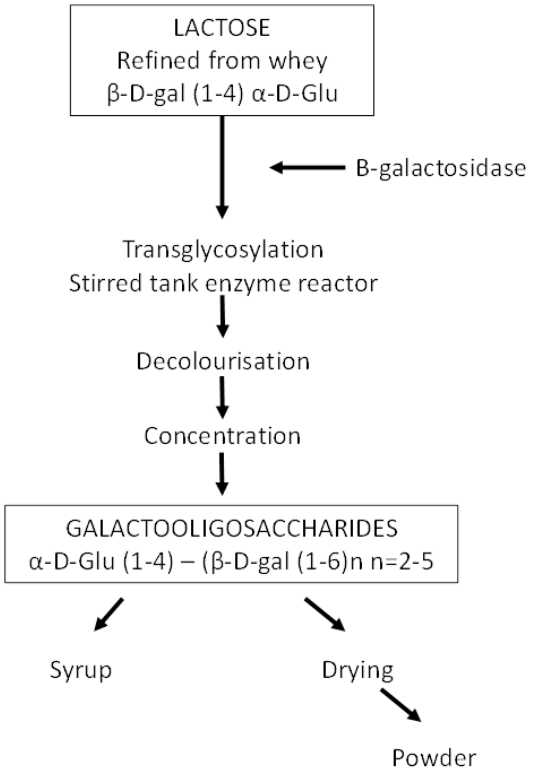

Figure 2. 17 Schematic showing production of GOS from lactose adapted from Angus, Smart and Shortt., (2005).

One way of increasing the specificity of the products and interaction with prebiotic bacteria in the gut may be by using β-GOS synthesized by bifidobacteria when manufacturing the product (Macfarlane, Steed and Macfarlane 2008). The prebiotic used in the current thesis was produced this way. A novel strain of *Bifidobacterium bifidum* NCIMB 41171 isolated from a faecal sample from a healthy human volunteer with an ability to express β-galactosidase activity was used in synthesis reactions for the production of GOS from lactose (Tzortzis, Goulas and Gibson 2005). Synthetic prebiotic mixtures produced by enzymes from initial probiotic organisms potentially confer greater selectivity on those organisms when ingested and then fermented by the colonic microflora (Depeint, et al. 2008). This product is now commercially available

as a prebiotic powder marketed as BiMUNO by Clasado and referred to as a trans-galactooligosaccharide (B-GOS).

The bifidogenic properties of B-GOS have been studied under a number of conditions. The prebiotic potential of B-GOS was initially assessed *in vitro* and in pigs *in vivo*. The *in vitro* study was a model of the gut whereby physicochemical conditions of the colon were replicated in a 3-stage fermenter representing the proximal, transverse, and distal colon (Tzortzis, et al. 2005). Following the addition of the B-GOS mixture the bifidobacterial numbers in the first two vessels representing the proximal and transverse colon increased ($p < 0.05$). In addition the study showed that the B-GOS mixture may also have potential therapeutic effects by reducing *Salmonella* and *E.coli* associated infections. Addition of B-GOS *in vivo* to the commercial diets of pigs increased the number of bifidobacteria ($p < 0.001$) and acetate concentration ($p < 0.001$) and decreased the pH ($p < 0.001$) compared to a control diet, and control diet with inulin. These data suggest that B-GOS has a greater prebiotic potential than inulin (Tzortzis, et al. 2005).

The prebiotic potential of this novel B-GOS mixture (BiMUNO) was studied in 30 healthy human volunteers. Participants were assigned to a sequence of 7 day treatments differing in the quantity of B-GOS received (0, 3.6, or $7g \cdot d^{-1}$) with a 7 day washout between treatments. Stool samples were collected before and after each intervention, and bacteria numbers were determined by fluorescent *in situ* hybridization. The novel B-GOS supplement significantly increased the bifidobacterial population ratio compared to the placebo ($p < 0.05$), and $7g \cdot d^{-1}$ was shown to significantly increase bifidobacteria numbers greater than a previous commercial GOS supplement ($p < 0.05$). Furthermore a dose dependent response between the bifidobacteria proportion and

novel B-GOS dose was observed ($p < 0.01$). This study showed that the novel B-GOS mixture had greater bifidogenic properties than previously commercially available supplements, and increased the bifidobacteria in a dose dependent response from 3.6 to 7.0 $g \cdot d^{-1}$. This study highlighted the bifidogenic properties of B-GOS in humans for the first time but the subsequent potential therapeutic impact of B-GOS on immune function and inflammation remained to be elucidated.

Consequently the effects of B-GOS on immune function and faecal microflora composition were assessed in a healthy elderly population (Vulevic, et al. 2008). Aging is associated with a reduced number of bifidobacteria within the gut and weakened immunity. Forty four elderly participants (age 69.3 ± 4.0 y) were randomly assigned to receive either a placebo or B-GOS treatment (5.5 $g \cdot d^{-1}$) for 10 weeks with a 4 week washout period between treatments under double-blind, cross-over conditions. Blood and faecal samples were collected prior to, during (5 week), and at the end of the treatment periods. The study showed that B-GOS significantly increased the numbers of beneficial bacteria, primarily bifidobacteria ($p < 0.01$) at the expense of less beneficial bacteria compared to baseline and placebo. Significant increases in phagocytosis, natural killer cell activity, and the production of anti-inflammatory cytokine IL-10, and reduction in pro-inflammatory cytokines IL-6, IL-1β, and TNF-α were observed. These data suggest that B-GOS administration in the elderly can positively alter the gut microflora and subsequently affect immune function (Vulevic, et al. 2008). The influence of B-GOS on asthma and airway inflammation remains to be elucidated. The proposed rationale for B-GOS supplementation in asthma is discussed in more detail in Chapter 6.

2.7. General Summary

Hyperpnoea through exercise or volitional breathing involving dry/cold air is an important trigger of EIB. Increased airflow will cause dehydration of the airway surface liquid, mucosal cooling, and epithelial disruption resulting in airway inflammation (Anderson and Kippelen 2005a). Airway inflammation is associated with an increase in chemokine activity with subsequently greater leukocyte migration to the site of inflammation. This results in the significant release of a number of pro-inflammatory mediators from a range of leukocytes. These include lipid derived eicosanoids from arachidonic acid metabolism (leukotrienes and prostaglandins) and a number of pro-inflammatory cytokines (TNF-α, IL-33 amongst others). These pro-inflammatory mediators are known to cause bronchial smooth muscle contraction, airway obstruction, and increased mucous production (Anderson and Daviskas 2000).

Conventional pharmacological treatment for EIB primarily involves the suppression of airway inflammation with chronic treatment involving inhaled corticosteroids and acute relief of symptoms with bronchodilators. However, these therapies do not consistently relieve symptoms in all individuals and long term use can lead to systemic side effects. Traditional treatment typically targets symptoms and therefore therapeutic strategies which target the underlying inflammation are warranted. Research has highlighted the effectiveness of high dose omega 3 polyunsaturated fatty acids (ω3-PUFA) in reducing airway obstruction and eicosanoid accumulation associated with EIB (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010) but further research is warranted to establish more achievable dose levels.

Furthermore epidemiological research has highlighted that an increase in asthma prevalence has run parallel with alterations in Western lifestyles (Bach 2002) (Hygiene hypothesis), which has recently been expanded to include alterations in the gut microbiome. Significant levels of dysbiosis of the gut microbiota have been found in asthmatic individuals and this highlights the potential that the gut microbiota may be a significant therapeutic target in the treatment of asthma and EIB (Noverr and Huffnagle 2004). Beneficial manipulation of the gut microbiota can be achieved through the feeding of pre- and probiotics and this area warrants further investigation to establish if this manipulation can have a systematic benefit for adult sufferers of asthma and EIB.

3. Chapter 3 – General Methods

3.1. Participants

Participants were recruited through adverts placed in the Nottingham Trent University, local sports clubs, leisure centres and running shops; and online postings on relevant forums (Runners World, Cycling Weekly, and TriTalk). All participants provided written informed consent for each experimental study (Appendix 3, Appendix 5, and Appendix 7) following approval from the Nottingham Trent University Human Research Ethics Committee. A self-reporting medical questionnaire (Appendix 1) confirmed participants were non-smokers, free from illness (excluding asthma), and injury, and where not taking medication other than asthma related medication detailed in section 3.1.1. All participants completed a 48 h estimated diet record prior to their first laboratory visit which was then replicated on all subsequent conditions. Participants were instructed to adhere to their habitual training programme and not undergo any dramatic changes in their training schedule throughout the duration of the studies, and were asked not to partake in strenuous exercise 48 h prior to testing days. On test days participants abstained from caffeine (8 h prior) and alcohol (24 h prior) and arrived at the laboratory at least 2 h post-prandial.

3.1.1. Participant medication requirements

Asthma and HIB diagnosis was performed through spirometery (section 3.3) and eucapnic voluntary hyperpnoea (EVH) (section 3.4). In preparation for this testing participants using asthma medication were instructed to cease their medication prior to each EVH challenge (inhaled corticosteroids and leukotriene modifiers: 4 days (American Thoracic Society 2000); inhaled long acting β2 agonists: 2 days (Cockcroft and Swystun 1997); anti-histamines: 2 days; inhaled short acting β2 agonists: the day of

the test (Ahrens, et al. 1984, Greenspon and Morrissey 1986); and were tested at least 2 weeks following recovery from any chest or upper respiratory tract infection (Dickinson, McConnell and Whyte 2011, Anderson, et al. 2001).

3.2. Anthropometric Measurements

Body mass was measured using a calibrated electronic scale (SECA 877 Scale, SECA, Birmingham, UK) to the nearest 0.1 kg, whilst participants wore lightweight clothing and were barefoot (Roger, Eston and Reilly 2009). Height was measured with a portable stadiometer (SECA stadiometer, SECA, Birmingham, UK) with participants standing barefoot, with heels together, looking straight ahead, and arms relaxed by their sides (Roger, Eston and Reilly 2009).

3.3. Pulmonary Function – Dynamic Spirometery

Pulmonary function was conducted in accordance with ERS guidelines (Miller, et al. 2005, Wanger, et al. 2005) using a pneumotrac spirometer (Vitalograph, Buckinghamshire, UK) calibrated with a 3 L syringe. Participants performed lung function manoeuvres standing upright and wearing a nose clip. Measures were taken from three flow-volume loops, to determine forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1), with the two highest values being within 0.100 L of each other, and the highest taken for subsequent analysis (Miller, et al. 2005). Baseline FEV_1 had to be within 65% of predicted based on normative predictive equations (Knudson, et al. 1983), for participants to undergo the EVH challenge (Argyros, et al. 1996). Following the EVH challenge two reproducible flow-volume loops were measured, with the highest acceptable value recorded at each interval.

3.4. Bronchial Provocation Test – Eucapnic Voluntary Hyperpnoea

The EVH challenge is used as alternative for exercise challenges to identify exercise induced bronchoconstriction in athletes and recreationally active individuals (Anderson, et al. 2001). The bronchoconstriction associated with EVH is commonly termed hyperpnoea induced bronchoconstriction (HIB). EVH is the method of bronchoprovocation used within this series of studies subsequently the term HIB will be used for the experimental chapters' 4, 5, and 6.

The EVH challenge required participants to maintain a target $\dot{V}_E$ of 30 x baseline FEV_1 for 6 min, equivalent to 85% of an individual's maximal voluntary ventilation (Argyros, et al. 1996, Hurwitz, et al. 1995) (Figure 3. 1). Participants breathed through a flanged mouthpiece (Series 9060, Hans Rudolph, Missouri, USA) connected to a flow sensor (ZAN variable orifice pneumotach; Nspire Health, Oberthulba, Germany) that was calibrated using a 3 L syringe. Gas concentrations were measured using fast responding laser diode absorption spectroscopy sensors, which were calibrated using gases of known (5% CO_2, 15% O_2, balance N_2) concentration (BOC, Guilford, UK), and ventilatory and pulmonary gas exchange variables were determined breath-by-breath (ZAN 600USB; Nspire Health, Oberthulba, Germany). A two-way non-rebreathing valve (2700 Series, Hans Rudolph) was connected distally to the flow sensor and the inspiratory port was connected via a 1.2 m length of corrugated tubing (internal diameter = 35 mm) to a 150 L capacity Douglas bag. Participants inspired from the Douglas bag, which was continuously filled with gases of known concentration (21% O_2, 5% CO_2, balance N_2). The inspired gas was at room temperature (19-21°C) and of low humidity (<3%). Participants received real-time

visual feedback of their $\dot{V}_E$ throughout EVH and continuous monitoring of end-tidal CO_2 confirmed that eucapnia (end-tidal CO_2 between 35-45 mmHg) was maintained. Following the EVH challenge participants remained in the laboratory until their FEV_1 was within 10% of their baseline measurement. The test-retest reproducibility of the bronchoconstrictive response to EVH in asthmatic individuals was assessed.

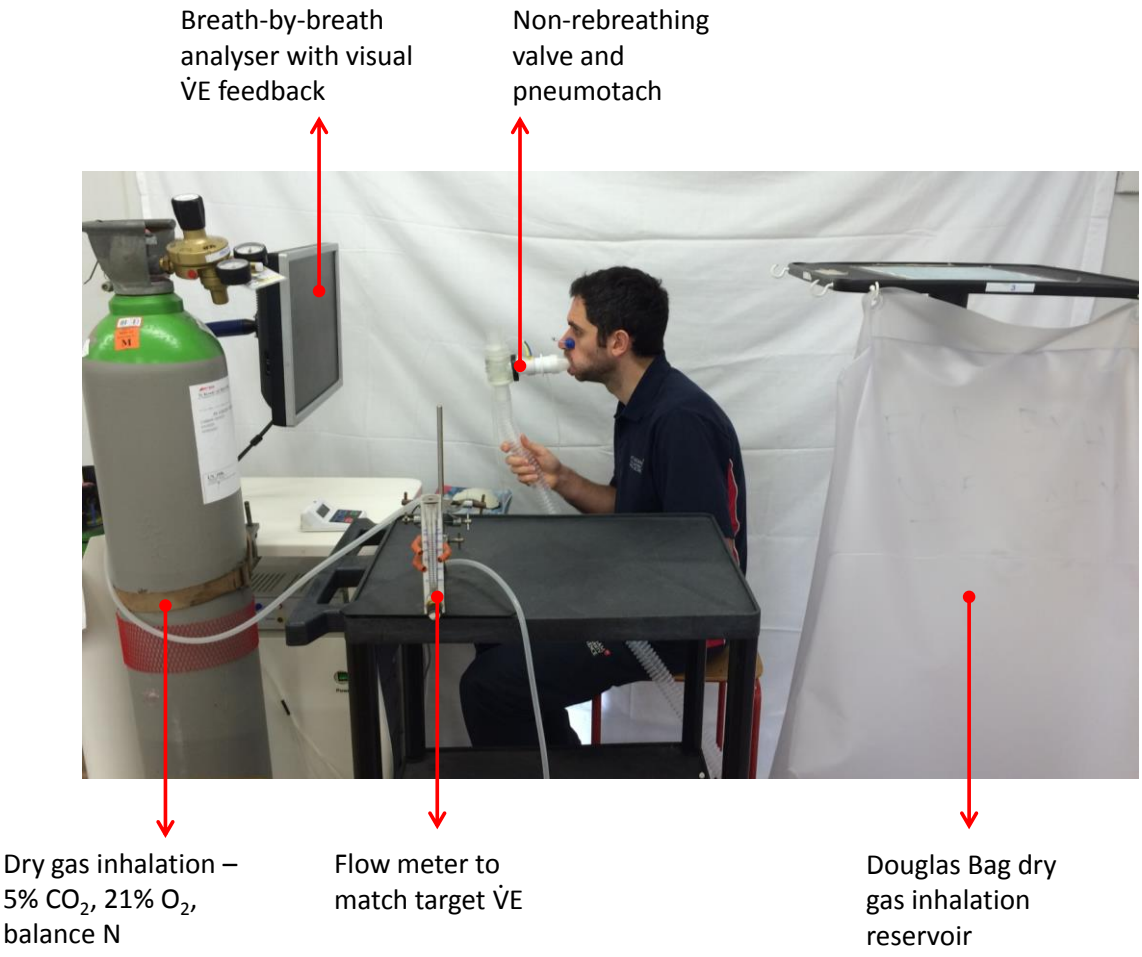

Figure 3. 1 Eucapnic voluntary hyperpnoea testing set up.

3.5. Fraction of Exhaled Nitric Oxide (F_ENO) – Aerocrine NIOX Mino

Measurement of fractional nitric oxide in exhaled breath (F_ENO) is a simple non-invasive method of quantifying airway inflammation, and provides a complementary

measure to the assessment of asthma (Dweik et al. 2011) and was conducted for the studies in Chapter 5 and 6. F_ENO was measured at baseline prior to the EVH challenge on all visits to the laboratory in the asthmatic participants only using a handheld monitor (Niox MINO, Aerocrine, Sweden). Participants were instructed to inhale through the machine to maximum inhalation, and then an exhalation of 50 $mL \cdot s^{-1}$ was maintained for 6 seconds using a visual target (Dweik, et al. 2011). Previous research has established the reliability of the handheld Niox MINO comparing it to the standard stationary chemiluminescence unit (Khalili, Boggs and Bahna 2007). One hundred and ten patients (6-86 years) completed three exhalations with the standard unit and NIOX MINO. Intrasubject F_ENO levels showed no significant difference between the measurements ($p > 0.05$); with a strong correlation between F_ENO measurements by the standard unit and the NIOX MINO ($r = 0.98, p < 0.0001$). The intrasubject F_ENO difference between the standard and the MINO was -0.5 ppb, which was not statistically significant ($p > 0.01$) (Khalili, Boggs and Bahna 2007).

3.6. Blood sampling

Venous blood was drawn from a vein in the antecubital fossa region of the forearm, via a 21 gauge needle (BD value-set, BD, Plymouth, UK). Chapter 5 required the collection of 20 mL drawn into plain syringes (no anticoagulant) of whole blood prior to the EVH testing protocol for the determination of neutrophil fatty acid phospholipid profiles. Chapter 6 required the collection of 20 mL of whole blood prior to EVH and at 15 min, 60 min, and 24 h post EVH for the determination of cytokine and chemokine concentrations. Upon completion of each blood sample, the needle was removed and medical tissue was applied under firm pressure to avoid any superficial haematoma.

3.7. Enzyme linked immunosorbant assay (ELISA)

The ELISA has become the 'gold standard' in measuring systemic cytokine and eicosanoid concentrations from human serum, plasma or urine. ELISA is a biochemical technique that detects the presence of an antigen (cytokine or eicosanoid) in a sample (Figure 3. 2). The technique of running an ELISA is simple and reliable, and with the development of many commercially available kits it allows for the measurement of many systemic cytokines and eicosanoids. ELISAs were used for the determination of urinary eicosanoid 9α, 11β-PGF_2 and serum cytokine TNF-α, and serum CRP and IgE in chapters 5 and 6.

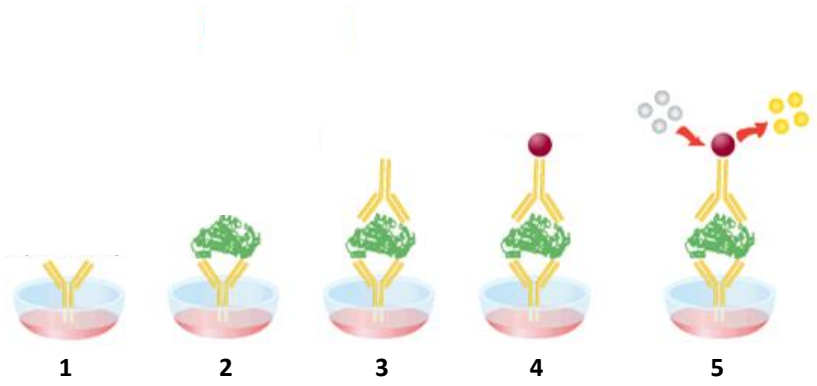

Figure 3. 2 Sandwich enzyme linked immunosorbant assay (ELISA). 1) Surface is prepared to which a known quantity of capture antibody (specific for metabolite being measured) is bound; 2) the antigen containing sample (e.g., serum) us added to the plate; 3) a specific antibody is added and binds to the antigen (sandwich); 4) enzyme-linked secondary antibodies are applied; and 5) a chemical is added to be converted by the enzyme into a colour of fluorescent signal and the absorbency signal of the plate wells is measured to determine the quantity of the metabolite.

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4. Chapter 4 – Reproducibility of the bronchoconstrictive response to eucapnic voluntary hyperpnoea

4.1. Introduction

Asthma is a significant public health issue and affects a large proportion of the population (Section 2.1.1). It's estimated that up to 90% of individuals with asthma and 10 to 50% of a given elite athlete population can experience EIB (Naguwa, Afrasiabi and Chang 2012), and asthma is the most common chronic medical condition reported among Olympic athletes (prevalence of 7-8%) (Kippelen, et al. 2012). Exercise induced bronchoconstriction (EIB) is a phenotype of asthma that is characterized by a transient airway narrowing during or on the cessation of exercise (McFadden Jr and Gilbert 1994, Anderson and Holzer 2000, Suman, et al. 1999), and it is generally accepted that airway drying and changes in airway osmolality trigger the response (Rundell and Jenkinson 2002). For more details on the pathophysiology of EIB see section 2.2. Whilst EIB is prevalent amongst physically active asthmatics and competitive sportsmen and women, the risk of acute bronchoconstriction in those undertaking exercise can be reduced through early detection and suitable treatment along with reproducible monitoring of the condition (Carlsen, et al. 2008).

Despite EIB's high prevalence rates there still remains a significant problem with diagnosis. The largest issue is forming an accurate diagnosis of EIB from exercise induced respiratory symptoms without any confirmatory objective testing. EIB typically presents itself with a variety of nonspecific symptoms such as, shortness of breath, wheeze, and tightness in the chest. In athletes, EIB was historically assessed by self-reported symptoms and clinical history (Larsson, et al. 1993, Heir and Oseid 1994). The lack of specificity and sensitivity of symptoms make diagnosis using this information alone extremely inaccurate

and can result in either false positive or false negative results (Rundell, et al. 2001a, Holzer, Anderson and Douglass 2002, Parsons 2009). Previous research has shown that only 35% of participants who presented with suggestive symptoms of EIB actually had objectively diagnosed EIB using pulmonary function and bronchial provocation challenge testing (Parsons, et al. 2007). Similarly it has been documented that 45% of athletes who complain of respiratory symptoms during exercise were found to be EIB negative after bronchial provocation testing (Rundell, et al. 2001a). These data highlight the need for confirmatory objective testing to diagnose EIB; however there is no gold-standard bronchoprovocation test. A number of bronchoprovocation methods are available, and this in part may explain why so many individuals are diagnosed without any objective testing (Parsons 2009).

Both direct and indirect challenge tests are employed as objective testing (section 2.4). Direct challenge tests involve the use of inhaled pharmacological agents such as histamine and methacholine to act directly on airway smooth muscle receptors to induce a bronchoconstriction. Methacholine is widely used as a screening test for asthma, but its value for diagnosing EIB is limited. Methacholine challenge tests have been shown to have low sensitivity for confirming EIB in athletes (Holzer, Anderson and Douglass 2002). These direct challenge tests asses for nonspecific airway hyperresponsiveness, and are not specific for the mechanisms that trigger EIB (Parsons 2009). Indirect bronchial provocation challenge tests that mimic the breathing rates and mechanisms associated with EIB may have greater sensitivity to diagnosing EIB in physically active asthmatics (Weiler, et al. 2007); these include exercise tests and eucapnic voluntary hyperpnoea (EVH). EVH is an

alternative to laboratory and field based exercise challenge tests (section 2.4.1.1). It was originally developed, standardised, and validated by members of the US army (Eliasson, et al. 1992, Argyros, et al. 1995, Argyros, et al. 1996) and later adopted to identify EIB in elite athletes (Anderson, et al. 2001).

The EVH challenge is recommended by the International Olympic Committee-Medical Commission to diagnose EIB in elite athletes (Weiler, et al. 2007, Fitch, et al. 2008) to allow for certification for the use of World Anti-Doping Agency prohibited asthma medication (World Anti-Doping Agency). An EVH challenge test comprises 6 minutes voluntary hyperpnoea breathing a dry gas (5% CO_2, 21% O_2, balance N_2). This hyperpnoea mimics the breathing rates associated with intense aerobic exercise, and the hyperpnoea induced bronchoconstriction (HIB) results from a similar pathogenesis as EIB (Anderson, et al. 2001). Following EVH, a reduction in forced expiratory volume in 1 s (FEV_1) of $\geq$10% is commonly accepted as a diagnosis of EIB/HIB (Anderson, et al. 2001). EVH has been shown to have greater sensitivity than other objective tests such as the methacholine challenge (Holley, et al. 2012) particularly in athletes and military personnel (Dickinson, McConnell and Whyte 2011, Argyros, et al. 1996), and results in fewer false-negative EIB diagnoses compared to exercise challenges (Rundell, et al. 2004, Mannix, Manfredi and Farber 1999). Furthermore, EVH can diagnose EIB in previously undiagnosed and asymptomatic athletes and is the recommended challenge of choice in elite athletes (Dickinson, McConnell and Whyte 2011, Molphy, et al. 2013, Dickinson, et al. 2006).

The target ventilation for the 6 minute EVH challenge is established from baseline pulmonary function, and set as 30 x FEV_1. Target minute ventilation ($\dot{V}_E$) of 30 x FEV_1 is maintained for 6 min and has been shown to have high EIB diagnostic sensitivity (Argyros, et al. 1996, Hurwitz, et al. 1995). However, the duration and target minute ventilation ($\dot{V}_E$) of the EVH challenge can directly impact on the bronchoconstrictive response (Argyros, et al. 1996) and, therefore, should be closely controlled. Factors that may influence pulmonary function in asthmatics include the level of dependence upon medication, the level of asthma control, and exposure to triggers prior to assessment (Borsboom, et al. 1999, Ryan, et al. 1982, Hruby and Butler 1975). Influencing the pulmonary function testing will subsequently impact upon the target $\dot{V}_E$ and consequent bronchoconstrictive response to EVH. Therefore an understanding of the day-to-day variability in pulmonary function testing and EVH response is warranted. In competitive swimmers, EVH has been shown to elicit consistent reductions of FEV_1 when assessed over 2 consecutive days (95% limits of agreement: 6%) (Stadelmann, Stensrud and Carlsen 2011). However, the long-term reproducibility of the bronchoconstrictive response to EVH has not been documented but is important given the variability in symptoms, pulmonary function, and biomarkers associated with asthma in response to a variety of triggers (Frey, Maksym and Suki 2011).

Previous research assessing reproducibility of pulmonary function, and responses to dry gas exercise challenges have used intraclass correlation coefficients (ICCs) to establish the reliability (Dahlen, et al. 2001, Hofstra, et al. 1997). ICCs are commonly used to evaluate reliability; however these focus on a correlation between measures. Correlations can only establish an association between variables, and do not show agreement between

two measures. For example a high correlation coefficient may exist when one value is almost twice the other (Figure 4. 1a). The strong correlation allows for prediction of one measure based on the other, but the actual agreement in the absolute values are non-existent, whereas if the measured values are similar then you achieve both correlation and agreement (Figure 4. 1b). Therefore it is important to understand the level of reproducibility in the bronchoprovocation response to EVH and conduct statistical methods to ensure confidence can be taken from the results.

When attempting to monitor changes in severity of EIB it is important to understand the clinical significance of methods and therefore statistics that utilise reproducibility statistics and the calculation of the smallest measurable change are warranted (Bland and Altman 1996, Hopkins 2000). With reductions in FEV_1 following EVH used to diagnose hyperpnoea induced bronchoconstriction, it is important to ascertain the reproducibility of such reductions and the smallest measurable change in these reductions. This will allow for greater confidence when assessing interventions that aim to reduce the severity of EIB. If changes in post EVH FEV_1 exceed the smallest measurable change then it is likely to be of physiological importance. Understanding the long-term reproducibility of the 6 min EVH test may thus improve the monitoring and evaluation of EIB management and intervention strategies.

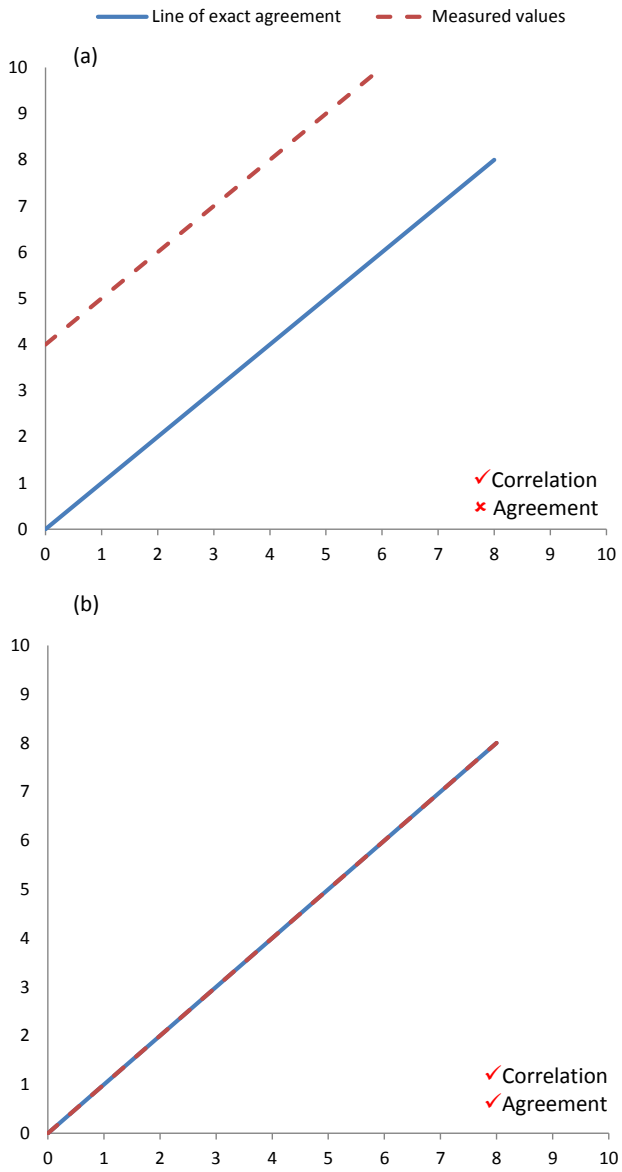

Figure 4. 1. Graphical representations of correlation and agreement

Thus, the aim of this study was to evaluate the short- (21 days) and long- (70 days) term test-retest reproducibility of the bronchoconstrictive response to a 6 min EVH protocol in asthmatic EIB sufferers and non-asthmatic-non-EIB controls. It was hypothesised that both asthmatic EIB sufferers and non-asthmatic controls would receive reproducible diagnoses in response to an EVH challenge over 21, and 70 day periods.

4.2. Methods

4.2.1. Participants

Initially twenty eight recreationally active (completing 4-6 hours of aerobic exercise a week) males (14 asthmatics and 14 non-asthmatics) provided written informed consent and were assessed for eligibility (Figure 4. 2). Following eligibility, two cohorts of 16 participants were deemed suitable to take part in a short-term and/or long-term study of EVH reproducibility. Each cohort of 16 participants comprised 8 asthmatic HIB positive and 8 non-asthmatic HIB negative controls; 8 participants (4 HIB positive and 4 controls) were common to both cohorts (Figure 4. 2). Inclusion criteria for HIB positive participants were a ≥10% reduction in FEV_1 following EVH (Parsons, et al. 2013) and a GP diagnosis of asthma. The studies were approved by the Nottingham Trent University Human Ethics Committee, and all procedures were conducted in accordance with the Declaration of Helsinki.

Throughout the study, participants adhered to their usual training regime and were asked not to partake in strenuous exercise 48 h prior to testing. On test days, participants abstained from caffeine and alcohol and arrived at the laboratory at least 2h post-prandial. Asthmatics were instructed to cease their medication prior to each EVH challenge (inhaled corticosteroids and leukotriene modifiers: 4 days; inhaled long acting β2 agonists: 2 days; anti-histamines: 2 days; inhaled short acting β2 agonists: the day of the test) and were tested at least 2 weeks following recovery from any chest or upper respiratory tract infection (Dickinson, McConnell and Whyte 2011, Anderson, et al. 2001).

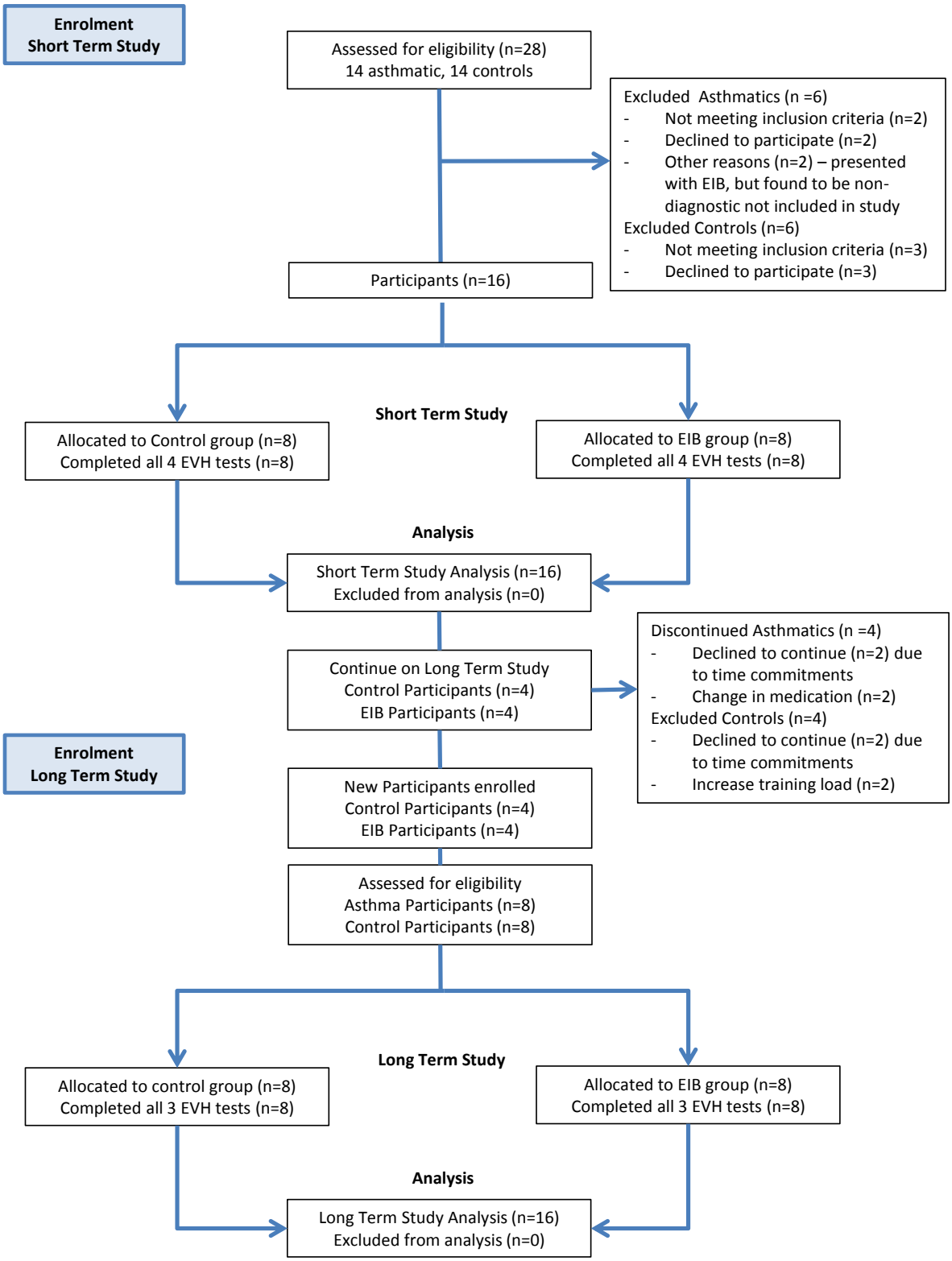

Figure 4. 2 Consort diagram – flow of participants through both short-term and long-term studies.

4.2.2. Experimental design

Following familiarization with the EVH protocol, the short-term reproducibility study comprised 4 EVH challenge tests each separated by 7 days. The long-term reproducibility study comprised 3 EVH challenge tests each separated by 35 days. Tests were conducted at the same time of day for each participant.

4.2.3. Pulmonary function and EVH

Pulmonary function (forced vital capacity (FVC) and FEV_1) and the EVH protocol were conducted following the methods outlined in Chapter 3. Following each EVH challenge, pulmonary function was re-evaluated in duplicate at 3, 6 and 16 min, and the highest values recorded at each interval were used for subsequent analysis (Parsons, et al. 2013). A reduction in FEV_1 of $\geq$10% following EVH was deemed positive of HIB (Anderson, et al. 2001).

4.2.4. Statistical analysis

Statistical analyses were performed using SPSS for Windows version 22 (SPSS Inc., Chicago, IL, USA). One way repeated measure ANOVAs were performed to assess changes in pre-EVH FEV_1 and FVC over the course of both short-term and long-term studies in HIB and control participants. Between-group comparisons (HIB vs. control) of baseline pulmonary function and $\%\Delta FEV_1$ were made using independent samples t-tests. To assess changes in FEV_1 and FVC as a result of EVH, one-way repeated measures ANOVA followed by Bonferroni adjusted t-tests were conducted to compare pre-EVH values to the 3, 6, and 16 min time points post EVH for each day in both HIB and control participants. Bonferroni adjusted pairwise comparisons assessed differences between the

post EVH time points for FEV_1 and FVC at day 0 for both short-term and long-term studies. If no difference was evident ($p > 0.05$) the post-EVH values were averaged to produce a mean response to EVH which was subsequently followed by the calculation of absolute changes (mL). The $\%\Delta FEV_1$ was calculated and a reduction of 10% was deemed diagnostic.

Within participant coefficients of variation (CV) were calculated for pulmonary function measures pre and post EVH. Measurement error otherwise known as the within-subject standard deviation was calculated as the square root of the within-subjects error generated by SPSS repeated measures analysis of variance (Bland and Altman 1996). Reproducibility was calculated from measurement error multiplied by 2.77 ($\sqrt{2}$ x 1.96) (Bland and Altman 1996). This states that the difference between two measurements for the same participant is expected to be less than 2.77 x measurement error for 95% of a pair of measurements (Bland and Altman 1996). Subsequently the smallest measurable change was calculated from the reproducibility value divided by 2 (Hopkins 2000). These reproducibility statistics were calculated for pre-EVH FVC and FEV_1, the post EVH FEV_1 and FVC, and the absolute change in FEV_1 and FVC (mL) post EVH. In HIB participants only, the area under the curve for $\%\Delta FEV_1$ during the 16 min period after EVH was calculated using the trapezoidal rule ($AUC_{0\text{-}16}\%\Delta FEV_1$). All data are reported as mean ± SD. Statistical significance was set at $p < 0.05$.

4.3. Results

Participant characteristics and pre-EVH pulmonary function measures are shown in Table 4. 1. Baseline FEV_1 was always ≥65% of predicted (Knudson, et al. 1983) in all participants, who were therefore able to undergo all EVH challenge tests (Argyros, et al. 1996). HIB participants in the short-term and long-term studies achieved their target $\dot{V}_E$ on all occasions (120 ± 24 vs 120 ± 20; p >0.05 and 114 ± 24 vs 115 ± 22; p >0.05 respectively). The lower baseline FEV_1 in the HIB group meant that the target $\dot{V}_E$ during EVH was lower compared to the control group in both the short- and long-term studies (p < 0.05). Control participants achieved their target $\dot{V}_E$ on all occasions for both short- and long-term studies (141 ± 12 vs 139 ± 12; p >0.05 and 139 ± 11 vs 137 ± 9 p >0.05). All HIB participants experienced a ≥10% reduction in FEV_1 after each EVH challenge test receiving a positive diagnosis on each occasion (Figures 4.1 and 4.2).

Table 4. 1. Participant characteristics for both short- and long-term studies. * significant difference between HIB and Control group (p < 0.05). % predicted from (Knudson, et al. 1983)

	Short-term study		Long-term study	
	HIB group	Control group	HIB group	Control group
Age (yr)	28 ± 7	25 ± 3	31 ± 9	26 ± 4
Height (m)	1.77 ± 0.06	1.81 ± 0.06	1.75 ± 0.02	1.81 ± 0.05
Body mass (kg)	75.83 ± 0.90	79.30 ± 7.86	72.75 ± 9.63	77.75 ± 8.58
Baseline FVC (L)	5.14 ± 0.90	5.56 ± 0.42	4.68 ± 0.69	5.22 ± 0.40
% predicted	100 ± 12	102 ± 7	94 ± 10	97 ± 6
Baseline FEV_1 (L)	4.03 ± 0.80*	4.71 ± 0.40	3.79 ± 0.80*	4.63 ± 0.39
% predicted	93 ± 15	103 ± 6	91 ± 14	102 ± 5

In both short- and long-term studies pre-EVH FVC did not differ between HIB and control groups, whereas FEV_1 was consistently lower in the HIB group when compared to the control group (p < 0.05) (Table 4. 1). No difference in pre-EVH FEV_1 and FVC were

found between days for the short- and long-term studies in either the HIB or control groups ($p > 0.05$).

HIB participants at day 0 of the short-term study exhibited a significant reduction in FVC from pre-EVH to post-EVH ($p < 0.05$), no difference was evident between the three post-EVH time points ($p > 0.05$) which were averaged for reproducibility statistics (Table 4.3). Bonferroni pairwise adjusted comparisons in the mean post-EVH FVC between day 0, 7, 14, and 21 revealed no significant differences ($p > 0.05$). The absolute fall in FVC did not differ between the four short-term days ($p > 0.05$). In the long-term study a significant reduction in FVC from pre-EVH to post-EVH occurred at 3 and 6 minute time points ($p < 0.05$) at day 0. The mean of 3 and 6 minute time points did not differ between days 0, 35, and 70 ($p >0.05$). The absolute fall in FVC did not differ across the three long-term days ($p >0.05$).

In the short term study FEV_1 differed significantly from pre-EVH to post-EVH ($p < 0.05$) at all-time points in the HIB participants (3, 6 and 16 minutes). Bonferroni pairwise adjusted comparisons revealed no difference was evident between these post-EVH time points ($p >0.05$) which were subsequently averaged for each day (Table 4.2). There was no difference in post-EVH FEV_1 between days ($p >0.05$). The absolute fall in FEV_1 did not differ between days ($p >0.05$). The fall in FEV_1 in HIB participants was always greater than 10% meaning a consistent diagnosis was achieved through EVH over the 4 short-term and three long term days (Figure 4. 3 and Figure 4. 4).

Control participants exhibited no reductions in FVC following the EVH protocol in both the short- and long-term studies ($p > 0.05$). Post-EVH FVC did not differ over the

course of the 4 short-term days ($p > 0.05$), and the 3 long-term days ($p > 0.05$). In the short-term study FEV_1 differed from pre-EVH to post-EVH time points ($p < 0.05$). Bonferroni pairwise comparisons revealed post-EVH FEV_1 had reduced but no difference was evident between the 3, 6, and 16 minute time points ($p > 0.05$). In the long-term study pre-EVH FEV_1 differed to the 3, and 6 minute time points ($p < 0.05$), but not 16 minutes post EVH ($p > 0.05$) at day 0. The 3 and 6 minute time points were averaged to establish the post EVH absolute change in FEV_1. In both the short- and long-term studies the reductions in post-EVH FEV_1 were below the 10% diagnosis leading to a consistent non-diagnosis in all control participants. Table 4. 2 shows the group means for $\%\Delta FEV_1$ for both short- and long-term studies. The severity of the bronchoconstrictive response as assessed by $AUC_{0\text{-}16}\%\Delta FEV_1$ in HIB participants did not differ over the 4 short-term days, and 3 long-term days ($p > 0.05$) (Table 4. 2).

Table 4. 2– Bronchoconstrictive response to EVH over both short- and long-term studies for HIB and control groups (mean ± SD).

SHORT –TERM		Day 0		Day 7		Day 14		Day 21		Mean of 4 sessions	
		% change	Absolute change (L)	% change	Absolute change (L)	% change	Absolute change (L)	% change	Absolute change (L)	% change	Absolute change (L)
EIB Group	Post EVH ΔFEV1	-27 ± 18	-0.97 ± 0.51	-27 ± 20	-1.00 ± 0.62	-27 ± 19	-0.96 ± 0.56	-26± 19	-0.94 ± 0.61	-27 ± 18	-0.97 ± 0.55
Control Group	Post EVH ΔFEV1	-2 ± 1	-0.11 ± 0.06	-2 ± 2	-0.10 ±0.09	-2 ± 2	-0.11 ± 0.09	-2 ± 2	-0.11 ± 0.08	-2 ± 2	-0.11 ±0.08
EIB Group	Post EVH ΔFVC	-11 ± 13	-0.51 ± 0.54	-16 ± 18	-0.74 ± 0.79	-14 ± 15	-0.66 ± 0.66	-14 ± 14	-0.61 ± 0.56	-14 ± 14	-0.63 ± 0.62
Control Group	Post EVH ΔFVC	-1 ± 1	-0.05 ± 0.07	-1 ± 1	-0.05 ± 0.04	-2 ± 2	-0.09 ± 0.09	0 ± 1	-0.01 ± 0.05	-1 ± 1	0.05 ± 0.07
LONG-TERM		Day 0		Day 35		Day 70				Mean of 3 sessions	
		% change	Absolute change (L)	% change	Absolute change (L)	% change	Absolute change (L)			% change	Absolute change (L)
EIB Group ΔFEV_1 (%)	Post EVH ΔFEV1	-25 ± 18	-0.85 ± 065	-25 ± 16	-0.73 ± 0.47	-25 ± 16	-0.74 ± 0.62			-25 ± 16	-0.77 ± 0.56
Control Group	Post EVH ΔFEV1	-2 ± 2	-0.10 ± 0.08	-2 ± 2	-0.09 ± 0.07	-3 ± 2	-0.13 ± 0.07			-2 ± 2	-0.10 ± 0.07
EIB Group	Post EVH ΔFVC	-19 ± 16	-0.85 ± 0.65	-17 ± 12	-0.73 ± 0.47	-17 ± 15	-0.74 ± 0.62			-18 ± 14	-0.77 ± 0.56
Control Group	Post EVH ΔFVC	-1 ± 2	-0.04 ± 0.09	-2 ± 2	-0.10 ± 0.10	-1 ± 2	-0.04 ± 0.09			-1 ± 2	-0.06 ± 0.10

The within-participant CVs and reproducibility of pre-EVH FVC and FEV_1 and change in FVC and FEV_1 post EVH are shown in Table 4. 3. The within participant CV for the post EVH change in FEV_1 for HIB participants in the short-term was 11.5%, the measurement error was 118 mL and the reproducibility was 328 mL, and smallest measurable change was 164 mL. The within participant CV was 8.5% in the HIB participants over the long term study for post EVH change in FEV_1. The measurement error was 63 mL, the reproducibility was 175 mL, and smallest measurable change was 88 mL.

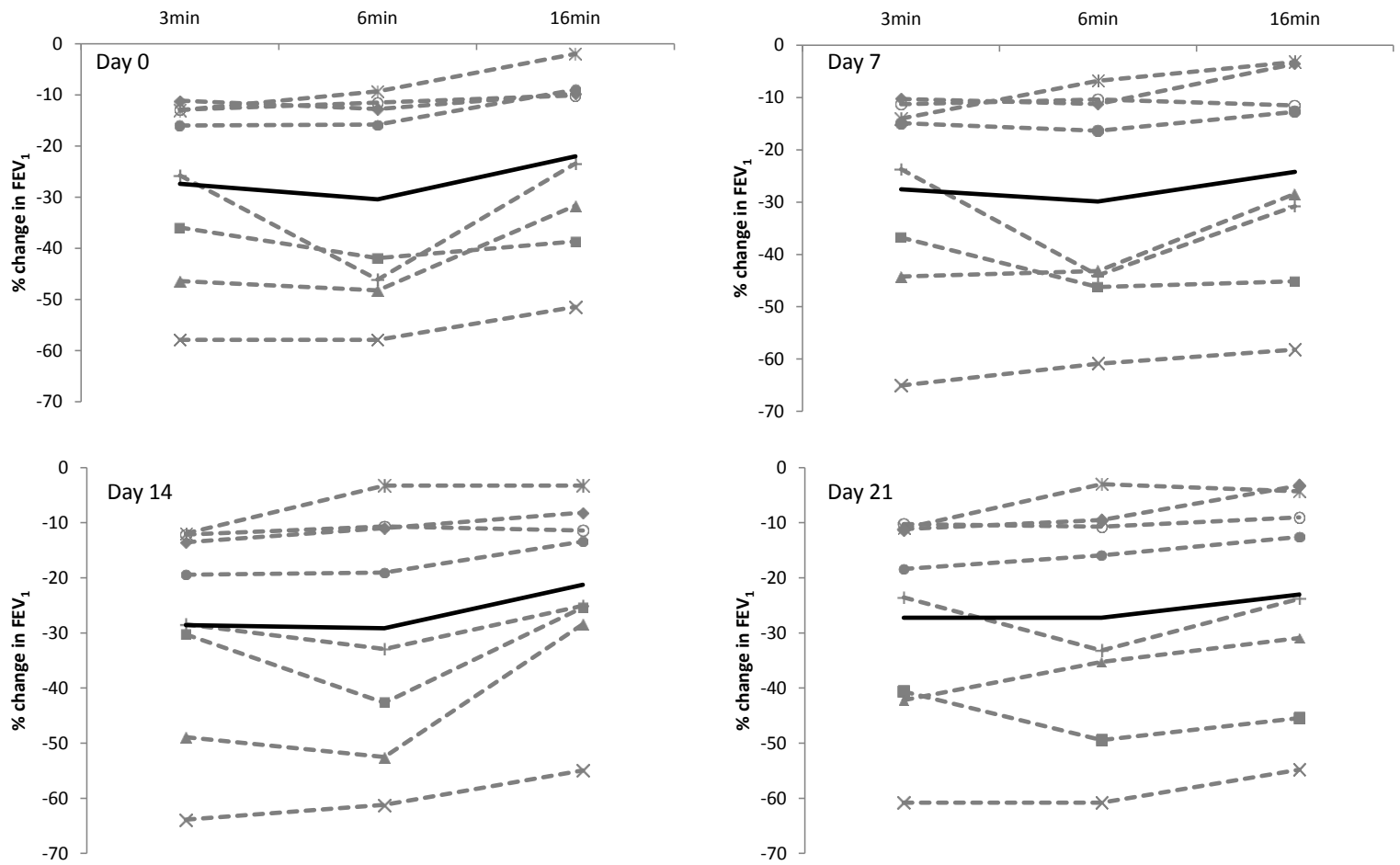

Figure 4. 3 Individual (grey dotted lines) and mean (black line) percentage change in FEV_1 following EVH for HIB participants over the four short-term sessions. No difference was found in %change FEV_1over the 21 days ($p > 0.05$)

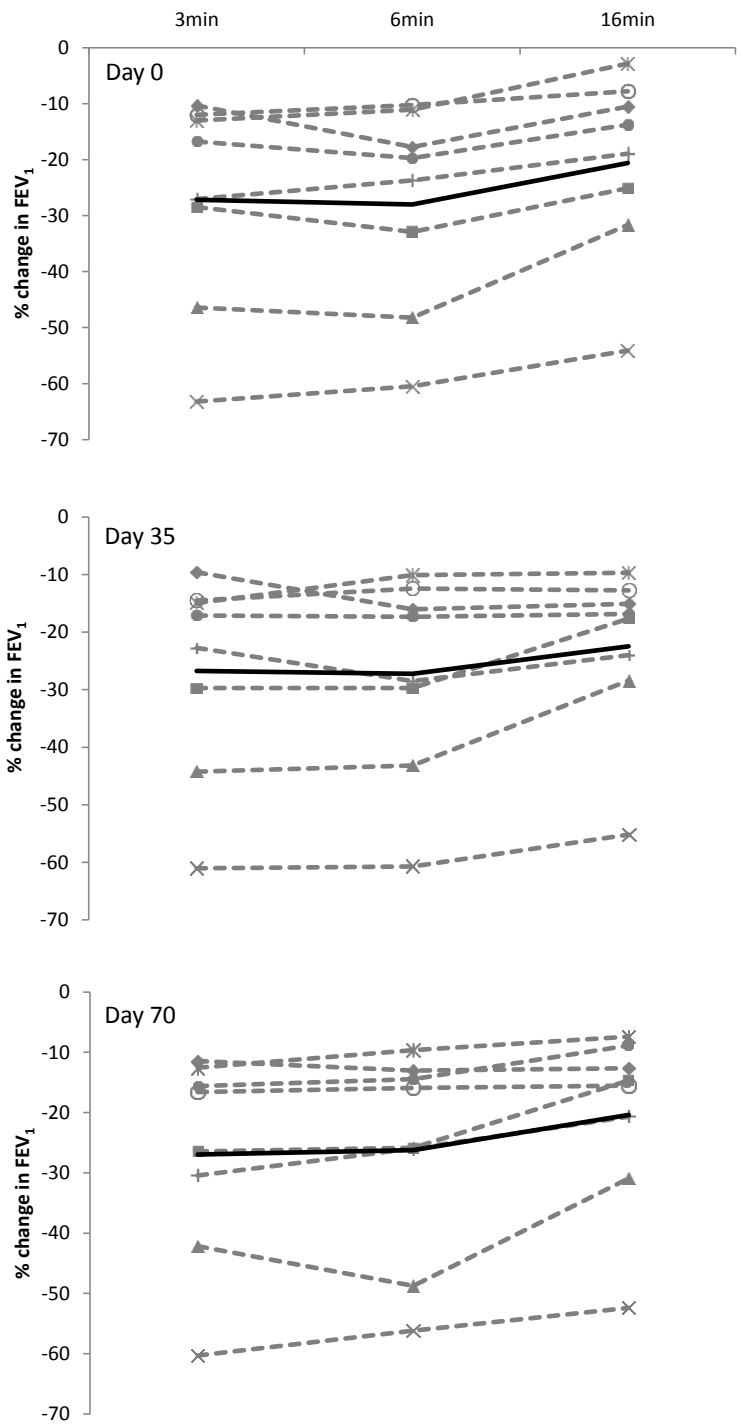

Figure 4. 4 Individual (grey dotted lines) and mean (black line) percentage change in FEV_1 following EVH for HIB participants over the three long-term sessions. No difference was found in %change FEV_1over the 21 days ($p > 0.05$)

Table 4. 3 Reproducibility in pre-EVH pulmonary function, and change in FEV_1 post EVH for control and HIB participants, and change in FVC for HIB participants over both short- and long-term studies. SMC, smallest measurable change.

SHORT-TERM	Mean ± SD Within participant CV (%)	Measurement Error (mL)	Reproducibility (mL)	SMC (mL)
CTRL Pre-EVH FEV_1	2.0 ± 0.9	100	277	139
EIB Pre-EVH FEV_1	2.2 ± 1.3	95	263	131
CTRL Change in FEV_1 post-EVH	29.0 ± 13.7	45	124	62
EIB Change in FEV_1 post-EVH	11.5 ± 4.1	118	328	164
CTRL Pre-EVH FVC	1.3 ± 0.3	77	215	107
EIB Pre-EVH FVC	3.2 ± 3.3	182	503	252
CTRL Change in FVC post-EVH	-	-	-	-
EIB Change in FVC post-EVH	40.5 ± 32.5	130	361	181
LONG-TERM				
CTRL Pre-EVH FEV_1	1.6 ± 0.8	85	235	118
EIB Pre-EVH FEV_1	2.2 ± 1.7	89	246	123
CTRL Change in FEV_1 post-EVH	28.3 ± 17.0	32	88	44
EIB Change in FEV_1 post-EVH	8.5 ± 4.5	63	175	88
CTRL Pre-EVH FVC	2.0 ± 1.2	115	319	159
EIB Pre-EVH FVC	3.1 ± 2.2	143	395	198
CTRL Change in FVC post-EVH	-	-	-	-
EIB Change in FVC post-EVH	24.1 ± 15.6	164	455	228

4.4. Discussion

The main finding of the present study is that EVH incorporating real-time visual feedback of $\dot{V}_E$ and end-tidal PCO_2 induces a highly reproducible bronchoconstrictive response, over both short- term (21 days) and long-term (70 days) periods, in physically active asthmatics with HIB. We also show that baseline pulmonary function is reproducible in this population of asthmatics, and non-asthmatic controls.

The present study showed no significant between day differences in the bronchoconstrictive response as measured by both absolute drop in FEV_1 (mL) and $\%\Delta FEV_1$ following the EVH protocol in the asthmatic participants. The reproducibility

of the change in FEV_1 post EVH was 328 mL and 175 mL for the short- and long-term studies respectively. This suggests that the fall in FEV_1 following EVH over two different testing sessions is expected to vary less than 328 mL for 95% of paired of observations over a 70 day period (Bland and Altman 1996). This is significant as the mean absolute fall in FEV_1 in the asthmatics was greater at ~770 – 970 mL highlighting the EVH protocol induces a reproducible level of bronchoconstriction. The smallest measurable post-EVH change in FEV_1 was 164 and 88 mL for the short- and long-term studies respectively. To the authors knowledge this is the first time that the smallest measurable change in the fall in FEV_1 post-EVH has been calculated from repeated EVH challenges. With the smallest measurable change at 88 mL it suggests that alterations in the post-EVH fall in FEV_1 that are greater than this are likely to be of physiological significance. The current protocol allows confidence in monitoring changes greater than 88 mL to be detected. With the average fall in FEV_1 in the HIB participants >770 mL it suggest EVH is likely to be sensitive to changes in the bronchoconstrictive response that may be a result of treatment interventions. Having an understanding of the smallest measurable change in the FEV_1 response to EVH allows future research to discriminate physiological changes from any inherent variability in the EVH protocol. Interventions to treat HIB that show alterations in the fall in FEV_1 above 88 mL could indicate a significant physiological effect in the response to EVH in physically active asthmatics.

The current study has reported reproducibility in applicable values of mL, previous research has assessed reproducibility through the use of intraclass correlation coefficients (ICCs). Previous research has reported ICCs for the $\%\Delta FEV_1$ following EVH to be 0.72 and 0.57 obtained using a dry-air exercise challenge (Dahlen, et al.

2001, Hofstra, et al. 1997). Although not presented, ICCs for the %ΔFEV$_1$ were calculated in the current study as ≥0.98, which is higher than those previously reported. The data presented in Table 4. 3 suggest that EVH induces a reproducible bronchoconstrictive response in physically active asthmatics. The low SMC for post-EVH change in FEV$_1$ supports previous research that suggests EVH is also more sensitive in diagnosing EIB than (non-dry-air) exercise challenges, which are more difficult to standardize due to potential variability in exercise intensity and duration, and inspirate moisture content (Rundell, et al. 2004, Rundell, et al. 2000). Not standardising these factors and/or using an exercise challenge (e.g. an incremental / ramp protocol) that evokes inadequate bronchoprovocation (Anderson and Brannan 2011) may result in false negative EIB diagnoses (Rundell and Slee 2008) due, in part, to release of bronchodilating substances (e.g. prostaglandin E$_2$) at low exercise intensities (Manning, Watson and O'Byrne 1993, Pavord and Tattersfield 1995). Bronchoprovocation tests based on field-based exercise challenges also lack utility due to difficulties in standardizing exercise intensity, $\dot{V}_E$, inspirate temperature, humidity and air quality (Anderson 2011, Anderson 2011, Rundell and Slee 2008, Rundell, et al. 2004). The present study demonstrates that the bronchoconstrictive response to EVH is reproducible over both the short- and long-term and therefore may be a more useful challenge than exercise when evaluating and monitoring treatment interventions for asthma and EIB/HIB.

The reproducibility of the evoked bronchoconstrictive response in the present study may be explained by the “square wave” nature of the ventilatory response during EVH, which permits rapid achievement of the target $\dot{V}_E$. The total pulmonary ventilation over the 6 min EVH period is therefore likely to be higher than that achieved

during an exercise protocol where $\dot{V}_E$ increases progressively over time (Anderson, et al. 2001). The target $\dot{V}_E$ during EVH, which was well tolerated in the present study, approximates 85% of maximal voluntary ventilation (Anderson, et al. 2001), which is thought to represent that which can be maintained for at least 6 min whilst also stimulating a bronchoconstrictive response (Anderson, et al. 2001). Pre-EVH pulmonary function showed no differences over the 4 short-term and 3 long-term days, the mean within-participant CVs were <3% and exhibited a low measurement error, and good reproducibility (Table 4. 3). Consistent pre-EVH pulmonary function tests allows for a highly reproducible target $\dot{V}_E$ for the EVH challenge. In the present study, two of the asthmatics presenting with GP diagnosed EIB experienced a fall in FEV_1 of <10% and therefore did not meet the EIB group inclusion criterion (Figure 4. 1). This is consistent with reported EIB misdiagnosis (inspiratory stridor may, for example, be confused with wheeze) (Rundell and Spiering 2003) and highlights the utility of EVH in accurately identifying EIB. However, it must be acknowledged that in certain cases EVH can fail to diagnose EIB in some athletes (Dickinson, McConnell and Whyte 2011). EIB is highly heterogeneous and additional factors such as medication and asthma control level, or specific activities/environments and triggers can influence the diagnosis. Furthermore some athletes with inspiratory stridor may be diagnosed with EIB on symptoms (Rundell and Spiering 2003), but would subsequently be EVH negative and non-responsive to β2-agonist medication.

The use of a breath-by-breath analyser (Figure 3. 1) in the present study provided participants with a visual target and real-time feedback of $\dot{V}_E$. Previous EVH protocols have used a metronome to pace breathing frequency and a dry gas meter to determine cumulative ventilation (Anderson, et al. 2001). Not providing real-time

feedback to the participant may compromise standardization of the EVH challenge. Additionally, since hypocapnia evokes bronchoconstriction in asthmatics (Suman, et al. 1999, Sterling 1968), breath-by-breath analysis allows continual monitoring of end tidal PCO_2 and maintenance of eucapnia (defined as end tidal PCO_2 of 35-45 mmHg) all EVH challenge tests in the current study maintained end-tidal PCO_2 within this range. The apparatus used in the present study may have therefore improved validity and reproducibility of the EVH challenge and subsequent HIB response. It must be acknowledged that EVH causes hyperpnoea induced bronchoconstriction as an experimental model of the breathing rates associated with exercise that results in EIB. EVH is a relatively severe bronchoprovocation challenge compared to laboratory and field exercise tests, and some athletes who are HIB diagnostic from EVH may not notice symptoms during training and competition (Dickinson, McConnell and Whyte 2011). However, the protocol in the current study is highly sensitive and reproducible and if an individual does present HIB then it suggests they are at risk of EIB during or following exercise and may benefit from pharmacological and non-pharmacological treatment options. The effectiveness of subsequent treatment interventions can be confidently and effectively monitored by alterations in the bronchoconstrictive response through the use of EVH.

In conclusion, this study confirms the efficacy of a 6 min EVH protocol as a bronchoprovocation test. The study has demonstrated excellent within-participant test-retest reproducibility for EVH and the evoked changes in pulmonary function in HIB participants, over both short- (21 days) and long-term (70 days) periods. The EVH protocol described in the present study thus provides a valuable tool for monitoring HIB and assessing the efficacy of interventions aimed at reducing HIB.

5. *Chapter 5 – The effects of omega 3 polyunsaturated fatty acid supplementation dose level (6.2 g·d^{-1} vs 3.1 g·d^{-1}) on the severity of hyperpnoea induced bronchoconstriction*

5.1. Introduction

Airway inflammation is associated with the release of a number of lipid derived pro-inflammatory mediators which include leukotrienes and prostaglandins generated from arachidonic acid. These pro-inflammatory eicosanoids are known to cause bronchial smooth muscle contraction and airway obstruction (Anderson and Daviskas 2000) (see section 2.2.5).

Traditional asthma and EIB treatment with β2-agonists typically targets symptoms alone, and long-term use of inhaled corticosteroids can have systemic side effects (section 2.5.4). Therefore therapeutic strategies which aim to reduce the release of pro-inflammatory mediators such as the arachidonic acid metabolites in an attempt to reduce airway inflammation are warranted. Dietary supplement use is popular in both the general and asthmatic populations and a nutritional approach to treating asthma is appealing to reduce the reliance upon medication that can have adverse side effects. A recent US National Health and Nutrition Examination Survey revealed that approximately 50% of people in both these groups reported using dietary supplements in the last 30 days. The survey also found that omega-3 polyunsaturated fatty acid (ω3-PUFA) supplementation is used by 6.7% of asthmatics as a nutritional therapy (Ma and Xiao 2011) .

The effectiveness of high dose ω3-PUFA supplementation in reducing airway obstruction and eicosanoid accumulation associated with EIB has been demonstrated previously (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010, Mickleborough, et al. 2013), however, the optimal dose level of ω3-PUFA for alleviating EIB in physically active asthmatics is yet to be determined and warrants further investigation.

5.1.1. ω3-PUFA and EIB

The first study to investigate the effects of supplementation with ω3-PUFA on symptoms of asthma and response to exercise provided equivocal results (Arm, et al. 1988). Twenty five mild asthmatics (responsive to exercise and histamine challenge) were randomised to receive either ω3-PUFA (3.2 $g \cdot d^{-1}$ EPA and 2.2 $g \cdot d^{-1}$ DHA) or identical in size and shape placebo capsules (olive oil) for 10 weeks under double blind conditions. Airway responsiveness to histamine and exercise, diurnal peak expiratory flow, symptom scores, and bronchodilator use were measured. Neutrophil fatty acid composition was evaluated by gas chromatography as an indication of compliance to the supplementation regime. The ω3-PUFA supplementation produced a greater than 10 fold increase in the EPA content of the neutrophil phospholipids, and a 50% inhibition of total leukotriene B_4 generation ($p < 0.01$) in stimulated neutrophils. Despite the changes in phospholipid fatty acid content and suppression of pro-inflammatory eicosanoid production, there was no significant change in airway responsiveness following either the histamine, or exercise challenge, in the asthmatics. The results indicated that neutrophil function could be suppressed in asthmatics following high dosages of ω3-PUFA but did not improve the severity of disease. The authors attributed the results to the fact that neutrophils may not play a major role in the pathogenesis of asthma, or the level of change in neutrophil function was not of a level to alter disease severity (Arm, et al. 1988). The study failed to acknowledge additional roles of other inflammatory cell types and provide data on the physical activity of their asthmatic population.

In contrast, two key studies have shown improvements in EIB after exercise challenge testing following 3 weeks of high doses of ω3-PUFA (3.2 $g \cdot d^{-1}$ EPA and 2.2 $g \cdot d^{-1}$), with FEV_1 improving to non-diagnostic levels, coupled with reduced

inflammatory markers (Mickleborough, et al. 2003, Mickleborough, et al. 2006). In 2003, Mickleborough and co-workers assessed the effectiveness of 3-weeks supplementation of ω3-PUFA on EIB in elite athletes. Ten elite athletes with mild EIB (~15% drop in FEV_1 post exercise) and 10 non-EIB controls completed the randomised, double-blind, placebo-controlled (olive oil) crossover study (normal diet vs placebo vs ω3-PUFA) with a two-week washout between interventions. EIB was assessed via exercise challenge testing, and a drop in FEV_1 of ≥10% was considered diagnostic of EIB.

In participants with EIB, intervention with ω3-PUFA improved post exercise pulmonary function compared with participant's normal diet, and placebo interventions. Following the 3 week ω3-PUFA intervention, asthmatic participants exhibited no reduction in FEV_1 after an exercise challenge -3 ± 2% ($p > 0.017$, *Bonferroni adjusted*) suggesting participants had become non-diagnostic. However, on the placebo intervention and their normal diets they remained diagnostic recording $\%\Delta FEV_1$, of -14.5 ± 5% and -17.3% ± 6% respectively ($p < 0.017$, *Bonferroni adjusted*) (Mickleborough, et al. 2003). Urinary eicosanoids and circulating immunoreactive cytokines as markers of airway inflammation were reduced following the ω3-PUFA intervention. At day 21 of the ω3-PUFA intervention urinary LTE_4 excretion was significantly reduced prior to exercise and 15 minutes post exercise by 19.4 $pg \cdot mg^{-1}$ creatinine and 13.1 $pg \cdot mg^{-1}$ creatinine respectively ($p < 0.017$) compared to pre supplementation LTE_4 concentration of 56.9 ± 13.3 $pg \cdot mg^{-1}$ creatinine (Mickleborough, et al. 2003). Day 21 of the ω3-PUFA intervention resulted in reductions of mean urinary excretion of 9α, 11β-$PGF_{2\alpha}$ prior to exercise of 16.8 $ng \cdot mg^{-1} \cdot mmol\ creatinine^{-1}$ and by 13.9 $ng \cdot mg^{-1} \cdot mmol\ creatinine^{-1}$ at 15 minutes post exercise compared with the pre supplementation level of 53.2 ± 12.4 $ng \cdot mg^{-1} \cdot mmol\ creatinine^{-1}$ ($p < 0.017$)

(Mickleborough, et al. 2003). Following the ω3-PUFA intervention both TNF-α, and IL-1β decreased by 22.6 ± 6.3% ($p < 0.017$), and 22.5 ± 6.7% ($p < 0.001$) respectively compared to day 0 of the intervention. IL-1β significantly reduced by 15.1 ± 4.7% at 15 minutes post exercise ($p < 0.017$) compared with pre-ω3-PUFA diet values (Mickleborough, et al. 2003).

Mickleborough et al. (2003) were the first to show a significant therapeutic effect of high dose ω3-PUFA in physically active asthmatics with improvements in pulmonary function and attenuation in inflammatory markers. These findings are in contrast to those previously shown by Arm et al. (1988). These conflicting findings are surprising considering the longer supplementation period with identical daily dosages used by Arm et al. (1988). The differences may be attributed to the participant's activity, and athletic ability levels. Mickleborough et al. (2003) studied collegiate or national level athletes ($\dot{V}O_{2max}$ = 61.7 ± 7.9 $mL \cdot kg^{-1} \cdot min^{-1}$) compared to non-athletic participants in the Arm et al (1988) study (VO_{2max} not reported). It has been suggested that higher level athletes are more likely to suffer with EIB than non-athletic asthmatics due to inspiring larger quantities of unconditioned air in both competition and training which may predispose this population to more severe EIB. There is evidence of airway remodelling in athletes with EIB (Sue-Chu, et al. 2000, Sue-Chu, et al. 1999, Karjalainen, et al. 2000, Karjalainen, et al. 2000), and some athletes are non-responsive to inhaled corticosteroids (Sue-Chu, et al. 2000). This indicates the varying pathogenesis of EIB, which may be more severe in physically active and athletic asthmatics.

Mickleborough et al. (2006) were able to support their earlier 2003 findings in a study where participants consumed ω3-PUFA intervention (3.2 g EPA and 2.0 g DHA daily) and placebo intervention (olive oil) for 3 weeks in a randomised double-blind

cross-over study. The authors found similar protective effects of ω3-PUFA in recreationally active asthmatics (Mickleborough, et al. 2006). This study continued to support the notion of a reduction in pro-inflammatory mediator release as a mechanism of action through the collection of induced sputum as a more direct measure of airway inflammation. Pulmonary function, induced sputum differential cell count percentage, pro-inflammatory eicosanoid metabolites (LTC_4 – LTE_4 and PGD_2) and cytokines (IL-1β, and TNF-α) were assessed pre and post exercise challenge. All participants exhibited EIB on their normal diet and placebo intervention. However, following the ω3-PUFA intervention, the mean reduction in FEV_1 after the exercise challenge was below diagnostic levels (%ΔFEV_1 -8.1 ± 0.19%) (Mickleborough, et al. 2006). The ω3-PUFA intervention resulted in large reductions in pre-exercise sputum LTC_4-LTC_4, PGD_2, IL-1 and TNF-α by 74%, 93%, 71%, and 95% respectively compared to the placebo intervention, this was also observed at 1 h and 24 h after the exercise challenge compared to placebo and normal interventions. There were positive correlations between the severity of EIB and percentages of post-exercise sputum eosinophils and neutrophils when on the placebo intervention and normal diet highlighting their role in pathogenesis of EIB (Mickleborough, et al. 2006). These findings corresponded with increased neutrophil phospholipid content of EPA (~20 fold increase) and DHA (~4 fold increase) on the ω3-PUFA ($p < 0.05$), and reduced content of linoleic acid and arachidonic acid ($p < 0.05$) (Mickleborough, et al. 2006). This study showed for the first time that fish oil supplementation reduced airway inflammation in recreationally active asthmatics. The mechanisms for this are likely to be related to the reduced production of cysteinyl leukotrienes and cytokines. With the cysteinyl leukotrienes thought to be the most important pro-inflammatory mediators causing EIB in asthmatics (O'Byrne

2000), the two Mickleborough studies highlight a strong rationale for the use of ω3-PUFA supplementation in the treatment of EIB.

More recently research has emerged in support of Mickleborough's previous work, showing ω3-PUFA to be as effective in EIB treatment as the pharmacological intervention of montelukast, a leukotriene antagonist (Tecklenburg-Lund, et al. 2010). In a randomised cross-over design, 3 weeks of 10mg·d^{-1} montelukast was equally as effective in treating EIB when compared to 3 weeks of ω3-PUFA alone (3.2g EPA and 2.0g DHA per day). Prior to the interventions, post EVH maximum percentage drop in FEV_1 was -18.4 ± 2.1%. Following interventions, post EVH FEV_1 percentage drop had improved to -9.3 ± 2.8% on fish oil, and -11.6 ± 2.8% on montelukast; and combination treatment was -10.8 ± 1.7% ($p < 0.05$). This finding suggests that the efficacy of montelukast can be met via non-pharmacological means and is likely due to the anti-leukotriene properties of omega-3 PUFA. Recently, the marine lipid fraction of the green-lipped mussel has been shown to reduce airway inflammation and bronchoconstriction following an EVH challenge in active asthmatics (Mickleborough, et al. 2013). The supplement contained five main lipid classes including sterol esters, triglycerides, free fatty acids, sterols and polar lipids so elucidating the actions of solely EPA and DHA could not be made, but the findings suggest manipulation of lipid derived mediators has a positive effect on asthma.

Contrasting the consistent findings of Mickleborough et al., (2003; 2006) and Tecklenburg-Lund et al., (2010), a recent study has shown no benefit of 3 weeks of ω3-PUFA (4.0 g·d^{-1} EPA and 2.0 g·d^{-1} DHA) on pulmonary function responses to a mannitol test and markers of airway inflammation in physically active asthmatics (Brannan, et al. 2014). The difference in the outcomes is likely to be due to the bronchial provocation test employed. Inhaled mannitol is a hypertonic challenge and

has been suggested as an alternative for EVH (Holzer, et al. 2003). However, it is plausible that exercise/EVH and mannitol produce very slightly different bronchoconstrictive responses. Although both mannitol and dry-air hyperpnoea are known to alter the tonicity and volume of the airway surface liquid and produce bronchoconstrictive mediators, the generations of airways recruited and affected may differ. Mannitol is known to not cause any disruption to the airway epithelium unlike dry-gas hyperpnoea (Romberg, Bjermer and Tufvesson 2011) and EVH and mannitol can produce different levels of bronchoconstriction in physically active asthmatics (Holzer, et al. 2003, Sue-Chu, et al. 2010).

Recent research has started to explore the effects of solely EPA or DHA upon airway inflammation and EIB. Interestingly, a recent study failed to show any benefit of consuming DHA in isolation on the severity of EIB (Head and Mickleborough 2013). The randomised, double-blind, placebo controlled crossover trial failed to show any improvement of lung function after EVH or markers of airway inflammation following 3 weeks of 4 $g \cdot d^{-1}$ DHA in nine asthmatics with EIB. This suggests that either a higher or longer dose of DHA is needed; or that a combination of EPA and DHA are required; or that EPA is solely effective in attenuating EIB in physically active asthmatics. If a combination of EPA and DHA is most effective then understanding the effects of a lower dose would be beneficial in improving the adherence and uptake of ω-3 PUFA as a treatment option for EIB. Despite the latest study findings producing equivocal results, the consensus suggests a potential benefit of ω3-PUFA on EIB in physically active asthmatics, however a number of questions still remain.

The previous research (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010), used very large dosages of ω3-PUFA (5.2g ω3-PUFA; 3.2 g EPA and 2.0g DHA) equating to ~10 capsules a day. This is significantly higher

than the UK recommended daily intake of 0.45 $g \cdot d^{-1}$ (Scientific Advisory Committee on Nutrition/Committee on Nutrition/Committee on Toxicity 2004) and there is a large cost implication with such a high dose. In addition, there is the potential for problems with adherence to these dosages and a potential side effect of gastrointestinal discomfort (Van de Rest, et al. 2008). These high doses are likely to have been set as a proof of concept, with the high dose saturating the phospholipid membrane of inflammatory cells. It is plausible that dose levels could be reduced but still have beneficial effects.

As such the aim of the current study was to investigate the effectiveness of a lower omega-3 PUFA dose on hyperpnoea induced bronchoconstriction and markers of airway inflammation.

5.2. Methods

5.2.1. Participants

Twelve male asthmatics and 10 male non-asthmatics (controls) recreationally active (completing 6-8 hours of aerobic exercise per week) participants volunteered for the study and provided written informed consent (Appendix 5 - Chapter 5 Informed Consent Form). Participants with a history of taking ω3-PUFA and antioxidant supplements above the RDI, or regularly consuming more than one fish meal a week were excluded from the study prior to eligibility assessment in accordance with the inclusion criteria outlined in the participant information sheet (Appendix 4 - Chapter 5 Participant Information Sheet). All volunteers underwent an initial screening session to determine the presence of hyperpnoea induced bronchoconstriction as an experimental model of EIB. Participants were excluded if resting FEV_1 values were less than 65% of predicted (Argyros, et al. 1996, Knudson, et al. 1983). Participants presenting to the study with a GP diagnosis of asthma and a history of shortness of breath and wheezing after exercise had to have a $\geq$ 10% reduction in FEV_1 following bronchoprovocation with eucapnic voluntary hyperpnoea to be diagnostic (Anderson, et al. 2001) and included in the study. Two asthmatics were found to be non-diagnostic with a < 10% reduction in FEV_1 following EVH and were excluded from the study; two controls declined to participate further. Two asthmatic participants withdrew from the study during the high dose ω3-PUFA intervention due to gastrointestinal distress leaving a total of 8 HIB participants (age 30 ± 9 Yr; height 1.77 ± 0.03 m; body mass 75.6 ± 9.4 kg) and 8 non asthmatic non-HIB controls (age 25 ± 9 Yr; height 1.81 ± 0.06 m; body mass 78.0 ± 9.0) to complete the study (Figure 5. 1). Independent samples t-tests revealed no between group differences for age, height, or weight ($p > 0.05$).

Current medication of the eight HIB participants was (n = 5 Salbutamol; n = 2 Salbutamol, Salmeterol, and Budesonide with Formoterol, n=1 Salbutamol and Beclomethasone). All participants had well controlled asthma and were on step 1-3 on the stepwise treatment of asthma (National Asthma Education and Prevention Program 2007). The study was approved by the Nottingham Trent University Human Ethics Committee, and all procedures were conducted in accordance with the Declaration of Helsinki.

Throughout the study participants were instructed to adhere to their usual training regime and were asked not to partake in strenuous exercise 48 h prior to testing. On test days participants abstained from caffeine and alcohol and arrived at the laboratory at least 2 h post-prandial. Asthmatics were instructed to cease their medication prior to each EVH challenge (inhaled corticosteroids [Budesonide, Formoterol, Beclomethasone]: 4 days; inhaled long acting β2 agonists [Salmeterol]: 2 days; inhaled short acting β2 agonists [Salbutamol]: the day of the test) and were tested at least 2 weeks following recovery from any chest or upper respiratory tract infection (Dickinson, McConnell and Whyte 2011, Anderson, et al. 2001).

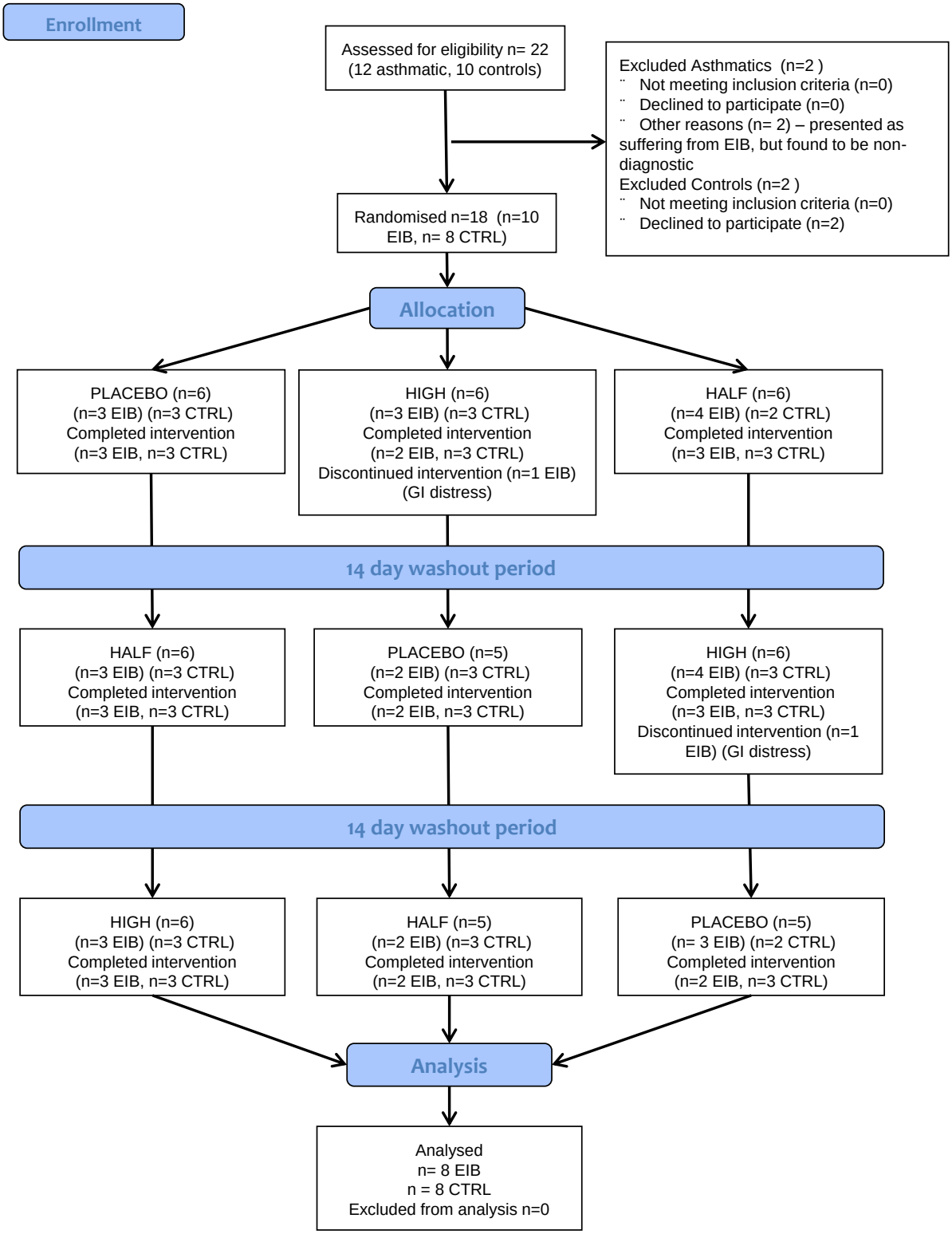

Figure 5. 1 CONSORT diagram of participant flow through the study

5.2.2. Experimental Design and Protocol

The study was conducted as a randomised, double-blind, placebo controlled cross-over design over fourteen weeks per participant. All participants entered the study on their normal diet after which they were randomised to receive in a counter-balanced order, one of the following supplement conditions for 21 days:

• ω3-6.2 $g \cdot d^{-1}$– 8 ω-3 capsules equalling 6.2 g (3.7 g EPA and 2.5 g DHA) per day

• ω3-3.1 $g \cdot d^{-1}$ – 4 ω-3 capsules equalling 3.1 g (1.8 g EPA and 1.3 g DHA) + 4 placebo capsules per day

• Placebo (PLA) – 8 placebo capsules containing medium chain triglyceride GTCC per day

Supplement capsules were identical in size, shape, taste and colour (CRODA Europe Ltd, East Yorkshire, UK). Participants followed a 2-week washout period (normal diet) between conditions (Mickleborough, et al. 2003, Mickleborough, et al. 2006). To standardise dietary intake prior to each testing session participants completed a food diary for their first intervention that was subsequently repeated prior to each session. This was also standardised for the first two days of each intervention.

At Day 0 and Day 21 of each intervention, participants attended the laboratory for the assessment of HIB by eucapnic voluntary hyperpnoea (EVH). Pulmonary function was assessed pre-EVH and at 3, 6, 16, 20, and, 30 minutes after EVH. A drop in FEV_1 following EVH of ≥10% compared to pre EVH values confirmed the presence of HIB (Anderson, et al. 2001). Pre EVH at day 0 and day 21 of each intervention a venous blood sample was drawn from the antecubital vein for the assessment of neutrophil phospholipid fatty acid content. In addition at day 0 and day 21 of each

intervention prior to EVH, HIB participants only provided a fraction of exhaled nitric oxide (F_ENO) sample. F_ENO was not assessed in controls as values are not elevated in non-atopic non-asthmatic individuals (Franklin, et al. 2003, Jouaville, et al. 2003). Both HIB and control participants provided urine samples before and at 12, 60, and 90 minutes post EVH for the determination of the urinary concentration of the pro-inflammatory eicosanoid 9α, 11β-PGF_2 (a PGD_2 metabolite).

5.2.3. Pulmonary function, EVH challenge, and F_ENO

Pulmonary function, EVH, and F_ENO were conducted in accordance with the procedures outlined in Chapter 3. EVH provides a reproducible bronchoconstrictive response (Chapter 4) so allows for accurate monitoring of responses to dietary interventions. Following the EVH challenge, two reproducible flow-volume loops were measured at 3, 6, 16, 20, and 30 min, with the highest acceptable value recorded at each interval (Parsons, et al. 2013).

5.2.4. Urinary 9α, 11β-PGF_2 Concentration

Participants were asked to provide ~20 mL urine samples prior to EVH and at 12, 60, and 90 min post the EVH protocol. Participants were instructed to arrive at the laboratory in a euhydrated state having consumed 500 mL of water 60 min prior to arrival. Participants drank water *ab libitum* pre and post the EVH challenge to ensure urine samples could be collected. Urine samples were immediately separated into 2 mL eppendorf tubes and frozen at −80°C until further analysis.

Urinary concentration of 9α, 11β-PGF_2 was determined by enzyme-linked immunosorbent assay (ELISA) (Caymen Chemicals, Ann Arbor, Michigan, USA). The 9α, 11β-PGF_2 antibody cross-reacted with 2,3 dinor-11β-PGF_2 (10%), 11β-13,14-dihydro-15-ketoPGF_2 (0.5%) and below 0.01% for all other primary eicosanoids metabolites. Intra-assay coefficient of variation was 4%, and Inter-assay coefficient of variation was 5%. No samples were below the lowest level of detection. The concentration of 9α, 11β-PGF_2 was adjusted for creatinine concentration which is used to normalise the concentration of urine samples and account for variable levels of hydration amongst samples (Cone, et al. 2009). Urinary creatinine concentration was measured using a clinical analyser (ABX Pentra 400; Horiba, Northampton, UK) and ABX Pentra creatinine kit using the kinetic Jaffe reaction (Junge, et al. 2004). The corrected 9α, 11β-PGF_2 was expressed as ng/mg·mmol·g^{-1} creatinine.

5.2.5. Neutrophil phospholipid fatty acid analysis

The neutrophil isolation protocol is a purification procedure to isolate neutrophils from 20 mL of whole human blood and was adapted from previous descriptions of the method (Eggleton, Gargan and Fisher 1989, Nauseef 2007). Twenty mL of blood was drawn into a syringe from a vein in the antecubital fossa region of the forearm and immediately transferred to a 50 mL sized conical tube containing 4 mL anticoagulant acid-citrate-dextrose (ACD - 250 mL of ultrapure water containing, 7.36 g citric acid; 14.71 g sodium citrate; 9.91 g dextrose). The conical tube was gently inverted several times to mix. 12 mL of 6% Dextran and 0.9% NaCl solution (250 mL of ultrapure water containing, 15.00 g 100,000 molecular weight Dextran and 2.25 g NaCl) was pipetted into the ACD/blood mixture and gently inverted several times to mix. This mixture was equally distributed between four 15 mL conical tubes and left to

stand at room temperature for 60 minutes. This dextran sedimentation stage causes the majority of the red blood cells to sediment to the bottom of each 15 mL conical tube while the mononuclear cells remain suspended in solution.

After 60 minutes the yellowish supernatant containing the leukocytes and lymphocytes was pipetted from each 15 mL conical tube and transferred to a 50 mL conical tube. This was centrifuged (MSE Harrier 18/80, MSE, London, UK) at 1150 rpm for 12 minutes at 4°C using a low brake. Following centrifugation, hypertonic lysis was conducted to remove any remaining red blood cells and platelets. The supernatant from the centrifuged solution was discarded. The remaining pellet was re-suspended in 12 mL of ice-cold ultra-pure water and, after 20 seconds 4 mL of 0.6 M KCl (250 mL ultra-pure water containing 11.18 g KCl) was added and mixed several times. This solution was diluted to 50 mL with phosphate buffered saline (PBS) and spun at 1300 rpm for 6 minutes at 4°C using a high brake. This step was repeated until no red blood cells remained. The remaining pellet containing the mononuclear cells was re-suspended in 2.5 mL of PBS. This cell suspension was layered over 3mL of Ficoll-Hypaque (Sigma-Aldrich, Dorset, UK) solution in a 15 mL conical tube; this then was spun at 1500 rpm for 30 minutes at 4°C using a low brake. The Ficoll sedimentation allowed the separation of the neutrophil cells (sinking to the bottom of the conical tube) from the remaining mononuclear cells which remain suspended in solution. The solution was removed and the neutrophil cell pellet was re-suspended in 2 mL of Hanks balanced salt solution (HBSS) (Sigma-Aldrich, Dorset, UK). The neutrophil cell suspension was stored under nitrogen in 1.5 mL eppendorf tubes at −80°C until further analysis by gas chromatography.

5.2.5.1 Determination of fatty acid phospholipid profile by gas chromatography

Phospholipids were extracted from the neutrophils using previously described methods (Bligh and Dyer 1959) through collaboration at Reading University. Fatty acid composition was analysed by gas chromatography. EPA, DHA, linoleic acid (ω6-PUFA), and arachidonic acid (ω6-PUFA) were expressed as a percentage of total fatty acid content.

5.2.6. Assessment of compliance

Participant's received unlabelled pots of supplements that had been randomly numbered by a colleague of the investigator. The pots were labelled and the contents of each pot was counted and recorded by the colleague of the investigator to ensure double-blind conditions were maintained. Participants received the pots and instructions to consume a total of 8 capsules a day (4 in the morning, and 4 in the evening) for 21 days. Participants were contacted several times throughout the duration of the interventions to monitor progress. On completion of the intervention participants returned the pots and the remaining capsules were counted by the colleague of the investigator. Compliance was expressed as the discrepancy between the expected numbers of returned capsules and the actual number used using the following equation: %compliance = (actual\expected) x 100. Compliance to all interventions was >85%. In addition to this, compliance was also was assessed through the analysis of the neutrophil phospholipid fatty acid contents to establish changes in the fatty acid phospholipid profiles (section 5.2.5).

5.2.7. Statistical analysis

The severity of the bronchoconstrictive response to EVH was assessed as the area under the curve for $\%\Delta FEV_1$ during 30 min post-EVH ($AUC_{0\text{-}30}\%\Delta FEV_1$). In addition $AUC_{0\text{-}30}FEV_1$, and $AUC_{0\text{-}30}FVC$ were also calculated using trapezoidal integration.

Data were analysed using the statistical software SPSS for Windows version 22.00 (SPSS Inc., Chicago, IL, USA). The data were initially assessed for normality using Shapiro-Wilks test and assessed for skewness and kurtosis. To confirm the presence of HIB, the drop in FEV_1 post EVH was assessed through one-way repeated measures ANOVA followed by a-priori Bonferroni adjusted *post-hoc* t-tests to compare pre-EVH values to post. Three-way repeated measures ANOVAs were used to analyse the effects of intervention (Placebo vs 6.2 g·d-1 vs 3.1 $g \cdot d^{-1}$) and day (Day 0 vs Day 21) with a between subject factor of group (HIB v Control) for $AUC_{0\text{-}30}\%\Delta FEV_1$, $AUC_{0\text{-}30}FEV_1$, $AUC_{0\text{-}30}FVC$. Within-group changes were further explored by analysing the HIB and control groups separately using a two-way repeated measures ANOVA. To establish the between intervention differences at Day 0 and Day 21 significant *F* ratio was followed by a-priori Bonferroni adjusted *post-hoc* t-tests to establish where significant differences occurred between conditions (Placebo, 6.2 $g \cdot d^{-1}$, 3.1 $g \cdot d^{-1}$) and time points (Day 0 and Day 21).

The response of urinary concentration 9α, 11β-PGF_2 to the EVH test was initially assessed by a one-way repeated measures ANOVA for time (Pre-EVH vs, 12 vs 60 vs 90 min Post–EVH) for each day (Day 0 and Day 21) within each group (HIB and Control). Subsequently a four-way repeated measure ANOVA to establish the effects of intervention (Placebo vs 6.2 g vs 3.1 g), day (Day 0 vs Day 21) and time (Pre-EVH vs Post-EVH), with a between subject factor of group (HIB vs Control) was

completed. This was followed by three-way repeated measure ANOVAs for each group (HIB and CONTROL) to analyse the effects of intervention (Placebo vs 6.2 g vs 3.1 g), day (Day 0 vs Day 21), and time (Pre-EVH vs Post-EVH). In the HIB group only two-way ANOVAs for each day (DAY 0 and DAY 21) analysed the effects of intervention (Placebo vs 6.2 g vs 3.1 g) and time (Pre-EVH vs Post-EVH). At day 21 in HIB group a one-way ANOVA at each time point (Pre-EVH and Post-EVH) assessed the effects of intervention (Placebo vs 6.2 g vs 3.1 g) followed by Bonferroni adjusted pairwise comparisons.

A two-way repeated measures ANOVA assessed differences in pre-EVH F_ENO within each condition (Day 0 versus Day 21) for the HIB participants only followed by Bonferroni adjusted paired t-tests. Two-way repeated measures ANOVA assessed differences in the fatty acid composition within HIB and control groups and to ensure complete washout prior to each intervention, followed by *post-hoc* Bonferroni adjusted pairwise comparisons. Independent samples t-test assessed differences in fatty acid composition between the asthmatic and control groups at day 0 of each intervention. Statistical significance was set as $p < 0.05$, all data are presented as mean ± SD unless otherwise stated.

5.3. Results

5.3.1. Pulmonary Function

There were no significant differences at Day 0 between each condition for pulmonary function, bronchoconstrictive response to EVH, and markers of airway inflammation within HIB and control participants ($p > 0.05$), suggesting there was no carry over effect after each two-week washout period (Table 5.1). Pre-EVH FEV_1 and FVC, and maximum $\%\Delta FEV_1$ post-EVH at Day 0 for each condition are shown in Table 5.1 for HIB and control participants. At Day 0, asthmatic participants demonstrated HIB for Placebo, 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ interventions with EVH causing a significant reduction in FEV_1 ($p < 0.01$). Maximum reductions in FEV_1 of -28 ± 18%, -28 ± 87%, and -27 ± 17% always occurred at 6 minute post EVH at Day 0 for Placebo, 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ respectively. At Day 0 of each intervention, the mean post-EVH $\%\Delta FEV_1$ was always greater than 10% for up to 30 minutes.

Table 5.1 Participant characteristics for HIB and control groups Mean (±SD). * Significant difference between HIB and Control participants ($p < 0.05$).

Pulmonary Function	HIB Participants (n=8)			Control Participants (n=8)		
Pre-EVH Day 0	Placebo	6.2 $g \cdot d^{-1}$	3.1 $g \cdot d^{-1}$	Placebo	6.2 $g \cdot d^{-1}$	3.1 $g \cdot d^{-1}$
FEV_1 (L)	3.79 ± 0.82	3.76 ± 0.87	3.72 ± 0.84	4.66 ± 0.45*	4.63 ± 0.41*	4.65 ± 0.45*
% predicted	91.4 ± 13.1	90.8 ± 12.8	89.9 ± 15.1	102.5 ± 5.5	102.2 ± 5.9	102.2 ± 5.5
FVC (L)	4.66 ± 0.70	4.71 ± 0.66	4.62 ± 0.72	5.25 ± 0.51*	5.20 ± 0.42*	5.20 ± 0.43*
% predicted	97.4 ± 9.3	96.1 ± 11.4	97.3 ± 6.4	98.7 ± 7.5	97.9 ± 6.6	96.7 ± 6.5
Post-EVH Day 0						
Maximum $\%\Delta FEV_1$	-28 ± 18	-28 ± 18	-27 ± 17	-3 ± 2	-3 ± 2	-3 ± 3
$AUC_{0\text{-}30}\%\Delta FEV_1$	583.0 ± 447.2	570.2 ± 394.2	528.2 ± 402.2	45.6 ± 41.3*	35.2 ± 49.1*	35.2 ± 49.1*

The three-way repeated measures ANOVA for $AUC_{0\text{-}30}\%\Delta FEV_1$ revealed a significant intervention x day x group interaction, ($p < 0.001$). Two-way repeated measures ANOVA for the control group revealed no main or interaction effects ($p > 0.05$). Subsequent two-way repeated measures ANOVA for the HIB group revealed a main effect for intervention ($p < 0.002$), and day ($p < 0.001$) and an intervention x day interaction ($p < 0.01$). Subsequent one-way repeated measures ANOVA in the HIB group revealed that $AUC_{0\text{-}30}\%\Delta FEV_1$ at Day 21 was significantly less following the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ interventions when compared to placebo ($p < 0.05$), but there was no difference between 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ($p > 0.05$). The greatest reductions in FEV_1 at 6 minutes had improved to -19 ± 15%; and -18 ± 14% following the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ interventions respectively (Figure 5.2A). The $AUC_{0\text{-}30}\%FEV_1$ values for the control group were less compared to the HIB group at Day 0 and Day 21 across all interventions ($p < 0.05$) (Figure 5.2B). Individual and mean severities of HIB as assessed by $AUC_{0\text{-}30}\%\Delta FEV_1$ are shown in Figure 5.3 for HIB participants. A three-way repeated measures ANOVA for $AUC_{0\text{-}30}FVC$ revealed a significant day x group interaction ($p < 0.05$). Subsequent one-way between group ANOVA found $AUC_{0\text{-}30}FVC$ was significantly greater in the control group compared to the HIB group at both Day 0 and Day 21 for all interventions ($p < 0.05$).

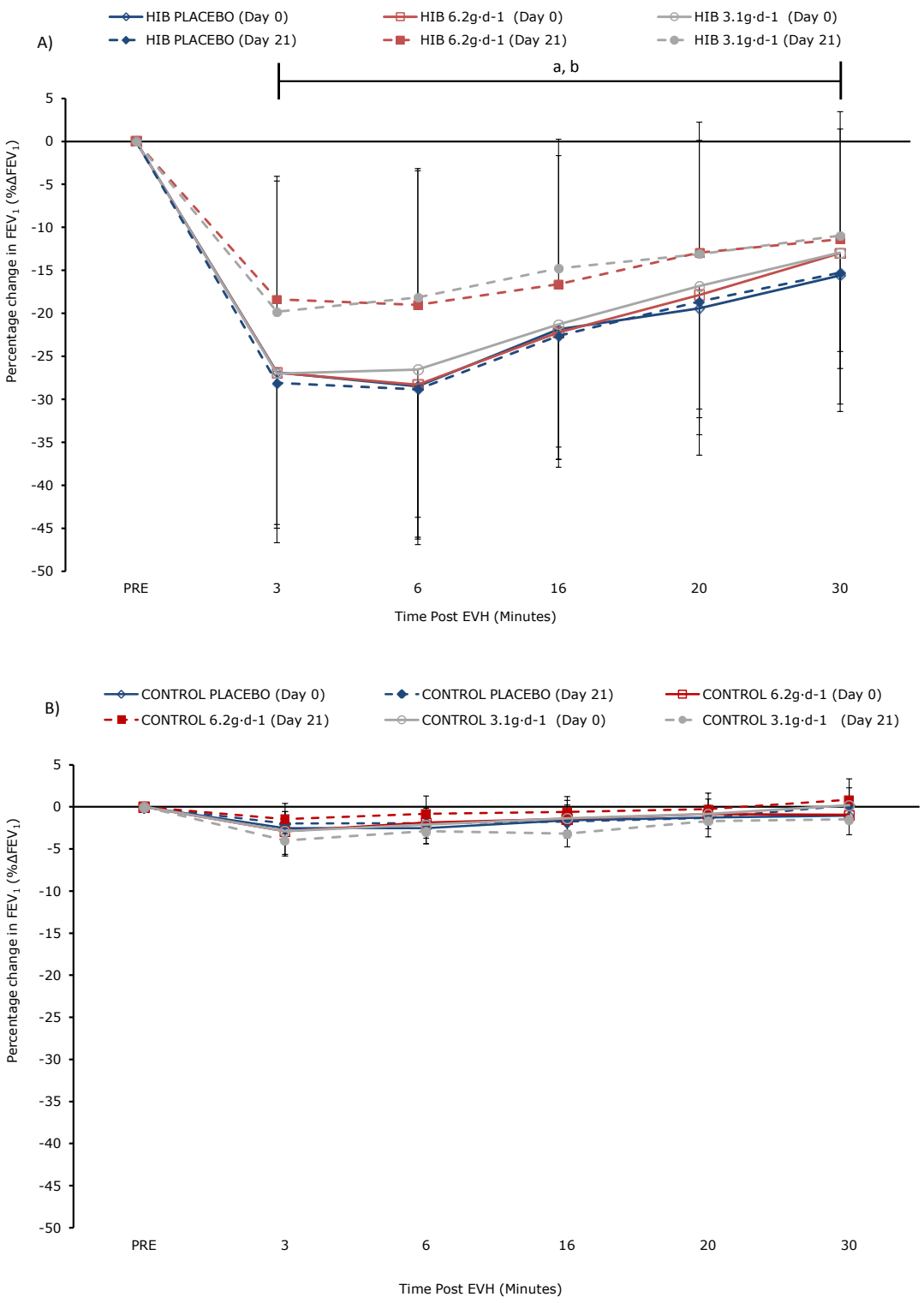

Figure 5.2 The percent change in FEV_1 from baseline to post EVH A) in HIB participants and B) in controls interventions (Mean ± SD). a) Significant reduction in $AUC_{0\text{-}30}\%\Delta FEV_1$ at Day 21 6.2 $g{\cdot}d^{-1}$; b) significant reduction in $AUC_{0\text{-}30}\%\Delta FEV_1$ at Day for 21 3.1 $g{\cdot}d^{-1}$ ($p < 0.05$).

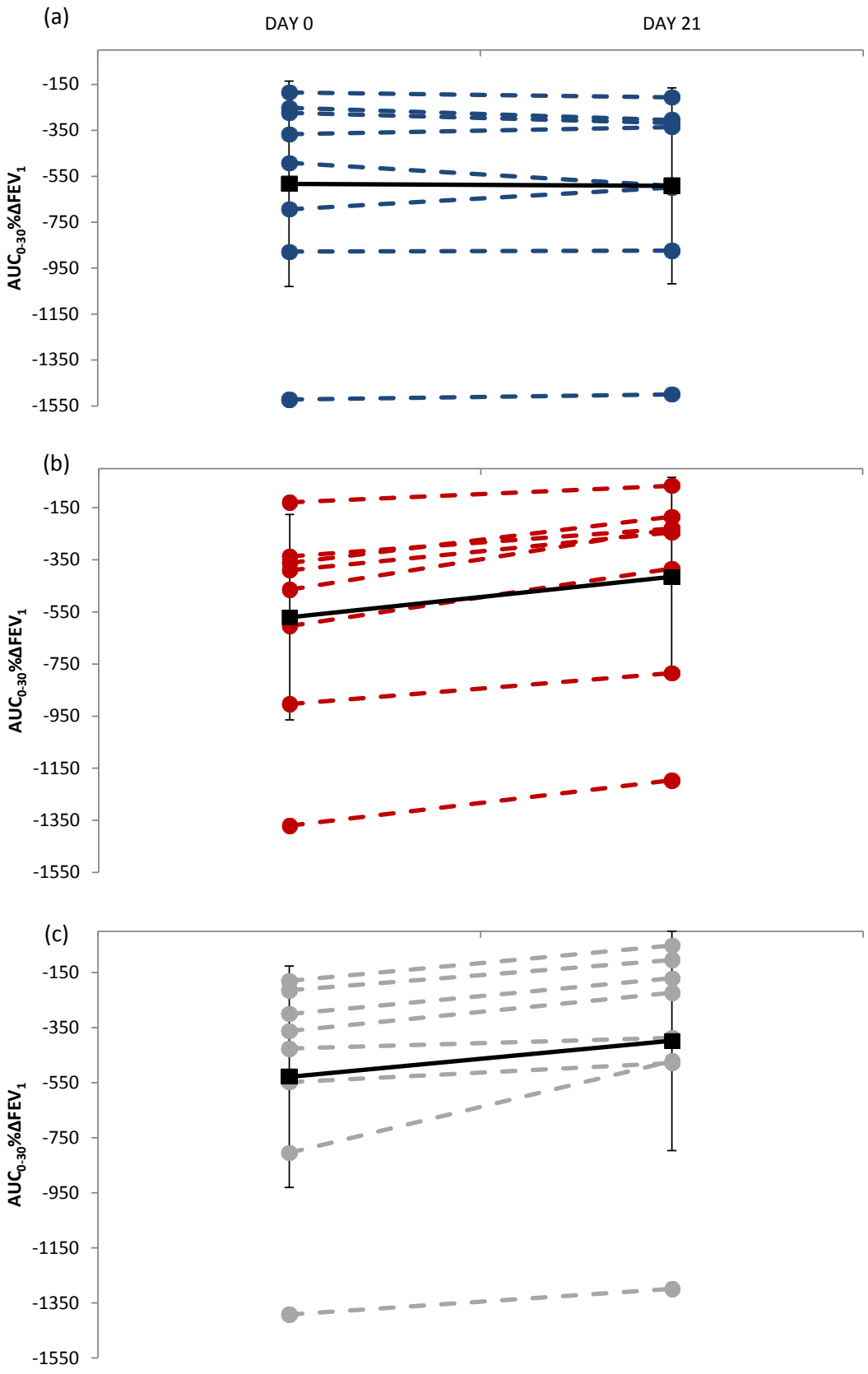

Figure 5.3 HIB asthmatic participants only, individual severity of HIB measured by $AUC_{0\text{-}30}\%\Delta FEV_1$ from Day 0 to Day 21. Black line indicates mean. (a) Placebo intervention; (b) 6.2 $g \cdot d^{-1}$ intervention and (c) 3.1 $g \cdot d^{-1}$ intervention.

5.3.2. Fraction of Exhaled Nitric Oxide (F_ENO)

Pre-EVH F_ENO values are shown in Figure 5. 4. A two-way repeated measures ANOVA revealed a significant intervention x day interaction (p <0.05). One-way repeated measures ANOVA for day 0 revealed no differences in F_ENO between interventions (p > 0.05), significant differences were evident at day 21 (p < 0.05). Subsequent Bonferroni pairwise comparisons revealed differences between placebo and 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions ($p < 0.05$), but no difference between ω3-PUFA interventions. Within intervention paired t-tests were used to establish intervention changes in F_ENO. Both 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions resulted in a significant reduction in the level of F_ENO at Day 21 when compared to Day 0 (p < 0.017). No significant changes were evident following the placebo intervention.

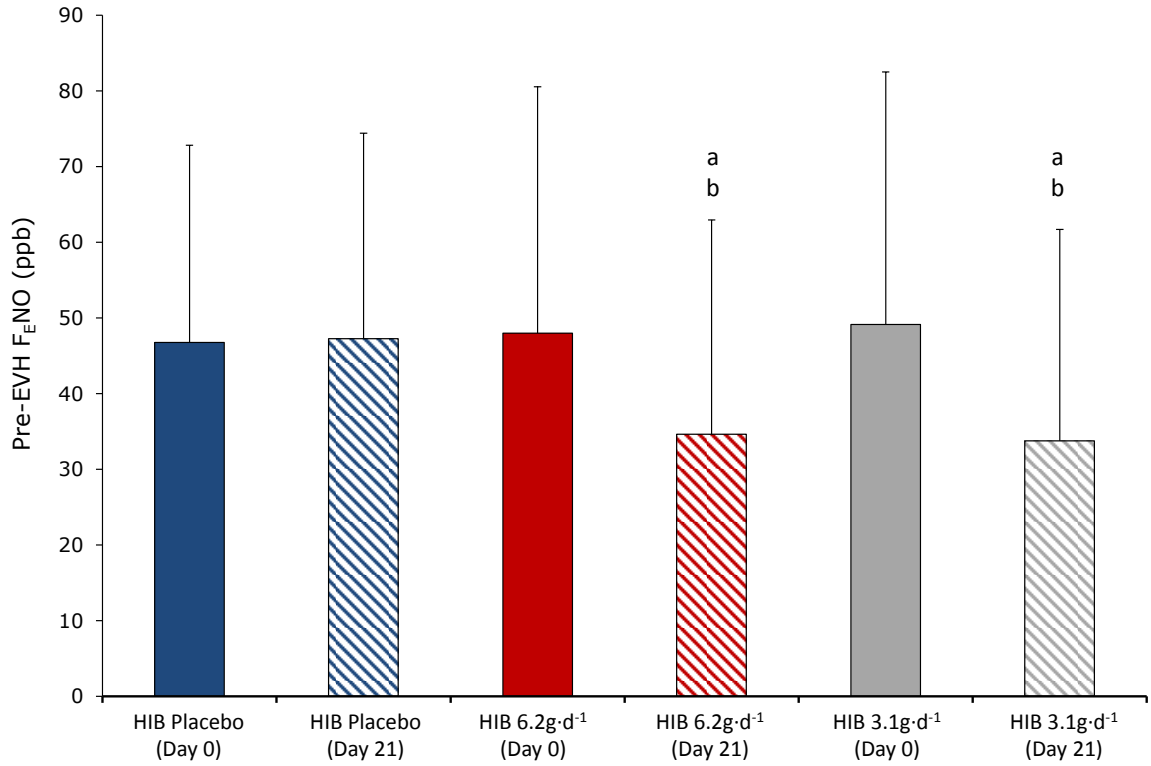

Figure 5. 4. Pre EVH fraction of exhaled nitric oxide in HIB participants (Mean ± SD). a) significant reduction in F_ENO concentration post intervention (p < 0.017).

5.3.3. Urinary 9α, 11β-PGF_2 concentration

Within-day one-way repeated measures ANOVA revealed a difference in urinary 9α, 11β-PGF_2 concentrations for pre- to post-EVH in the HIB group ($p < 0.05$). No difference between post EVH values were found which were subsequently averaged (Figure 5. 5). Control participants showed no changes in urinary 9α, 11β-PGF_2 concentration over the course of the study. The four-way repeated measures ANOVA revealed a number of significant ($p < 0.05$) interaction effects (intervention x group; time x group; intervention x day; intervention x time; day x time). The three-way repeated measures ANOVA in the HIB asthmatics revealed significant main effects for intervention, day, and time ($p < 0.05$), and a significant intervention x day x time interaction ($p < 0.05$). Two-way ANOVA for day 0 in the HIB asthmatics revealed a main effect for time ($p < 0.05$), but no interaction x time interaction. For day 21 there was a main effect for time and intervention ($p < 0.05$), and intervention x time interaction ($p < 0.05$). Subsequent one-way ANOVAs at both Day 0 and Day 21, followed by Bonferroni adjusted pairwise comparisons revealed a significant ($p < 0.05$) difference for Placebo vs 6.2 g·d-1, Placebo vs 3.1 g·d-1, but no difference for 6.2 g·d-1 vs 3.1 g·d-1 ω3-PUFA interventions (Figure 5. 5A). Independent samples t-tests revealed no difference in the HIB asthmatics urinary 9α, 11β-PGF_2 concentration at day 21 of 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions when compared to the control participants.

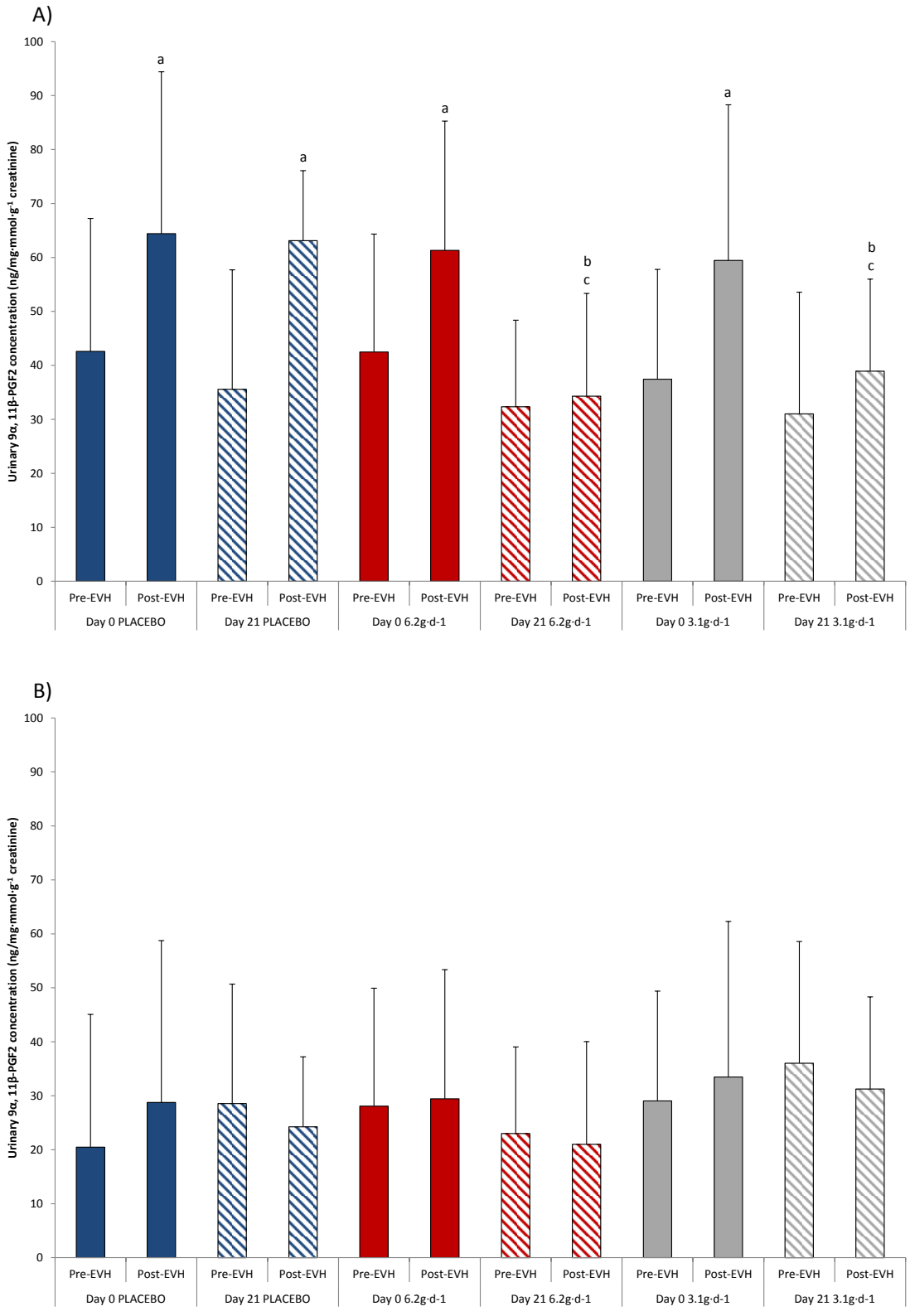

Figure 5. 5 (A) Urinary 9α, 11β-PGF_2 concentration in HIB participants (B) Urinary 9α, 11β-PGF_2 concentration in control participants (Mean ± SD). a significant difference Pre-EVH to Post-EVH ($p < 0.05$); b significant difference day 0 vs day 21 within the same intervention ($p < 0.05$); c significant difference compared to placebo for same time point ($p < 0.05$). (Mean ± SD).

5.3.4. Neutrophil phospholipid fatty acid content

The neutrophil phospholipid fatty acid content was assessed in both HIB and control participants (Table 5. 2) and expressed as a percentage of total fatty acid content. Between group comparisons at day 0 of each intervention revealed HIB participants had a greater percentage of arachidonic acid within the neutrophil phospholipid membranes compared to the control participants ($p < 0.05$). No significant differences ($p > 0.05$) were observed for linoleic acid, arachidonic acid, EPA, and DHA, when comparing day 0 to day 21 in the placebo intervention for both HIB and control participants. Following the 6.2 $g \cdot d^{-1}$ ω3-PUFA intervention in the HIB group the EPA content increased to 1.39 ± 0.38% ($p < 0.05$), and the DHA content increased to 2.39 ± 0.31% ($p < 0.05$). Conversely the content of AA decreased to 5.37 ± 0.80% ($p < 0.05$); there was no change in the content of linoleic acid ($p > 0.05$). The 3.1$g \cdot d^{-1}$ ω3-PUFA intervention resulted in a significant increase in the EPA content to 1.05 ± 0.31% ($p < 0.05$), with a concurrent reduction in arachidonic acid to 5.26 ± 0.99% in the HIB participants ($p < 0.05$). No significant changes ($p > 0.05$) in DHA, and linoleic acid were observed after the 3.1$g \cdot d^{-1}$ ω3-PUFA dose in the HIB group. In the control group, the 6.2 $g \cdot d^{-1}$ ω3-PUFA dose resulted in significant increases in the content of EPA and DHA ($p < 0.05$) and reductions in arachidonic acid ($p < 0.05$), and the 3.1 $g \cdot d^{-1}$ ω3-PUFA dose increased the content of DHA and reduced arachidonic acid ($p < 0.05$).

Table 5. 2 Phospholipid fatty acid composition of neutrophil extracts expressed as a percentage of total fatty acid content at day 0 and day 21 of each intervention in HIB asthmatics and control participants Mean (±SD). a Significant difference between HIB and Control participants ($p < 0.05$); b significant difference between day 0 and day 21 within condition.

Intervention	18:2 ω6 Linoleic Acid		20:4 ω6 Arachidonic Acid		20:5 ω3 Eicosapentaenoic Acid		22:6 ω3 Docosahexaenoic Acid	
	HIB	Controls	HIB	Controls	HIB	Controls	HIB	Controls
PLACEBO								
Day 0	6.46 ± 0.87	5.14 ± 1.26	6.52 ± 0.82 [a]	5.18 ± 1.02	0.72 ± 0.17	0.80 ± 0.19	2.15 ± 0.33	1.98 ± 0.31
Day 21	5.78 ± 0.82	5.25 ± 0.66	6.34 ± 0.62	5.09 ± 1.34	0.75 ± 0.19	0.79 ± 0.22	2.20 ± 0.37	2.14 ± 0.45
6.2 g·d^{-1} ω3-PUFA								
Day 0	5.76 ± 0.80	5.58 ± 0.97	6.03 ± 0.78 [a]	5.20 ± 0.63	0.77 ± 0.19	0.70 ± 0.20	2.06 ± 0.33	2.02 ± 0.28
Day 21	5.96 ± 0.61	5.25 ± 0.81	5.37 ± 0.80 [b]	4.94 ± 0.56	1.39 ± 0.38 [b]	0.93 ± 0.12 [b]	2.39 ± 0.31 [b]	2.30 ± 0.30 [b]
3.1 g·d^{-1} ω3-PUFA								
Day 0	5.99 ± 0.59	6.24 ± 1.88	6.35 ± 0.92 [a]	5.73 ± 1.29	0.59 ± 0.29	0.73 ± 0.21	2.14 ± 0.40	1.83 ± 1.40
Day 21	5.80 ± 0.53	5.44 ± 0.73	5.26 ± 0.99 [b]	5.02 ± 1.01 [b]	1.05 ± 0.31 [b]	0.84 ± 0.10	2.29 ± 0.35	2.22 ± 0.26 [b]

5.3.5. Summary of key findings

- 6.2 $g \cdot d^{-1}$ ω3-PUFA and 3.1 $g \cdot d^{-1}$ ω3-PUFA were equal in reducing the severity of HIB ($AUC_{0\text{-}30}\%\Delta FEV_1$) when compared to placebo.
- 6.2 $g \cdot d^{-1}$ ω3-PUFA and 3.1 $g \cdot d^{-1}$ ω3-PUFA were equal in reducing F_ENO in HIB asthmatics as a marker of airway inflammation.
- 6.2 $g \cdot d^{-1}$ ω3-PUFA and 3.1 $g \cdot d^{-1}$ ω3-PUFA were equal in reducing the urinary concentration of 9α, 11β-PGF2 when compared to placebo.

5.4. Discussion

This study has demonstrated for the first time that lower doses of ω3-PUFA (3.1 $g \cdot d^{-1}$) are equally as effective in reducing the severity of hyperpnoea induced bronchoconstriction in recreational athletes as higher doses (6.2 $g \cdot d^{-1}$). Changes in the severity of HIB assessed by $AUC_{0\text{-}30}\%\Delta FEV_1$ were similar in both the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions. Both interventions resulted in equal reductions in resting levels of exhaled nitric oxide, and equal suppression of urinary 9α, 11β-PGF_2 concentration following the EVH challenge. No changes in pulmonary function and urinary 9α, 11β-PGF_2 concentration were evident in the control group throughout the study. The current findings suggest for the first time that a lower ω3-PUFA dose is effective in management of HIB, and could be used as a therapeutic option for physically active asthmatics. Furthermore compliance to the lower 3.1 $g \cdot d^{-1}$ could be greater due to the reduced cost and risk of gastrointestinal side effects.

Previously, 21 days treatment with high dose (~6 $g \cdot d^{-1}$) ω3-PUFA completely prevented the bronchoconstriction exhibited by asthmatics following an exercise challenge with mean post exercise $\%\Delta FEV_1$ becoming less than the diagnostic criteria of -10% (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010). In the current study $\%\Delta FEV_1$, remained diagnostic ($\geq$-10%ΔFEV_1) after both the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions in HIB participants and this may be due to differences in severity of disease and the bronchial provocation challenge used. HIB participants in the current study exhibited a greater maximum fall in FEV_1 of ~-30% (maximum range of -13 to -63%) compared to previous cohorts that report mean maximum $\%\Delta FEV_1$ of ~-20% in asthmatics prior to ω3-PUFA interventions (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010). This suggests that participants in the current study had greater HIB severity with

high heterogeneity; furthermore, EVH was used as the bronchial provocation challenge as opposed to an exercise challenge. This dry-gas hyperpnoea challenge mimics the breathing rates associated with exercise; it has been shown not only to be highly diagnostic, but also to elicit a greater bronchoconstrictive response when compared to exercise challenges (Rundell, et al. 2004, Mannix, Manfredi and Farber 1999). Consequently, the current study protocol may have elicited a greater bronchoconstriction accounting for the difference in recovery rate following ω3-PUFA between this and the previous research that used exercise as a challenge test (Mickleborough, et al. 2003, Mickleborough, et al. 2006).

Previous research typically shows 50-60% improvements in AUC%ΔFEV_1 following high dose ω3-PUFA (Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010). This is much greater than improvements shown from Day 0 to Day 21 in the current study with improvements in $AUC_{0\text{-}30}$%ΔFEV_1 of 27.2% and 24.7% for 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ interventions respectively. Similarly, this may be due to differences in severity of the bronchoconstriction induced between studies, and the high heterogeneity of response in the current participants. Further differences in the bronchoconstriction response of HIB participants between the current study and Mickleborough et al. (2003) and Mickleborough et al. (2006) were apparent. The %ΔFEV_1 peaked at 6 minutes post EVH in the current study whereas, previously, maximum reductions in FEV_1 occurred at 15 minutes post exercise (Mickleborough, et al. 2003, Mickleborough, et al. 2006). It may be that the square-wave nature of $\dot{V}_E$ achieved during EVH elicits a more rapid bronchoconstrictive response compared to exercise challenges. EVH is likely to produce greater cumulative ventilation over 6 minutes compared to exercise contributing to the higher levels of severity in bronchoconstriction and therefore reduced percentage improvements following the interventions in the current study.

The findings of the current study contrast those of the earliest ω3-PUFA intervention study by Arm et al (1988). After 10 weeks of 3.2 $g \cdot d^{-1}$ EPA and 2.2 $g \cdot d^{-1}$ DHA they found no improvements in airway hyperresponsiveness to a histamine challenge and no pulmonary function improvements in response to an exercise challenge in the asthmatic participants despite a 10-fold increase in neutrophil EPA. This increase in EPA is considerably greater than that in the current study and may be due to the longer intervention and lower initial dietary intakes of EPA allowing for a greater cumulative effect. The discrepancies in pulmonary function responses could be attributed to different bronchial provocation challenges and participant groups with Arm et al (1988) using a non-athletic population and a histamine challenge for airway hyperresponsiveness. Despite this, recent research has emerged in support in of Arm et al (1998) and contrasts our current findings (Brannan, et al. 2014). Three weeks of a daily dose of 4.0g EPA and 2.0g DHA intervention did not improve bronchial hyperresponsiveness to inhaled mannitol or suppress markers of airway inflammation (Brannan, et al. 2014).

The most obvious differences between the current study and that of Brannan et al. (2014) are the bronchoprovocation methods used and asthmatic population recruited. Inhaled mannitol is a hypertonic challenge and has been suggested as an alternative for EVH (Holzer, et al. 2003). The airway response is measured by changes in FEV_1 to increasing doses of mannitol, a reduction in FEV_1 of >15% from the pre challenge value is considered an abnormal response. Evidence suggests that inhaled mannitol mimics the airway response and components of HIB including the activation of mast cells and eosinophils (O'sullivan, et al. 1998, Brannan, et al. 2003), but equally so does EVH (Kippelen, et al. 2010a, Kippelen, et al. 2010b). It is plausible that EVH and mannitol are producing very slightly different bronchoconstrictive responses. Although

both mannitol and dry-air hyperpnoea are known to alter the tonicity and volume of the airway surface liquid and produce bronchoconstrictive mediators, the generations of airways recruited and subsequent level of inflammation may differ.

Mannitol powder is delivered from a dry powder inhaler, and *in-vitro* testing has shown 40-70% of the aerosol particles are less than 7μm in diameter which is small enough to reach the distal airways (Anderson, et al. 1997). However, it is acknowledged that only 13-31% of the dose inhaled is deposited into the lungs, and although this is enough to cause a positive test there is a large variation in peripheral to central deposition of mannitol into the lungs (Glover, et al. 2006) which could produce differing levels of bronchoconstriction when compared to EVH. Dry gas hyperpnoea as used in the current study closely mimics the ventilation rates associated with high intensity exercise; such high ventilation rates are known to result in the recruitment of smaller generations of airways in an attempt to condition the high volumes of inhaled air (Anderson and Kippelen 2008). These smaller airways are at increased susceptibility to injury and epithelial cell disruption (Anderson and Kippelen 2008, Kippelen and Anderson 2012). The high ventilation rates associated with exercise and dry-air hypopnoea are known to result in disruption of the airway epithelial cells, whereas mannitol does not cause any airway epithelial injury when urinary marker clara cell 16 was measured as a marker of epithelial airway injury (Romberg, Bjermer and Tufvesson 2011). Severe hyperpnoea causes high force generation on the airway epithelial surface causing sheer stress and increasing transepithelial pressure gradients (Kippelen and Anderson 2012). An injury-repair response may occur in athletes and physically active asthmatics causing a disruption in the epithelial cell layer which may alter the airway smooth muscle properties increasing hyperresponsiveness in this population (Kippelen and Anderson 2012). Although urinary clara cell 16 was not

measured in the current study it is plausible that the physically active asthmatics had a level of underlying airway injury which enhanced their susceptibly to the EVH challenge. Brannan *et al*. (2014) failed to provide any physical activity characteristics of their participants; this is an important description as the bronchoconstrictive response to EVH and mannitol is known to differ in athletic populations compared to non-elite and sedentary populations (Holzer, et al. 2003, Sue-Chu, et al. 2010).

It may be hypothesised that the differences in bronchial provocation challenges used, and the physical activity status of participants influences the severity of EIB and epithelial disruption, thus altering the subsequent responses to ω3-PUFA interventions. Long term physically active asthmatics may have an underlying level of airway injury as shown by higher levels of urinary clara cell 16 (Romberg, Bjermer and Tufvesson 2011, Bolger, et al. 2011); this may subsequently increase their responsiveness to EVH. Evidence regarding the ability of EPA and DHA to reduce airway epithelial cell inflammation is lacking. However, recent research has shown a significant reduction in urinary clara cell 16 following 3 weeks supplementation of an extract from the New Zealand Green Lipped mussels which contained 72 mg of EPA, 48 mg of DHA and mix of polyphenols (Mickleborough, et al. 2013). It is plausible that 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ dose ω3-PUFA in the current study may have influenced airway epithelial disruption to reduce the bronchoconstriction caused by EVH. Future research should include measures of urinary clara cell 16 in response to different ω3-PUFA doses in physically active asthmatics; this may prove to be a novel mechanism of therapeutic action.

Two participants in the current study dropped out having complained of gastrointestinal distress, following un-blinding of the study it was found they were on the higher 6.2 $g \cdot d^{-1}$ intervention, this further supports a rationale for reducing the effective dose of ω3-PUFA on asthma and HIB. Compliance to the interventions was

measured by assessing the fatty acid composition of neutrophil phospholipid cell membranes. Dietary intervention of the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA resulted in significant increases in the content of EPA, and the 6.2 $g \cdot d^{-1}$ dose increased the content of DHA at day 21 when compared to day 0 verifying compliance to the supplementation regimes.

The increases in EPA and DHA were in accordance with a reduction in the content of ω6-PUFA arachidonic acid following the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ interventions in the HIB group. Interestingly, the current study has shown that the HIB asthmatics had significantly greater levels of arachidonic acid at day 0 of the interventions compared to the control group. This is important because the arachidonic acid derived pro-inflammatory eicosanoids have consistently been associated with the pathogenesis of asthma and EIB (Hallstrand, et al. 2013). The pro-inflammatory eicosanoids of arachidonic acid are leukotriene B_4 (LTB_4) which is a potent neutrophil chemoattractant and pro-inflammatory mediator, and the cysteinyl series leukotrienes (LTC_4, LTD_4, and LTE_4), which produce smooth muscle contraction and bronchoconstriction (Manning, et al. 1990). Dietary intake influences arachidonic acid content and it may suggest discrepancies in lifestyles between the HIB asthmatics and control participants or the increase could be due to genetic variations in arachidonic acid metabolism although this remains to be fully understood in specific disease states (Simopoulos 2006, Lattka, et al. 2010). The dietary intervention of ω3-PUFA modifies the fatty acid profiles of phospholipid membranes of inflammatory cells such as neutrophil cells in the present study. The incorporation of EPA and DHA occurs in a dose response fashion at the expense of arachidonic acid (Calder 2012). The increase in EPA and DHA can then cause inhibition of the cyclooxygenase-2 and 5-lipoxygenase pathways from arachidonic acid to reduce the production of pro-inflammatory eicosanoids.

Following both the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ doses, arachidonic acid content was reduced in neutrophils phospholipids of the HIB participants. This is the first study to report a reduction in arachidonic acid content following a 3.1 $g \cdot d^{-1}$ (1.8g EPA and 1.3g DHA) dose of ω3-PUFA in HIB asthmatics. The phospholipid fatty acid composition of the neutrophil extracts presented in the current study differ considerably from those reported previously (Mickleborough, et al. 2003, Mickleborough, et al. 2006). The >6% arachidonic acid content in the current study at day 0 is lower than ~15-20% reported previously (Mickleborough, et al. 2003, Mickleborough, et al. 2006). These studies found a ~35-45% reduction in arachidonic acid which is greater than the 12-27% reductions reported in our current study. The differences suggest that the participants in current study had a lower dietary intake of arachidonic acid, subsequently, the response to the ω3-PUFA interventions in lowering the percentage of arachidonic is diminished slightly. Despite this it still shows significant increases in EPA and DHA content at the expense of arachidonic acid.

The 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions significantly reduced the urinary concentration of PGD_2 metabolite 9α,11β-PGF_2 at Day 21 compared to Day 0 post EVH. The increase in urinary 9α,11β-PGF_2 following EVH on the placebo, and at Day 0 of the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions is similar to that shown previously in response to exercise challenges in asthmatics, rising from a baseline of ~30-40 $ng \cdot mmol^{-1}$ creatinine up to ~80 $ng \cdot mmol^{-1}$ creatinine (Mickleborough, et al. 2003). The 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA interventions significantly attenuated the rise in post EVH 9α, 11β-PGF_2 urinary concentration at Day 21 when compared to Day 0 and Day 21 of the placebo intervention, the concentrations were subsequently similar to the control participants.

Urinary 9α,11β-PGF_2 is the primary metabolite of PGD_2 and has been reported to increase after exercise, hyperpnoea induced, and allergen induced bronchoconstriction (Mickleborough, et al. 2003, Tecklenburg-Lund, et al. 2010, Brannan, et al. 2003, Kippelen, et al. 2010b, Bochenek, et al. 2004). PGD_2 is a potent bronchoconstrictor that is primarily produced by activated mast cells and in smaller amounts by inflammatory eosinophil cells (O'Sullivan, et al. 1998, Dahlen and Kumlin 2004). Mast cell activation is significantly increased in asthmatics (O'Sullivan, et al. 1998), and the numbers of mast cells present in sputum samples of asthmatics is raised after exercise (Mickleborough, et al. 2006). The ω3-PUFA interventions reduced the 9α,11β-PGF_2 urinary concentration at Day 21 suggesting less degranulation of mast cells, thereby causing a reduction in PGD_2 and a suppressed inflammatory response to HIB. Despite this reduction in 9α,11β-PGF_2, there was not a complete inhibition of HIB in the asthmatic participants which supports the notion that the pathogenesis of asthma and EIB is multifaceted, suggesting PGD_2 suppression alone does not explain the full reduction in bronchoconstriction. This may be due to a time lag between urinary 9α,11β-PGF_2 and that occurring in the airways. Mickleborough et al., (2003) showed a steady increase in urinary 9α,11β-PGF_2 concentration following an exercise challenge in asthmatics receiving no dietary interventions, peaking at 120 minutes post challenge.

It makes sense that PGD_2 alone does not explain the full benefits of ω3-PUFA as there are numerous arachidonic acid derived eicosanoids, and pro-inflammatory cytokines implicated in its pathogenesis (Hallstrand and Henderson 2009) and these may also respond favourably to ω3-PUFA interventions. Although not measured in the current study previous research suggests that increases in EPA and DHA content following ω3-PUFA supplementation are sufficient to ameliorate pro-inflammatory cysteinyl leukotriene production (Mickleborough, et al. 2003, Mickleborough, et al.

2006, Tecklenburg-Lund, et al. 2010). The long chain ω3-PUFAs of EPA and DHA competitively inhibit the metabolism of arachidonic acid which is the precursor for 2-series prostaglandins and 4 series leukotrienes. ω3-PUFA reduces the levels of arachidonic by the partial replacement of arachidonic acid in the phospholipid membranes of inflammatory cells primarily with EPA that was provided from the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ doses. EPA is the preferential substrate of the 5-lipoxygenase pathway, therefore increasing the quantity of EPA in the phospholipid membranes of inflammatory cells inhibits the release of pro-inflammatory eicosanoids from arachidonic acid specifically the 4-series leukotriene's (Calder 2007). leukotriene B_4, is a potent neutrophil chemoattractant and proinflammatory mediator, and the cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) cause smooth muscle contraction and bronchoconstriction (Manning, et al. 1990). Urinary leukotriene E_4 concentration has previously been shown to significantly reduce following high dose ω3-PUFA supplementation (Mickleborough, et al. 2003). The EPA derived eicosanoids 5-series leukotrienes are much less biologically active than those produced from arachidonic acid. For example EPA-derived LTB_5 is 10- to 100-fold less potent as a neutrophil chemoattractant compared with LTB_4 (Lee, et al. 1984). Furthermore high dose ω3-PUFA supplementation has been shown to supress pro-inflammatory cytokine concentrations (TNF-α and IL-1β) in individuals with EIB (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010).

*In vitro c*omparisons of EPA and DHA have shown that EPA is more effective in inhibiting pro-inflammatory mediator production and transcription from lipolysaccharide stimulated alveolar macrophage cells isolated from human asthmatics. EPA significantly suppressed the production of TNF-α, PGD_2, IL-1β, and LTB_4 compared to treatment with DHA (Mickleborough, et al. 2009). In contrast other

research showed that DHA can reduce the production of IL-1β, and IL-6 to a greater extent than EPA (Weldon, et al. 2007). The contrasting findings may well be due to the different cell lines taken from asthmatic individuals. Weldon et al (2007) used human THP-1 macrophages from a monocytic leukaemia cell line whereas Mickleborough et al (2009) studied a more relevant cell line of asthmatic alveolar macrophages. These findings may suggest that the most effective therapeutic benefit of ω3-PUFA may be a combination treatment of EPA and DHA for asthma, although further research is required on appropriate cell lines.

The 3.1g·d^{-1} ω3-PUFA intervention provided less EPA and DHA (1.8 g·d^{-1} EPA and 1.3 g·d^{-1} DHA) than previous research studies but still attenuated the reduction in lung function following EVH and reduced the PGD_2 metabolite urinary 9α,11β-PGF_2. This is a low level of daily DHA in comparison to previous research (2.0g·d^{-1}) (Tecklenburg-Lund, et al. 2010, Brannan, et al. 2014) and although DHA may have similar immunomodulatory effects as EPA, it does not act by direct inhibition of arachidonic acid. *In vitro* DHA can decrease the release of arachidonic acid from the phospholipid membrane of inflammatory cells by decreasing phospholipase A_2 activity (Martin 1998). *In vitro*, studies also reveal that DHA can decrease the responsiveness of inflammatory cells to toll-like receptors and suppress the activation of nuclear factor kappa β (NF-$_K$β) and subsequent inflammatory gene transcription (Lee, et al. 2003, Lee, et al. 2004). Further research is required *in vivo* and *in vitro* in relevant cell lines to establish the immunomodulatory effects of EPA and DHA in isolation in a dose response manner.

There are additional mechanisms of action for ω3-PUFAs that involve specialist pro-resolving mediators that are derived from both EPA and DHA and are termed resolvins and protectins; these have gained significant interest (Serhan, Yacoubian and

Yang 2008, Serhan, et al. 2011). The first identified bioactive pro-resolving mediator was termed resolvin E1 (RvE1). RvE1 is generated from EPA and was initially isolated from exudates. *In vivo* RvE1 reduces inflammation and *in vitro* it blocks human polymorphonuclear leukocytes migration (Serhan, et al. 2000, Serhan and Chiang 2008). Intraperitoneal administration of RvE1 has been shown to dampen airway inflammation and hyperresponsiveness in a murine model of asthma (Aoki, et al. 2008). Subsequently administration of RvE1 to mice intraperitoneally both during sensitisation to, and challenge with ovalbumin resulted in a decrease in airway eosinophil and lymphocyte recruitment and a reduction in Th2 cytokine and airway hyperresponsiveness (Aoki, et al. 2010). In the current study the 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA doses provided 3.7 $g \cdot d^{-1}$ and 1.8 $g \cdot d^{-1}$ of EPA respectively which may have resulted in significant generation of RvE1 (Seki, Tani and Arita 2009). Previous research suggests that RvE1 acts on several phases of asthmatic inflammation and modulates its anti-inflammatory and pro-resolving effects on various cell types (Aoki, et al. 2008, Aoki, et al. 2010). Further research is required to fully establish the roles of EPA derived resolvins in human asthma and HIB. It highlights a more novel role aside from arachidonic acid metabolite suppression for the influence of EPA on asthma and HIB.

With EPA key in competitive inhibition of arachidonic acid and formation of RvE1, it is of interest to note that many studies have supported the notion that DHA is a more potent regulator of resolvin and protectin formation (Levy, et al. 2007, Serhan, et al. 2002). The metabolism of DHA can result in the synthesis of protectin D1 (Serhan, et al. 2002). Protectin D1 actively resolves inflammation by reducing pro-inflammatory signalling (Levy, et al. 2007). *In vivo* murine studies have shown that protectin D1 injection significantly decreased subsequent bronchoconstriction during a methacholine

challenge (Levy, et al. 2007). Moreover, *ex vivo* research demonstrates that adding DHA to homogenised murine lung tissue resulted in a significant increase in protectin D1 concentration, suggesting DHA can be converted to protectins in the respiratory tissue (Levy, et al. 2007). Despite these proposed roles of DHA in inflammation, supplementation of high dose DHA in isolation has produced equivocal results in human asthmatics (Head and Mickleborough 2013).

Supplementing nine adult asthmatics with 4.0 $g \cdot d^{-1}$ of DHA in isolation for 3 weeks did not significantly alter pre and post EVH pulmonary function, markers of airway inflammation, or DHA metabolite concentration when compared to placebo (Head and Mickleborough 2013). This suggests that DHA supplementation taken alone cannot improve HIB and there are a number of reasons why DHA taken in isolation may not have significantly altered pulmonary function and markers of airway inflammation. In the current study the 3.1$g \cdot d^{-1}$ ω3-PUFA dose provided just 1.3$g \cdot d^{-1}$ of DHA, whereas the Head and Mickleborough, (2013) study provided 4.0$g \cdot d^{-1}$. We found significant improvements in pulmonary function using a combination of 1.8$g \cdot d^{-1}$ EPA and 1.3$g \cdot d^{-1}$ DHA. This suggests that either EPA alone may have greater immunomodulatory properties in HIB, or that a combination of EPA and DHA are required for maximum effectiveness in physically active asthmatics suffering with EIB. There is a wealth of research to support the effectiveness of DHA in reducing inflammation, but this is primarily conducted on cellular or murine models of asthma (Levy, et al. 2007, Weldon, et al. 2007, Yokoyama, et al. 2000). The Head and Mickleborough (2013) study and our current study suggest that further work is required to establish the anti-inflammatory and inflammatory resolving properties of EPA and DHA *in vivo* with human asthmatics with EIB and further suggests that a combination treatment of EPA and DHA may be the most effective.

In the current study, we have shown that both 6.2 $g \cdot d^{-1}$ and 3.1 $g \cdot d^{-1}$ ω3-PUFA doses significantly reduced F_ENO levels after 21 days of intervention in physically active asthmatics with HIB. Fraction of exhaled nitric oxide is now commonly used as a marker of asthma control and an objective measure of airway inflammation (Sandrini, et al. 2010) with levels shown to be raised in asthmatics (Kharitonov, et al. 1994). In asthmatics, F_ENO is an indicator of eosinophilic airway inflammation, raised IgE levels and allergic sensitisation (Strunk, et al. 2003). Exact reference values for F_ENO are not recommended due to confounding factors and overlap between normal and asthmatic populations (Dweik, et al. 2011). Despite this, a number of studies show a relationship between F_ENO levels and EIB (Scollo, et al. 2000, ElHalawani, et al. 2003, Buchvald, et al. 2005). The results of the current study support previous findings that high dose ω3-PUFA alone and in combination with montelukast can reduce pre-hyperpnoea F_ENO in EIB sufferers from 42.5 ± 6.6 ppb to 25.5 ± 3.9 ppb; and 23.2 ± 3.4 ppb respectively (Tecklenburg-Lund, et al. 2010). To the authors knowledge only one other study has directly investigated the response of F_ENO in asthmatics to ω3-PUFA interventions (Moreira, et al. 2007). Fourteen days of ω3-PUFA consumption in female asthmatics failed to reduce levels of F_ENO, however the daily ω3-PUFA intake was markedly lower than the 3.1 $g \cdot d^{-1}$ dose in the current study (0.91 g EPA and 0.65 g DHA vs 1.8 g EPA and 1.3 g DHA). It is likely that this dose was not sufficient to raise levels of EPA and DHA in the phospholipid membranes of inflammatory cells to influence F_ENO levels. Additionally no biochemical measure of compliance to the supplementation regime was undertaken.

Nitric oxide is produced in the airway by the conversion of L-arginine to L-citrulline, which is catalysed by enzymes known as nitric oxide synthases (NOS) (Nathan and Xie 1994). Elevated levels of F_ENO in asthmatics likely results from

increased expression of the inducible nitric oxide synthase (iNOS) in T lymphocytes, macrophages, airway epithelial cells, and inflammatory cells, and can be induced by certain pro-inflammatory cytokines notably TNF-α *in vitro* (Hamid, et al. 1993, Robbins, et al. 1994). The increased expression in response to pro-inflammatory cytokines is thought to be via the activation of transcription factor nuclear factor kappa β (NF-$_K$β) (Barnes 1995), and, importantly, ω3- and ω6-PUFAs are ligands and modulators for NF-$_K$β. This suggests that ω3-PUFAs influence inflammatory processes via modulating cytokine expression and activation of NF-$_K$β (Schmitz and Ecker 2008). Interestingly, high dose ω3-PUFA has been shown to suppress circulating plasma TNF-α in EIB sufferers (Mickleborough, et al. 2003) which may in turn influence NF-$_K$β activity. Consequently a possible mechanism of action of ω3-PUFA on F_ENO levels may be from ω3-PUFA reducing pro-inflammatory cytokine expression resulting in less activation of NF-$_K$β and lower expression of iNOS. Furthermore, previous research has shown ω3-PUFA supplementation to be equal to montelukast in reducing levels of F_ENO (Tecklenburg-Lund, et al. 2010). Montelukast reduces levels of F_ENO in patients with mild chronic asthma and works as a leukotriene receptor antagonist blocking Cyst-LT receptors (Drazen 2002). This could suggest that ω3-PUFA has a similar anti-inflammatory role working as a cysteinyl leukotriene antagonist to modify airway inflammation and reduce F_ENO levels.

6.2 g·d^{-1} and 3.1 g·d^{-1} ω3-PUFAs modulate inflammatory processes via a multitude of mechanistic pathways to influence the airway inflammation associated with HIB. It is likely that a combination of both EPA and DHA is needed for maximum effect, with the current study supporting the notion that lower levels of combined EPA and DHA are useful although an exact dose response relationship remains to be elucidated.

5.5. Conclusion

In conclusion, this study has shown for the first time that 3.1g·d^{-1} ω3-PUFA doses are as effective as higher doses in attenuating HIB in physically active asthmatics. It highlights that lower doses represent a potentially beneficial treatment for physically active asthmatics suffering with EIB whilst reducing the burden of cost, compliance and potential for GI distress. It is clear that the combination of EPA and DHA can provide anti-inflammatory effects to reduce airway inflammation as assessed by F_ENO and urinary 9α,11β-PGF_2. Further *in vivo* research is required to establish an exact dose-response relationship for EPA and DHA to allow for a greater understanding into their individual and synergistic roles in suppressing airway inflammation. This will increase current understanding of their individual and dual modulation of the arachidonic acid derived eicosanoids. In addition, it will allow for further understanding into their roles in serving as pro-resolving agonists and inflammatory antagonists, and their influence upon the airway epithelium to restore respiratory homeostasis in asthmatics and EIB sufferers.

6. Chapter 6 – Prebiotic trans-galactooligosaccharide (B-GOS) supplementation reduces the severity of hyperpnoea induced bronchoconstriction and inflammatory markers in physically active asthmatics

6.1. Introduction

Despite the range of pharmaceutical interventions, asthma remains poorly controlled in 50% of asthmatics largely due to poor treatment adherence (Barnes 2010). In addition, β_2-agonists target symptom relief rather than treating the pathogenesis of the disease, and long term corticosteroid treatment can have undesirable side effects (section 2.5.4). These limitations highlight the need for novel therapeutics which importantly target the underlying inflammation and immune response. A novel approach to improve the treatment and management of asthma and exercise induced bronchoconstriction (EIB) is to positively manipulate the gut microflora through pre- or probiotic supplementation. This may positively alter immune responses in asthmatics. As highlighted in section 2.3.1 a dysregulated gut microflora increases susceptibility to asthma and inflammatory mediated conditions. Recent evidence suggests that the gastrointestinal commensal bacteria could be a therapeutic target to influence the immune and inflammatory responses beyond the gastrointestinal tract thereby mediating allergy and asthma (Noverr and Huffnagle 2004, McLoughlin and Mills 2011) (see section 2.6.2 for more details).

The gastrointestinal (GI) microbiota includes over 1000 identified bacterial species that colonize the mucosal surface of the gut (Human Microbiome Project Consortium 2012) and it is involved in host immune system maturation in both a local and systemic manner. The intestinal microbiota plays an important role in the development and regulation of gut-associated lymphoid tissue (GALT), ligands and cells within the GALT regulate T helper 1 (Th-1), T helper 2 (Th-2) and regulatory T cell (Treg) differentiation. Although expressed by different phenotypes, asthma and EIB are both considered to be driven by activated mast cells, eosinophils, and Th-2 lymphocytes that collectively produce pro-inflammatory mediators. These mediators, cytokines, chemokines, and eicosanoids contribute to persistent

airway inflammation and are implicated in the onset of EIB (Wenzel 2013, Zietkowski, et al. 2009, Zietkowski, et al. 2011, Wan and Wu 2007, Boyce 2008).

The increase in prevalence of asthma in western societies could be associated with alterations in the GI microbiota as a result of changes in lifestyle, poor diet, and increased antibiotic use (De Filippo, et al. 2010, Adlerberth and Wold 2009). Dysbiosis of the gastrointestinal and respiratory microbiota can increase the risk of developing asthma in childhood (Hilty, et al. 2010, Böttcher, et al. 2000). Modulation of the GI microbiota through dietary supplementation may directly or indirectly alter the immune and inflammatory response and therefore impact on airway inflammation associated with asthma and EIB. There are a number of supplementation methods that can result in positive modulation of the GI microbiota, including probiotic, prebiotics and synbiotics.

The concept that supplementation in sufficient quantities of certain microorganisms can confer direct benefit onto the host is described by the term ‘probiotics’ (Marco, Pavan and Kleerebezem 2006). Probiotics are broadly defined as living, non-pathogenic microorganisms (usually bacteria) that can exert positive health benefits onto the host (Reid 2005). Only a minority of bacterial species meet this definition and these are primarily from the *Lactobacillus* and *Bifidobacterium* genera (Howarth and Wang 2013). Within the human body the gastrointestinal tract is home to the largest quantities of lactobacilli and bifidobacteria, which act as commensal bacteria and contribute to the maintenance of a healthy intestinal immune system. A number of studies have now shown the benefits of probiotics on health and disease through the maintenance of healthy gut microbiota (Moro-García, et al. 2013, Demirel, et al. 2013, Whelan and Quigley 2013). Importantly, evidence is accumulating for the use of probiotic supplementation in the treatment of allergies and asthma (Gutkowski, et al. 2010, Sagar 2014, Rose, et al. 2010, Isolauri, et al. 2000, Forsythe,

Inman and Bienenstock 2007). This is discussed in more detail in section 6.1.3. Probiotic supplementation can beneficially alter the microbiota composition within the gut from a pathogenic predominance towards a more beneficial microbiotic composition (Howarth and Wang 2013). However due to the transient nature of probiotic colonization, the microbial composition of the microbiota will return to the original state if probiotic supplementation is ceased. Furthermore, supplementation with varying strains of probiotic bacteria can have varying impacts on the microbiota composition. Probiotics also have to compete against the established colonic communities potentially reducing the efficacy of treatment.

As such, prebiotics are arguably a more effective way to manipulate the gut microflora than probiotics (Macfarlane, Steed and Macfarlane 2008). As outlined in section 2.6.2 a prebiotic (usually an oligosaccharide) is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microbiota, that confers benefits upon host well-being and health (Gibson, et al. 2004).

The main mechanisms of action of prebiotics is to selectively stimulate the growth of commensal probiotic bacteria. The majority of prebiotic research has focused on inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) (Macfarlane, Macfarlane and Cummings 2006). GOS has been shown to have a greater prebiotic effect (a stronger bifidogenic effect) than short chain FOS (Bouhnik, et al. 2004) and sections 6.1.1 and 6.1.2 provide more detail about GOS supplementation. A combination of pro- and prebiotic bacteria is termed a synbiotic (Schrezenmeir and de Vrese 2001), and has been used in the management of a number diseases (Kolida and Gibson 2011). A synbiotic may benefit the microbiome of individuals with dysbiosis as it provides both a probiotic (to increase beneficial bacteria numbers) and a prebiotic to selectively stimulate the growth of beneficial bacteria to positively manipulate the gut microflora (Kolida and Gibson 2011).

6.1.1. The use of probiotics, prebiotics, and synbiotics in allergy and asthma

The health benefits of probiotics in relation to a variety of immune-mediated conditions including allergy, asthma and infections of the upper respiratory tract have been described previously (Gill and Prasad 2008, Vliagoftis, et al. 2008). In human trials, upper respiratory tract infections and the severity of allergic rhinitis is reduced following the use of probiotics (Vliagoftis, et al. 2008). This systematic review found nine of the twelve randomised control trials showed meaningful improvements in clinical outcomes of allergic rhinitis following the use of probiotics (which included *Lactobacillus casei*, *Lactobacillus acidophilus, Lactobacillus paracasei,* and *Bifidobacterium*); however, none of the twelve showed any positive effects on asthma. This may be due to the small scale nature of the studies and diverse asthma heterogeneity. Furthermore, the variety of probiotic strains used are likely to have variable effects upon the composition of the GI microbiota and thus the immune system.

Probiotics have been used as a treatment option in a number of research studies investigating paediatric asthma. Forty-six children (aged 4-10 years) with mild to moderate asthma were randomly allocated into matched groups to receive probiotic Trilac® capsules (1.6×10^9 lactic acid bacteria cells: *Lactobacillus acidophilus* – 37.5%, *Bifidobacterium bifidum* – 37.5% and *Lactobacillus delbrueckii subsp. Bulgaricus* – 25%) or placebo for 12 weeks (Gutkowski, et al. 2010). Asthma exacerbations and use of bronchodilators were significantly less in the children who received the probiotic capsules compared to those receiving placebo. Immunological parameters studied by flow cytometry differed between groups. There was an increase in the expression of HLA-DR, a monocyte activator, in the Trilac® group compared with the placebo. Mean percentage (range) of HLA-DR positive monocytes increased from baseline to week 12, 90 (72-98)% vs 96 (89-99)% respectively (*p*

= 0.004). The subset of percentage CD8/CD45RA+ cells (naive cell marker) decreased in peripheral blood mononuclear cells from children with atopic asthma after probiotic treatment from 28% (visit 1) to 25% (visit 4) (p =0.03). As HLA-DR is considered a marker of monocyte activation, and CD45RA is a naive cell marker, the authors suggested that the probiotic treatment led to the activation of monocytes and suppression of lymphocyte proliferation suggesting a potential immunogenic effect of the probiotic capsules (Gutkowski, et al. 2010). Current evidence suggests that asthma is characterised by a relative deficiency in Treg cell function, which allows Th-2 cells to proliferate. The Treg cell functioning can be measured through examination of Foxp3 expression in isolated PBMCs and with allergic asthma characterised by an imbalance in Th1, Th2, and Treg cells, the exact immunological effect could have been further explored in the Gutkowski et al. (2010) study through this examination of Foxp3 in the PBMCs. Foxp3 expression has been shown to be reduced in asthmatics (Provoost, et al. 2009), but may respond to probiotic treatment, as shown in a murine model of chronic asthma (Sagar, et al. 2014). Increases in Foxp3 expression allow for greater Treg cell functioning which benefits immune function in asthmatics by suppressing the dominance of Th2 cell expression and associated inflammation (Sagar, et al. 2014, Provoost, et al. 2009).

The use of synbiotics could confer a greater health benefit through the combination of both pro- and prebiotics. To date, two studies have explored the effects of synbiotics on allergy. Children with atopic dermatitis (AD) have an increased risk of developing asthma with approximately 40% of children with atopic dermatitis developing asthma in later childhood (Gustafsson, Sjöberg and Foucard 2000). Early intervention with a synbiotic supplement was investigated in ninety infants (<7 months) with AD to investigate the impact on asthma symptoms (Van der Aa, et al. 2011). The synbiotic was delivered in a hydrolysed

formula and included the combination of *Bifidobacterium breve* M-16V and a galacto/fructooligosaccharide mixture (Immunofortis®); this was compared to the same formula without the synbiotic mixture. At a one year follow up the prevalence of 'frequent wheezing' and 'wheezy noisy breathing apart from colds' was significantly lower in the synbiotic group compared with the placebo group. Significantly fewer children in the synbiotic group compared to the placebo group had started to use asthma medication (5.6% vs 25.6%, absolute risk reduction -20.1% CI -35.7% to -4.5%). However, no changes in total IgE levels were evident. The results suggest that this symbiotic reduced asthma-like symptoms in AD, although further research is needed to elucidate the mechanisms.

In allergic adult asthmatics receiving 4 weeks of synbiotics (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides: Immunofortis® and *Bifidobacterium breve* M-16V), treatment significantly increased (50%) morning and evening peak expiratory flow compared to placebo (Van de Pol, et al. 2010). The synbiotic was associated with a significantly lower increase in serum IL-5 24h after house dust mite challenge compared to the placebo (mean (± SE) increase in serum IL-5 at 24 h after challenge: from 2.5 (1.5) to 74.1 (28.4) $pg \cdot mL^{-1}$ after placebo vs from 1.4 (0.4) to 36.3 (18.7) $pg \cdot mL^{-1}$ after synbiotics, $p = 0.034$). Similar reductions were shown in *ex vivo* allergen-induced Th2-cytokine (IL-5, IL-4 and IL-13) production by PBMCs (Van de Pol, et al. 2010). However, treatment did not reduce bronchial inflammation as assessed by sputum eosinophil counts. The treatment with synbiotic was able to significantly reduce systemic production of Th-2-cytokines and improve peak expiratory flow. Although no direct reduction in bronchial inflammation was found, this suggests that the gut microflora may alter systemic inflammation, but not selectively reduce bronchial inflammation. To date, no research has

investigated strategies to manipulate the gut microflora in adult asthmatics suffering with EIB.

Since alterations in the gut microbiota can influence immune function and inflammation beyond the gastrointestinal tract (Noverr and Huffnagle 2004), it could be a novel therapeutic target for asthmatics suffering with EIB. With asthma and EIB known to be associated with over expression of Th2 cells, and suppression of Treg cells, therapeutic strategies to alter this balance warrant further investigation. Probiotics are known to alter T cell function and supress immune disorders (Kwon, et al. 2010), thus administration of a prebiotic supplement to increase the quantity of bifidobacteria in the GI tract of asthmatics could be a novel treatment option for EIB sufferers.

6.1.2. Prebiotic Trans-Galactooligosaccharide (B-GOS) and its influence on immune function and inflammation

As described in 2.6.2.1 B-GOS is highly bifidogenic favourably altering the commensal bacteria within the gut (Depeint, et al. 2008, Vulevic, et al. 2008) which may, in turn, have a subsequent impact on immune function. Previous research into the use of B-GOS has focused primarily on adult and elderly populations with little evidence for its impact on allergy and asthma. In relation to inflammation the effect of B-GOS on cytokine production in PBMCs from elderly participants has been investigated. Five weeks of supplementation with B-GOS ($5.5 g \cdot d^{-1}$) resulted in decreased production of TNF-α ($p < 0.01$) and IL-6 ($p < 0.05$), and increased production of IL-10 ($p < 0.05$) compared to baseline and placebo. At 10 weeks of supplementation B-GOS resulted in reductions of IL-1β ($p < 0.01$) compared to baseline, and TNF-α and IL-6 when compared to baseline and week 5, and when compared to

placebo ($p < 0.05$) (Vulevic, et al. 2008). Increased levels of TNF-α and IL-6, and suppression of IL-10 are implicated in the pathogenesis of asthma and EIB (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Wong, et al. 2001). If these cytokines can be modulated by B-GOS in the elderly it is possible that they may respond favourably in asthmatics who consume B-GOS. In addition to the alterations in pro- and anti-inflammatory cytokines, from faecal analysis the Vulevic, et al. (2008) study showed that the B-GOS supplement was able to significantly increase the number of beneficial probiotic bacteria within the gut of healthy elderly volunteers. If B-GOS can increase the number of beneficial bacteria in the gut it could be a novel therapeutic strategy for asthmatics.

The research suggests that prebiotics can increase the growth of probiotic bacteria in the gut including lactobacilli and bifidobacteria. These probiotic bacteria then make contact with underlying immune tissues and cells and can bring about strain-specific immunomodulation (Winkler, et al. 2007), including induced cytokine production in a concentration dependent manner (Marin, et al. 1998). This is shown by altered concentrations of pro and anti-inflammatory cytokines (Vulevic et al. 2008). Increasing the quantity of beneficial probiotic bacteria in response to B-GOS supplementation can help to regulate the balance between necessary and excessive immune responses. Prebiotics are fermented to varying degrees by the commensal probiotic bacteria (bifidobacteria and lactobacilli) with the immunomodulatory properties mainly attributed to specific microflora-dependent effects (Vulevic, et al. 2008). Probiotic bacteria can enhance phagocytosis, and alter Th1 responses associated with the release of pro-inflammatory cytokines, and simultaneously induce anti-inflammatory cytokines through regulatory T cells (Winkler, et al. 2007). In elderly participants, it appears that B-GOS can have significant immunomodulatory effects, primarily resulting in less pro-inflammatory cytokine production and increased anti-inflammatory

cytokine production (IL-10). The authors speculate that the mechanism of action for increased IL-10 production likely involves the nuclear transcription factor kβ pathway (Vulevic, et al. 2008). IL-10 is a significant immunoregulator with potent anti-inflammatory and immunosuppressive properties and the suppression of pro-inflammatory cytokines by IL-10 is regulated by the nuclear transcription factor kβ (Baldwin 1996). Nuclear transcription factor kβ can play a critical role in the chronic inflammation that underlies asthma and EIB (Barnes 1996) and can be activated by cytokine TNF-α, furthermore it can regulate the expression of chemokines CCL17, and CCL11 which are also implicated in asthma (Lukacs 2001, Berin 2002). If B-GOS can result in reductions in cytokines and chemokines that are implicated in asthma, serum levels of TNF- α, CCL17, and CCL11 could be suitable markers for establishing alterations in the inflammation associated with EIB and the efficacy of B-GOS in asthma trials. In addition to their effect on probiotic bacterial numbers in the gut and the resulting effect on the immune system some prebiotics are reported to bind to specific receptors on cells of the immune system suggesting that a direct interaction between prebiotics and the host is also a possibility (Vos, et al. 2007, Hoyles and Vulevic 2008).

Similar to asthmatic patients, the gut microbiota of overweight individuals has a reduced microbial diversity and is accompanied by increased levels of inflammation (Abrahamsson, et al. 2014, Vulevic, et al. 2013). A double-blind, randomised, placebo controlled cross over study assessed the use of B-GOS in 45 overweight adults through markers of metabolic syndrome, immune function, and alterations in the gut microbiota (Vulevic, et al. 2013). Participants received $5.5g \cdot d^{-1}$ B-GOS or placebo maltodextrin for 12 weeks. B-GOS was shown to increase the number of faecal bifidobacteria at the expense of less desirable groups of bacteria. Although the study found no significant changes in cytokine production from whole blood cultures, it did find reduced levels of plasma c reactive protein

(CRP) at 12 weeks of B-GOS intervention ($p < 0.0012$) which the authors attributed to a positive shift in gut inflammation. C reactive protein can indicate a level of low grade inflammation and is an important marker underlying many common diseases. Increases in serum CRP have been associated with airflow obstruction and airway inflammation and may serve as a surrogate marker of airway inflammation in asthma (Takemura, et al. 2006). Therefore if B-GOS treatment can reduce CRP levels in obese patients, and if it is associated with asthma it could be a useful marker in assessing the efficacy of B-GOS in EIB patients who experience greater levels of airway inflammation.

In general, the use of B-GOS for the treatment of inflammatory and immune diseases is a novel area. Since asthma is described as a chronic inflammatory disorder the use of B-GOS in the treatment of this disease warrants future investigation.

6.1.3. Rationale for prebiotic use in asthma and EIB

The research area of pre- and probiotic treatment for airway associated diseases is growing. To date it suggests that oral treatment to favourably manipulate the gut microflora may prove to be a novel therapeutic strategy to attenuate major characteristics of an asthmatic response including airway hyperresponsiveness, local cytokine influx and airway eosinophilia in adults and children (Sagar, et al. 2014, Gutkowski, et al. 2010, Vliagoftis, et al. 2008, Provoost, et al. 2009, Van de Pol, et al. 2010), and murine models (Sagar, et al. 2014). Therefore, it is possible that the gut microflora could be a significant therapeutic target for systemic inflammatory airway disorders (Gollwitzer and Marsland 2014, Nagalingam, Cope and Lynch 2013).

Thus the aims of the study were to investigate a 3 week intervention of a prebiotic trans-galactooligosaccharide (B-GOS) on the severity of hyperpnoea induced bronchoconstriction (HIB) in physically active asthma sufferers and non-asthmatic controls using a randomised double-blind placebo controlled cross-over design. Second it was to investigate markers of airway inflammation to gain an understanding into the systemic cytokine and chemokine levels of asthma participants with diagnosed HIB and their responses to B-GOS. Markers of airway inflammation to be measured included serum TNF-α concentration which is implicated in the pathogenesis of EIB, and shown to be supressed in response to B-GOS treatment and other nutritional interventions (Mickleborough, et al. 2003, Vulevic, et al. 2008, Mickleborough and Rundell 2005). The inflammatory cytokine IL-33 (section 2.2.2.2) is a newly discovered member of the interleukin-1 family of cytokines and is an important contributor to allergic responses and asthma (Prefontaine, et al. 2009). It is expressed by T helper 2 cells and mast cells following a pro-inflammatory stimulation both of which have been implicated in the pathogenesis of EIB (Wenzel 2013). To date, no research has quantified the serum levels of IL-33 in a cohort of physically active asthmatics with EIB and this warrants further investigation. The current study will also aim to measure serum chemokine levels CCL11, CCL17, and CCL5 which are previously underreported in this population of asthmatics. CCL11 and CCL17 are Th2 cell-specific chemokines, and amplify the recruitment of Th2 cells to sites of inflammation to generate a self-sustaining pro-inflammatory loop (Teran 2000, Bloemen, et al. 2007). This makes them an important target to reduce the airway inflammation associated with asthma and EIB (Garcia, Godot and Humbert 2005). Furthermore, serum C-reactive protein (CRP) and immunoglobulin E (IgE) will be measured in the asthmatic and control groups as markers of low-grade systemic

inflammation. These are known to be raised in asthmatics and CRP can respond favourably to B-GOS supplementation (Vulevic, et al. 2008).

6.2. Methods

6.2.1. Participants

Eleven asthmatic HIB positive (6 male, 5 female) and 11 non-asthmatic non-HIB (4 male, 5 female) recreationally active participants volunteered for the study and provided written informed consent. Prior to the study all participants had to meet the inclusion and exclusion criteria outlined in Appendix 6. All volunteers (HIB asthmatics and non-HIB controls) underwent an initial screening session to determine the presence of HIB. Participants were excluded if resting forced expiratory volume in 1 second (FEV_1) was less than 65% of predicted (Argyros, et al. 1996, Knudson, et al. 1983). Participants presenting to the study with a GP diagnosis of asthma and a history of shortness of breath and wheezing after exercise had to have a $\geq$ 10% reduction in FEV_1 following bronchoprovocation with eucapnic voluntary hyperpnoea to be diagnostic (Anderson, et al. 2001) and included in the study. One male asthmatic was found to be non-diagnostic (<10% reduction in FEV_1) following bronchoprovocation and excluded from the study. Two controls declined to participate further. The study was approved by the Nottingham Trent University Human Ethics Committee, and all procedures were conducted in accordance with the Declaration of Helsinki. A total of 19 participants; 10 HIB asthmatics (age 27 ± 9 Yr; height 1.72 ± 0.08 m; body mass 69.80 ± 9.0 kg) and 9 non-asthmatic non-HIB controls (age 26 ± 4 Yr; height 1.74 ± 0.10 m; body mass 72.2 ± 12.0) completed the study (Figure 6. 1). Independent samples t-test revealed no differences in age, height, or body mass between groups ($p > 0.05$).

Current medication of included HIB participants was n = 5 Salbutamol; n = 3 Salbutamol and Budesonide; n = 1 Salmeterol and Budesonide; n = 1 Salbutamol, Budesonide, and formoterol. All participants had well controlled asthma and were on step 1-3

of the stepwise treatment of asthma (National Asthma Education and Prevention Program 2007).

Throughout the study, participants were instructed to adhere to their usual training regime and were asked not to partake in strenuous exercise 48 h prior to testing. On test days, participants abstained from caffeine and alcohol and arrived at the laboratory at least 2 h post-prandial. Asthmatics were instructed to cease their medication prior to each EVH challenge (inhaled corticosteroids: 4 days; inhaled long acting β2 agonists: 2 days; inhaled short acting β2 agonists: the day of the test) and were tested at least 2 weeks following recovery from any chest or upper respiratory tract infection (Dickinson, McConnell and Whyte 2011, Anderson, et al. 2001).

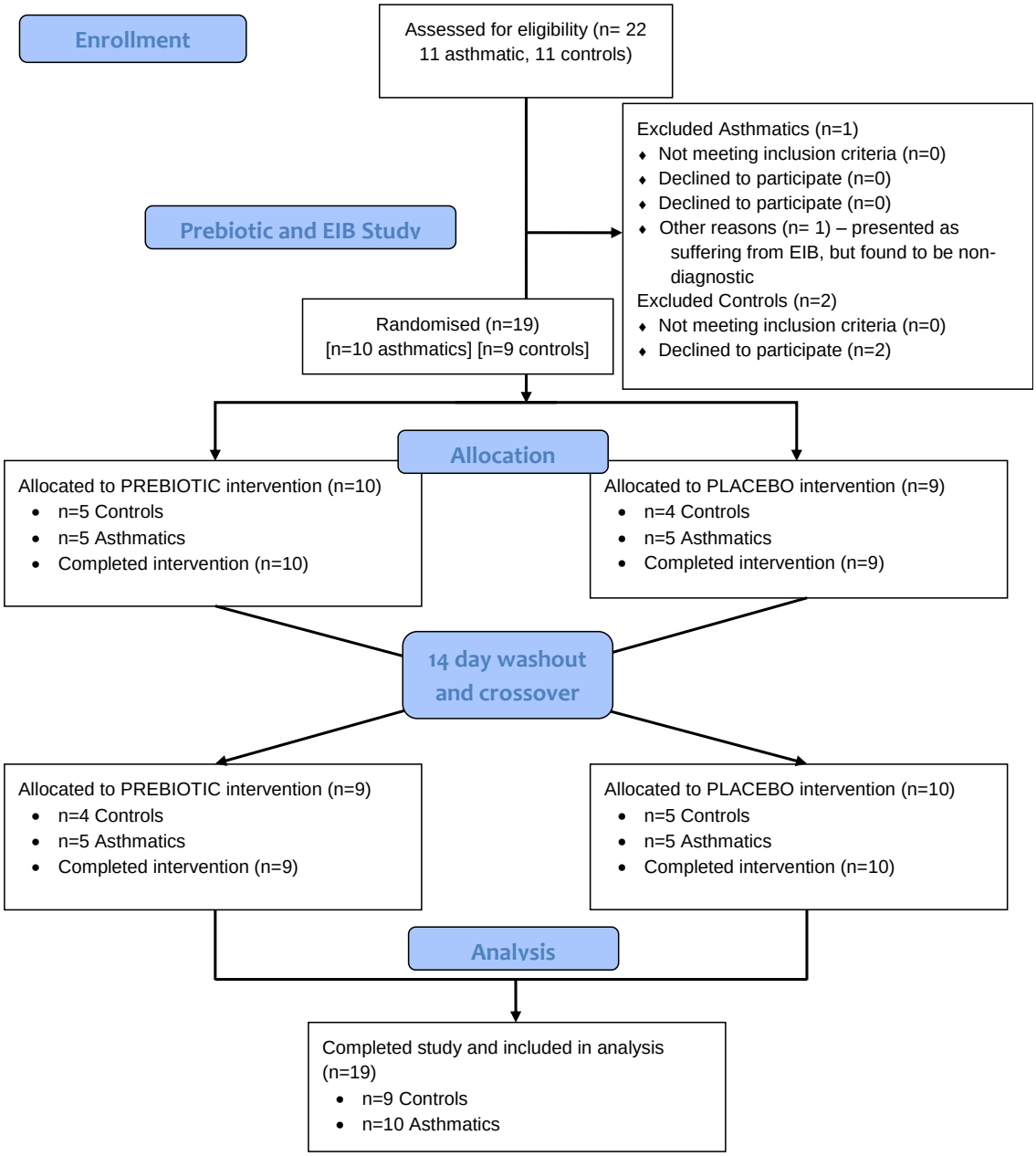

Figure 6. 1. CONSORT diagram of participant flow through the study.

6.2.2. Experimental design and protocol

The study was conducted as a randomised, double-blind, placebo controlled cross-over design over eight weeks per participant (Figure 6. 1). All participants entered the study on their normal diet after which they received in a randomised order one of the following interventions for 21 days:

Prebiotic (B-GOS) –5.5 $g \cdot d^{-1}$ (48% wt:wt of trans-galactooligosaccharide (B-GOS)) (Bi^2muno, Clasado, Milton Keynes, UK).

Placebo – Maltodextrin (Clasado, Milton Keynes, UK).

Placebo and prebiotic sachets were the same in shape, size and colour. Both contained white powder that was identical in taste, colour and texture. Participants followed a 2-week washout period (normal diet) between conditions. Prior to and for the first two days of supplementation all participants completed a food diary. This was then standardised for each condition and prior to every laboratory visit.

6.2.3. Pulmonary function, EVH, and F_ENO

Pulmonary function, EVH, and F_ENO (in EIB participants only) were conducted in accordance with the procedures outlined in Chapter 3. Following the EVH challenge, two reproducible flow-volume loops were measured at 3, 6, 10, 20, and 30 min, with the highest acceptable value recorded at each interval.

6.2.4. Collection of blood

Venous blood was drawn from a vein in the antecubital fossa region of the forearm via a 21 gauge needle (BD value-set, BD, Plymouth, UK). 20 mL of whole blood was collected at each time point (pre-EVH, 15 min, 60 min, and 24 h post EVH). Once drawn, it was immediately transferred into 5 mL serum separation tubes (Sanstedt AG & Co, Numbrecht, Germany). Tubes were gently inverted several times before being left to stand for 30 min at room temperature then centrifuged (Fisher Scientific Accuspin 1R, Fisher Scientific, Loughborough, UK) for 15 minutes at 1000 x g at 4°C. The serum supernatant was subsequently removed and stored in eppendorfs at −80°C until further analysis. After blood sampling, the needle was removed and medical tissue was applied under firm pressure to prevent a superficial haematoma.

6.2.5. Tumour necrosis factor-α (TNF-α) analysis

The concentration of serum TNF-α was assessed as a measure of systemic inflammation. Frozen serum samples were thawed and mixed by vortexing. Serum TNF-α concentration was determined in duplicate by high sensitivity ELISA (R & D Systems Europe., LTD, Abingdon, Oxford, UK). This ELISA did not cross-react with human IL-1β, IL-1α, IL-2-13, or TNF-β and was specific for the measurement of natural and recombinant human TNF-α. The minimum detectable dose of the assay was 0.106 $pg \cdot mL^{-1}$, intra-assay coefficient of variation was 11%, and inter-assay coefficient of variation was 10%, no samples were below the level of detection.

6.2.6. C-reactive protein (CRP) and Immunoglobulin E (IgE) analysis

Frozen serum samples were thawed and mixed by vortexing. Concentrations of serum CRP and IgE were assessed as measures of systemic inflammation and immune function and were determined by ELISA (Universal Biologicals, Cambridge, UK). CRP and IgE ELISAs did not cross react with other serum proteins, or immunoglobulins. The minimum detectable dose of the assays were 0.25 $ng \cdot mL^{-1}$ and 0.69 $ng \cdot mL^{-1}$ for CRP and IgE respectively. No samples were below the level of detection.

6.2.7. IL-33, CCL5, CCL11, and CCL17 analysis

Frozen serum samples were thawed, mixed by vortexing and spun at 10,000 x g for 5 min in order to remove any solid particles. Concentrations of serum CCL5, CCL11, CCL17 and IL-33 were determined through multiplex analysis (Luminex 100, Luminex Europe, Oosterhout, Netherlands) and Luminex screening assay plates (R & D Systems Europe., LTD, Abingdon, Oxford, UK). The Luminex multiplex technology allows for multiple protein profiling to occur simultaneously from a small volume of sample. The kits utilise colour-coded polystyrene microparticles that are coated with specific antibodies to specially recognise the analytes. The different analyte-specific beads are mixed and incubated with the serum sample. Captured analytes are subsequently detected using biotinylated detection antibodies and streptavidin-phycoerythrin conjugate. The Luminex 200 uses one laser to determine the colour of each bead, while a second laser determines the magnitude of the PE-derived signal, which is directly proportional to the amount of analyte bound. Multiplex technology has been used for the detection of multiple proteins including enzymes, cytokine, chemokines, and antigens in a number of disease states (Carson and Vignali 1999). All IL-33

and 53% of serum samples for CCL5 were found to be out of range for detection and so were excluded from further analysis.

6.2.8. Assessment of compliance

Participant's received two unlabelled bags containing a pre-counted number of sachets per intervention. Sachets were the same in shape, size and colour and had been blinded by the manufacturer (Clasado Ltd) and labelled L and T. Participants received 2 bags of the same labelled sachets and instructions to consume 1 sachet in the morning and 1 sachet in the evening dissolved in warm fluid for 21 days. Participants were contacted several times throughout the duration of the interventions to monitor progress. On completion of the intervention, participants returned the bags and the remaining sachets were counted. Compliance was expressed as the discrepancy between the expected numbers of returned sachets and the actual number used using the following equation: %compliance = (actual\expected) x 100. Compliance to the interventions was >88% indicating good compliance. The contents of the L and T sachets were not made available to the investigator until completion of the research study.

6.2.9. Statistical analysis

The area under the curve for pulmonary function measures $\%\Delta FEV_1$, FEV_1, and FVC ($AUC_{0\text{-}30}\%\Delta FEV_1$; $AUC_{0\text{-}30}FEV_1$; $AUC_{0\text{-}30}FVC$) were calculated over time for the 30 minute period post EVH using trapezoidal integration.

Data were analysed using statistical software SPSS for Windows version 22.00 (SPSS Inc., Chicago, IL, USA). The data were initially assessed for normality using Shapiro-Wilks test, and assessed for skewness and kurtosis. Three-way repeated measures ANOVAs were used to analyse the effects of intervention (Prebiotic vs Placebo) and day (Day 0 vs Day 21) with a between subject factor of group (HIB v Control) for $AUC_{0\text{-}30}$%ΔFEV_1, $AUC_{0\text{-}30}FEV_1$, $AUC_{0\text{-}30}FVC$. Within-group changes were further explored by analysing the HIB and Control groups separately using two-way repeated measures ANOVA. Within-group intervention x day interactions were analysed using a one-way ANOVA followed by *a-priori* t-tests to establish within intervention differences between Day 0 and Day 21. A significant *F* ratio ($p < 0.05$) was followed by *a-priori* paired samples t-tests within each group to establish differences between condition (Prebiotic and Placebo) and day (Day 0 and Day 21). Two-way repeated measure ANOVA (prebiotic vs placebo) and (Day 0 and Day 21) was used to assess differences in %ΔFEV_1 for each post EVH time point within each group.

The response of serum TNF-α to the EVH test was initially assessed by a one-way repeated measures ANOVA for time (Pre-EVH vs, 12 vs 60 vs 90 min post–EVH) for each day (Day 0 and Day 21) within each group (HIB and Control). Subsequently a four-way repeated measures ANOVA to establish the effects of intervention (Placebo vs B-GOS), day (Day 0 vs Day 21) and time (Pre-EVH vs Post-EVH), with a between subject factor of group (HIB vs Control) was completed. This was followed by three-way repeated measures ANOVAs for each group (HIB and Control) to analyse the effects of intervention (Placebo vs B-GOS), day (Day 0 vs Day 21), and time (Pre-EVH vs Post-EVH). In the HIB group only two-way ANOVAs for each day (Day 0 and Day 21) analysed the effects of intervention (Placebo vs B-GOS) and time (Pre-EVH vs Post-EVH). At day 21 in HIB group a one-way

ANOVA at each time point (Pre-EVH and Post-EVH) assessed the effects of intervention (Placebo vs B-GOS) followed by Bonferroni adjusted pairwise comparisons.

Serum CRP, IgE and chemokines (CCL11, CCL17) were not normally distributed (Shapiro-Wilks, $p < 0.05$). Subsequently, Friedman repeated measures test assessed responses in serum chemokine concentration to the EVH test (Pre-EVH vs 15 min vs 60 min vs 24 h). If no change was evident in response to EVH this was followed by Friedman repeated measures tests to assess the pre-EVH serum concentrations between day 0 and day 21 of the B-GOS and placebo interventions. Individual Wilcoxon signed rank tests with Bonferroni adjusted alpha level assessed within and between intervention differences. Effect sizes were calculated as the Z statistic divided by the square root of the n number where .1 is a small effect size, .3 is a medium effect size, and >.5 is a large effect size (Cohen 1988). Between groups analysis was conducted using a Kruskal-Wallis test and Bonferroni adjusted comparisons.

A two-way repeated measures ANOVA assessed differences in pre-EVH F_ENO within each condition (Day 0 versus Day 21) for the HIB participants only. Statistical significance was set as $p < 0.05$, all data are presented as mean ± SD unless otherwise stated.

6.3. Results

6.3.1. Pulmonary function and EVH challenge test

There were no significant differences at day 0 between each condition for pulmonary function values, response to EVH, and markers of airway inflammation within each group ($p > 0.05$), suggesting there was no carry over effect from the two-week washout period. Control participants had significantly higher pre-EVH FEV_1 values, and lower $\%\Delta FEV_1$, and $AUC_{0\text{-}30}\%\Delta FEV_1$ in response to EVH compared to HIB participants ($p < 0.05$) (Table 6.1).

The $\%\Delta FEV_1$ pre-EVH to post-EVH prior (day 0) to either the B-GOS or placebo interventions are shown in Table 6. 1. Twenty-one days of B-GOS supplementation in the HIB participants significantly improved the $\%\Delta FEV_1$ after EVH at the 3, 6, and 10 minute time points ($p < 0.05$), corresponding to improvements of 36 ± 17, 36 ± 19, and 43 ± 25% respectively (Figure 6. 2A). No significant changes in $\%\Delta FEV_1$ occurred following the placebo intervention ($p > 0.05$) and no changes were evident in the control group ($p > 0.05$) (Figure 6. 2B).

Table 6. 1 Pre- and post-EVH pulmonary function at day 0 of each intervention, Mean (±SD). *Significant difference between HIB and Control participants ($p < 0.05$).

Pre- and Post-EVH Pulmonary Function	**HIB Participants (n=10)**		**Control Participants (n=9)**	
Pre-EVH Day 0	**B-GOS**	**Placebo**	**B-GOS**	**Placebo**
FEV_1 (L)	3.43 ± 0.74	3.39 ± 0.70	3.88 ± 0.65*	3.89 ± 0.70*
% predicted	91.1 ± 13.8	90.2 ± 13.2	97.7 ± 9.2	97.7 ± 9.7
FVC (L)	4.33 ± 0.76	4.23 ± 0.86	4.72 ± 0.75	4.71 ± 0.83
% predicted	97 ± 10	95 ± 11	102 ± 8	102 ± 7
Post-EVH Day 0				
Maximum $\%\Delta FEV_1$,	-28 ± 14	-26 ± 15	-3 ± 3*	-3 ± 3*
$AUC_{0\text{-}30}\%\Delta FEV_1$	547 ± 385	495 ± 365	49 ± 65*	27 ± 55*

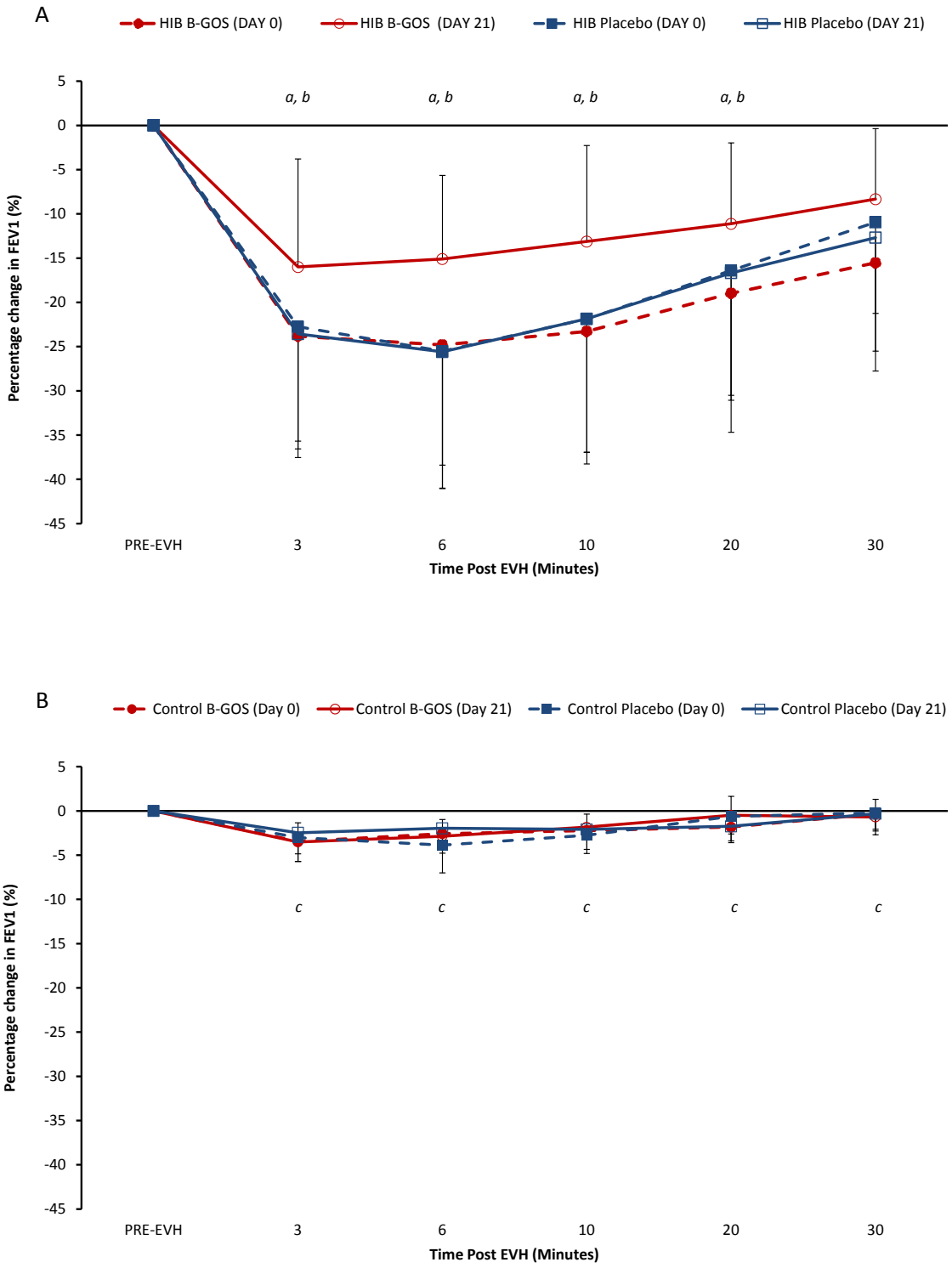

Figure 6. 2 A) The %ΔFEV$_1$ from Pre-EVH to post-EVH in participants with HIB for B-GOS and placebo conditions; B) The %ΔFEV$_1$ from baseline to post EVH in control participants for B-GOS and placebo conditions (Mean ± SD). *a* – significant difference within B-GOS intervention day 0 to day 21 ($p < 0.05$); *b* – significant difference at day 21 B-GOS vs placebo ($p < 0.05$) in HIB participants; *c* – significant difference between HIB participants and controls ($p < 0.05$).

The three-way repeated measures ANOVA for the $AUC_{0\text{-}30}\%\Delta FEV_1$ revealed a significant intervention x day x group interaction ($p < 0.01$). The $AUC_{0\text{-}30}\%\Delta FEV_1$ in the HIB participants differed between the interventions and between the days (two-way repeated measure ANOVA, intervention x day interaction, $p < 0.01$). A paired samples t-test revealed a reduction in $AUC_{0\text{-}30}\%\Delta FEV_1$ from day 0 to day 21 following the B-GOS intervention (582.53 ± 403.52 vs 345.44 ± 267; $p < 0.01$) equating to a 38 ± 18% improvement. No changes were evident following the placebo intervention ($p > 0.05$). No changes pre- to post-EVH in $AUC_{0\text{-}30}\%\Delta FEV_1$ at day 0 and day 21 for either intervention were evident for the control group.

The three-way repeated measures ANOVA for $AUC_{0\text{-}30}FVC$ revealed a significant intervention x day x group interaction ($p < 0.05$). The $AUC_{0\text{-}30}FVC$ in the HIB participants differed between interventions and days (two-way repeated measure ANOVA, intervention x day interaction, $p < 0.05$). A paired samples t-test revealed an increase in $AUC_{0\text{-}30}FVC$ from day 0 to day 21 following the B-GOS intervention (112.29 ± 24.94 vs 121.39 ± 25.56; $p < 0.01$) (Table 6. 2) corresponding to a 7% improvement in FVC. No significant changes were evident following the placebo intervention ($p > 0.05$). Control participants showed no differences in $AUC_{0\text{-}30}FVC$ ($p > 0.05$) on either of the dietary interventions.

Table 6. 2 Severity of HIB measured by $AUC_{0\text{-}30}\%\Delta FEV_1$; and $AUC_{0\text{-}30}FVC$ in HIB positive participants only. Mean ±SD *a* – significant difference compared to B-GOS day 0; *b* – significant difference compared to placebo day 21 ($p < 0.05$).

	B-GOS Day 0	**B-GOS Day 21**	**Placebo Day 0**	**Placebo Day 21**
$AUC_{0\text{-}30}\%\Delta FEV_1$	582.5 ± 403.5	345.4 ± 267.0 [a, b]	529.5 ± 304.0	543.5 ± 405.5
$AUC_{0\text{-}30}FVC$	112.9 ± 24.9	121.4 ± 25.6 [a ,b]	111.0 ± 31.3	110.2 ± 34.0

6.3.2. Fraction of exhaled nitric oxide (F_ENO)

In the HIB positive participants the two-way repeated measures ANOVA revealed no change in F_ENO after each intervention ($p > 0.05$) (Figure 6. 3).

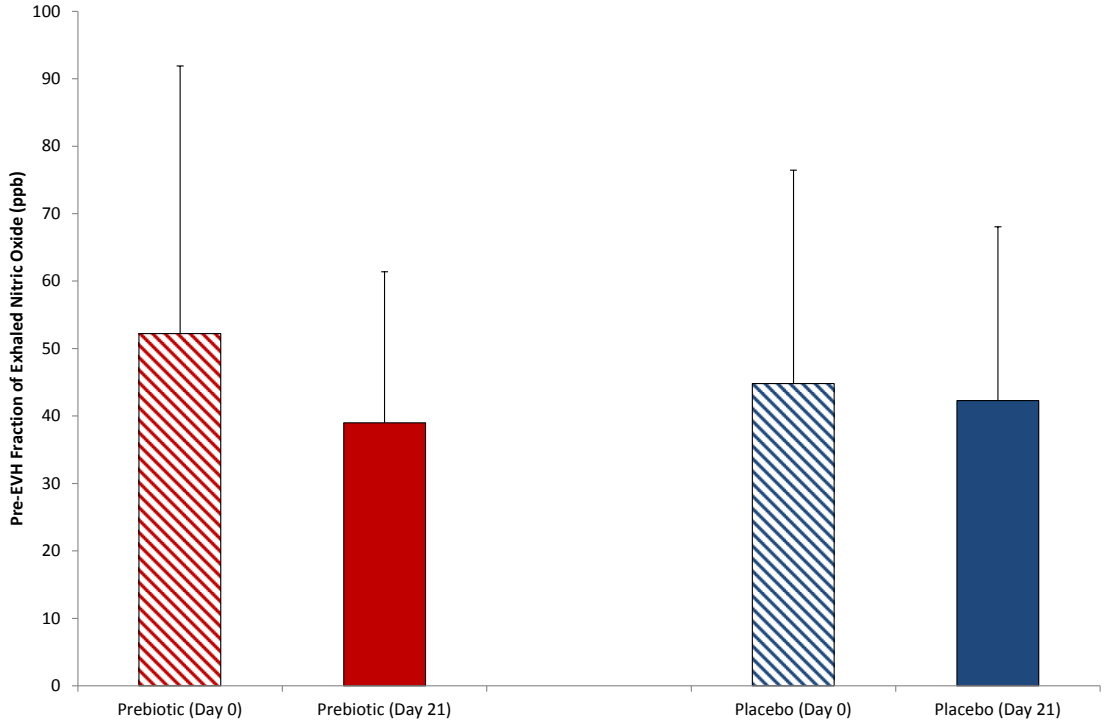

Figure 6. 3 Pre-EVH F_ENO in asthmatics only (Mean ± SD).

6.3.3. Serum cytokine concentration (TNF-α and IL-33)

Serum IL-33 concentrations were below the limit of detection and subsequently excluded from analysis. Figure 6. 5 panel A shows the serum TNF-α concentration prior to and post-EVH for HIB positive participants and panel B for control participants. Within-day one-way repeated measures ANOVAs revealed a difference in TNF-α concentration for prior to post-EVH in the HIB group, with no difference between post EVH values which were subsequently averaged (Figure 6. 4). Control participants showed no changes in TNF-α concentration over the course of the study. TNF-α concentration differed over the course of the study between HIB and control

participants (four-way repeated measures ANOVA, significant intervention x day x time x group interaction, $p < 0.05$). In HIB participants, serum TNF-α concentration differed between the placebo and B-GOS interventions (three-way repeated measures ANOVA, intervention x day x time interaction, $p < 0.05$). There was a significant within day effect in the HIB group ($p < 0.05$). For day 21 serum TNF-α concentration differed between interventions and time (intervention x time interaction, $p < 0.05$). Subsequent one-way ANOVAs at both Day 0 and Day 21, followed by Bonferroni adjusted pairwise comparisons revealed B-GOS resulted in a significant reduction in serum TNF-α concentration at day 21 for pre- and post-EVH, and compared to placebo day 21 ($p < 0.05$) (Figure 6. 5A). The post-EVH increase in TNF-α was suppressed by 36 ± 18% following the 21 days of B-GOS intervention compared to day 0.

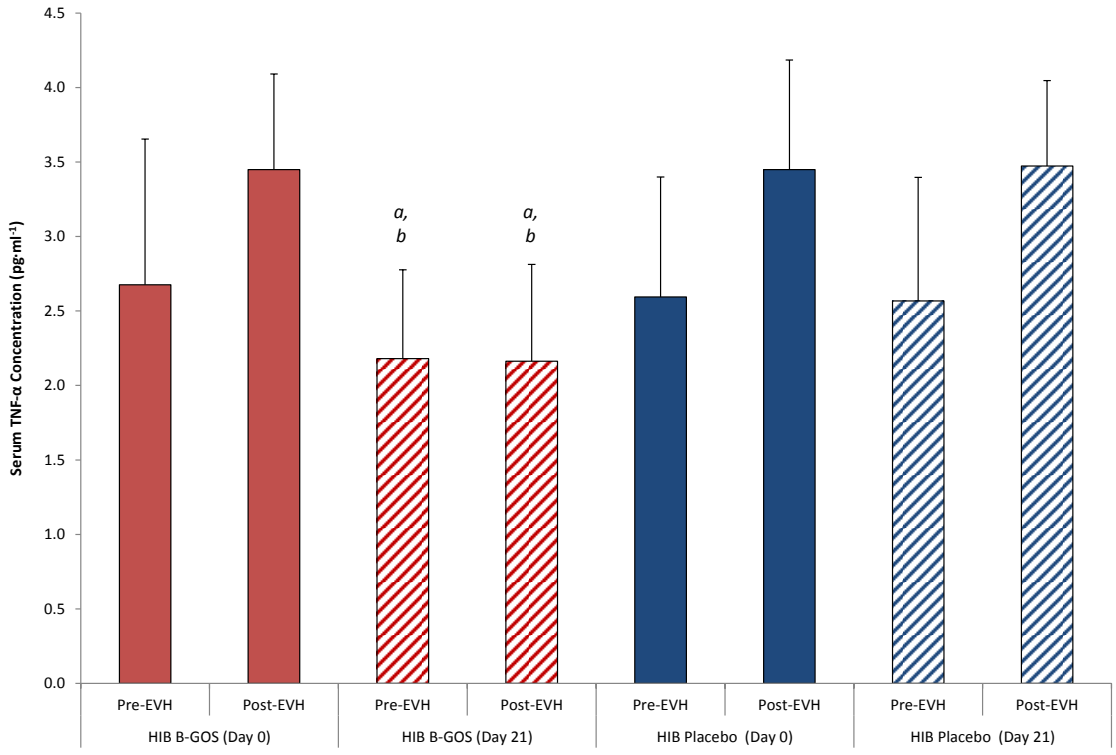

Figure 6. 4 Serum TNF-α concentration Pre- and post-EVH for HIB participants (Mean ± SD). *a* Significant difference B-GOS day 21 compared to B-GOS day 0 ($p < 0.05$). *b* significant difference B-GOS day 21 vs placebo day 21 ($p < 0.05$).

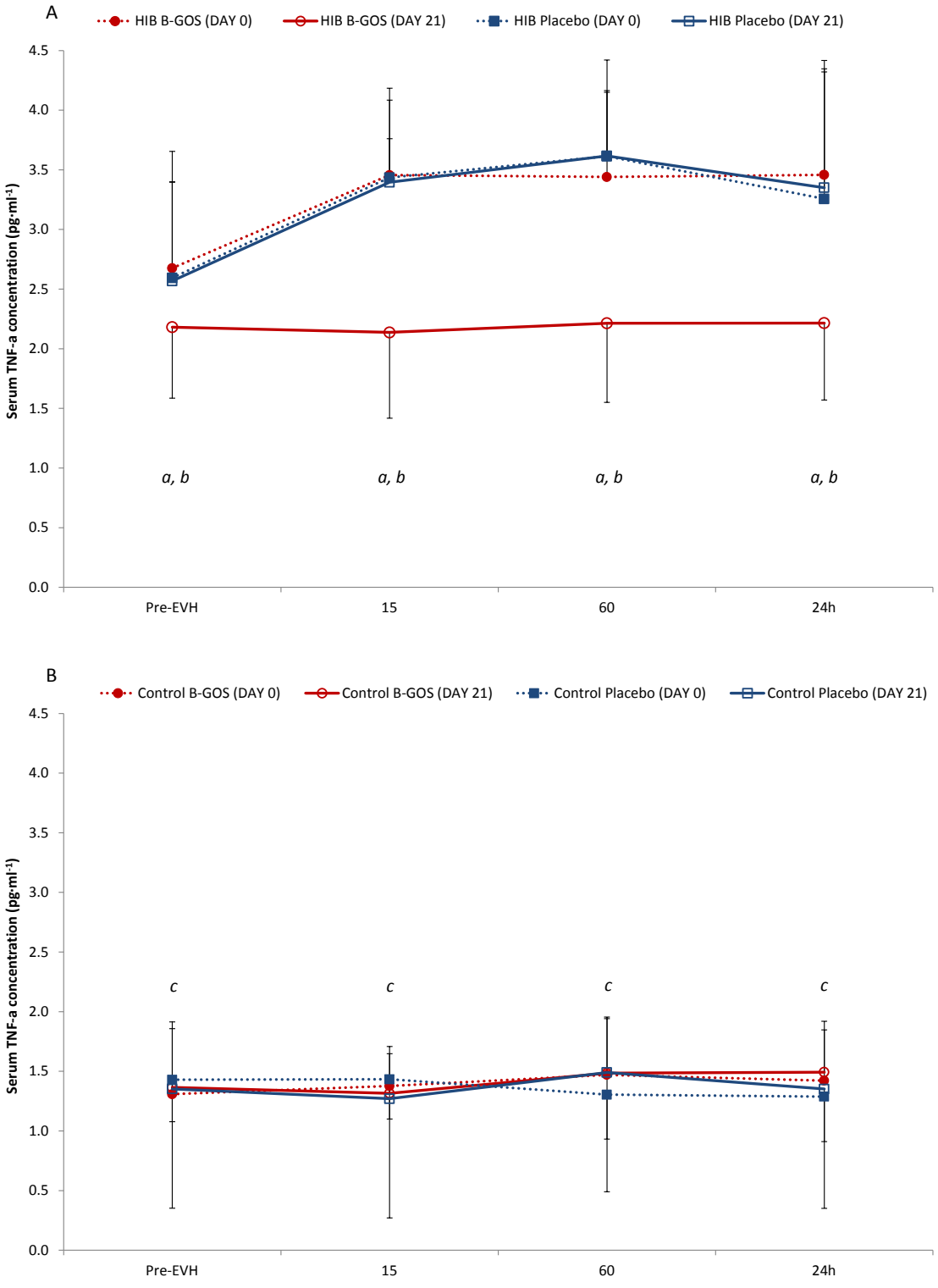

Figure 6. 5 Serum TNF-α concentration Pre- and post-EVH for HIB asthmatics (panel A) and controls (panel B (Mean ± SD). *a* – significant difference B-GOS day 21 compared to B-GOS day 0 B-GOS intervention ($p < 0.05$). *b* – significant difference B-GOS day 21 vs placebo day 21 in HIB asthmatics ($p < 0.05$). *c* – significant difference HIB asthmatics vs controls ($p < 0.05$).

6.3.4. Serum chemokine concentration

Friedman repeated measures test revealed no differences in pre- to post-EVH serum CCL17 concentrations in both asthmatic and control participants. Pre-EVH serum CCL17 concentrations differed over the course of the study ($p < 0.05$). In the HIB asthmatics, serum CCL17 concentration was reduced at day 21 of the B-GOS intervention when compared to day 0 (Wilcoxon signed rank tests with Bonferroni adjusted alpha level, $p < 0.0125$, with a large effect size, $r = .88$; see Figure 6. 6). In the HIB asthmatics, there was a trend for CCL17 day 21 in the B-GOS intervention to be less compared to day 21 in the placebo but it did not reach significance ($p = 0.06$), there was a large effect size however ($r = .61$). No changes were evident in the control group across the duration of the study. Serum CCL17 concentrations were greater in HIB asthmatics compared to controls at day 0 of the B-GOS, and day 0 and day 21 of the placebo intervention (Kruskall-Wallis test, $p < 0.0125$) see Figure 6. 6.

No changes in serum CCL11 concentrations were evident across the duration of the study in both the HIB asthmatics ($p > 0.05$), and control participants ($p > 0.05$). There was a trend for serum CCL11 to be greater in the asthmatics but this did not reach significance ($p = 0.07$) (Table 6. 3).

Table 6. 3. Serum CCL11 concentrations pre-EVH in HIB and control participants at day 0 and day 21 of each intervention. (Mean ± SD).

	B-GOS Day 0		B-GOS Day 21		Placebo Day 0		Placebo Day 21	
	HIB	CTRL	HIB	CTRL	HIB	CTRL	HIB	CTRL
Serum CCL11 ($pg \cdot mL^{-1}$)	201.02 ± 48.63	156.81 ± 72.58	201.90 ± 43.52	150.17 ± 58.86	214.18 ± 56.96	153.75 ± 72.02	219.84 ± 55.53	148.28 ± 34.67

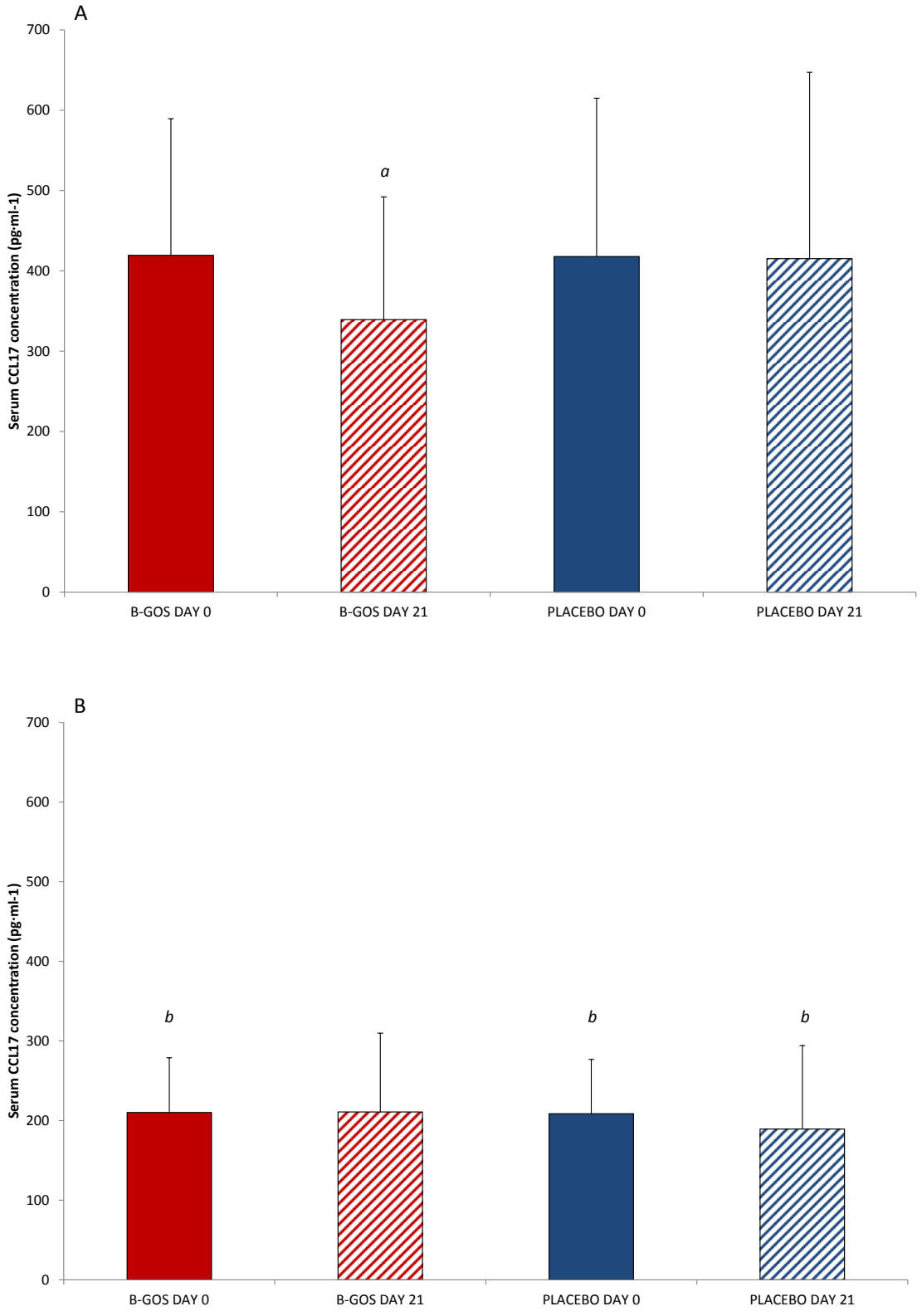

Figure 6. 6 Serum CCL17 concentrations in HIB (Panel A) and control groups (B) mean (± SD). *a* – significant difference day 0 to day 21 in HIB asthmatic group following B-GOS intervention; *b* – significant difference within-day between HIB and control group ($p < 0.0125$).

6.3.5. Serum CRP and IgE concentration

Friedman repeated measures ANOVAs revealed no change in serum CRP in response to EVH in both asthmatic and control participants. Pre-EVH serum CRP was significantly different between interventions and days in the HIB participants ($p < 0.05$). A-priori Wilcoxon signed rank tests revealed serum CRP had significantly reduced from day 0 to day 21 ($p < 0.0125$) in the HIB participants following the B-GOS intervention, with a large effect size ($r = 0.80$) (Figure 6. 7A). There was a trend for this reduction to differ from the placebo intervention but it did not reach significance ($p = 0.028$; effect size $r = 0.70$). No change was evident following the placebo intervention in the HIB participants ($p < 0.05$). Similarly, pre-EVH serum CRP was significantly different between interventions and days in the control participants ($p < 0.05$). A-priori Wilcoxon signed rank tests revealed serum CRP had significantly reduced from day 0 to day 21 following the B-GOS intervention in the control participants ($p < 0.0125$; effect size $r = 0.71$) (Figure 6. 7B). Again, there was a trend for day 21 in the B-GOS intervention to be reduced compare to day 21 of the placebo intervention but it did not reach significance ($p = 0.038$; effect size $r = 0.69$).

No significant changes in IgE over the course of the study in either the HIB or control groups were found. Kruskal-Wallis test revealed pre-EVH serum IgE concentrations were significantly greater in the HIB asthmatics when compared to control participants ($p < 0.0125$) see Table 6. 4.

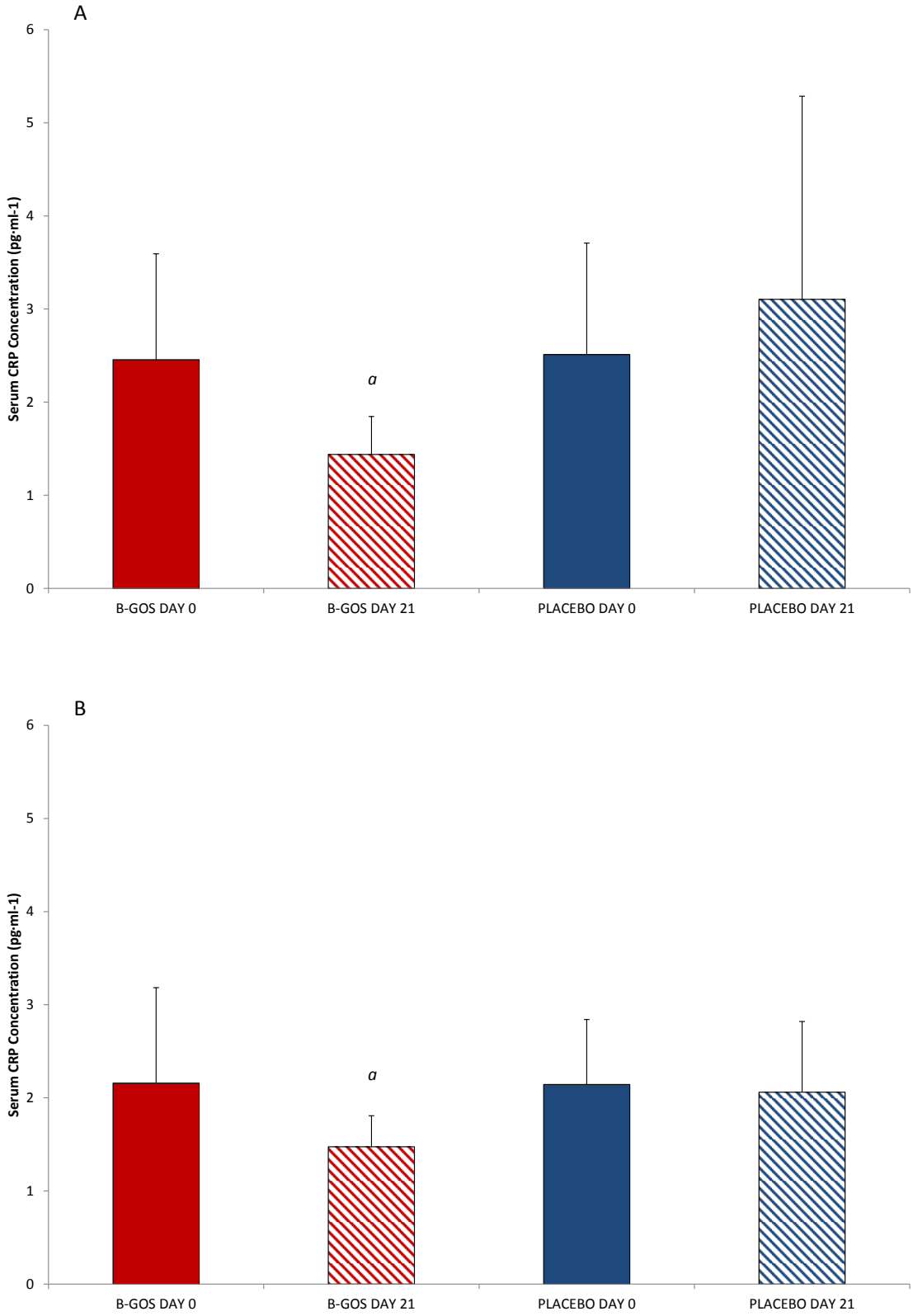

Figure 6. 7 Pre-EVH serum CRP concentrations in HIB asthmatics (Panel A) and control participants (Panel B) mean (± SD). *a* – significant difference day 0 to day 21 following B-GOS intervention ($p < 0.0125$).

Table 6. 4 Pre-EVH serum IgE (mean ± SD) in the HIB participants and control participants at day 0 and day 21 of both interventions. *a* – significant difference between HIB asthmatics and controls ($p < 0.0125$).

	B-GOS Day 0		B-GOS Day 21		Placebo Day 0		Placebo Day 21	
	HIB	Control	HIB	Control	HIB	Control	HIB	Control
Serum IgE ($KU \cdot L^{-1}$)	350 ± 174[a]	179 ± 129	357 ± 169[a]	153 ± 89	366 ± 177[a]	164 ± 105	364 ± 172[a]	174 ± 106

6.3.6. Summary of key findings

- 21 days of prebiotic B-GOS supplementation significantly attenuated the post-EVH decline in pulmonary function in the HIB participants.
- B-GOS supplementation reduced the concentration of pre- and post-EVH serum TNF-α in the HIB participants suggesting alterations in underlying immune function and inflammatory response to bronchoprovocation.
- B-GOS supplementation reduced the baseline serum concentrations of CCL17 in the HIB participants, and CRP in the HIB participants and controls. This suggests alterations in systemic inflammation and immune function may contribute to the reduced bronchoconstriction following EVH at day 21 of the B-GOS intervention.

6.4. Discussion

This study has demonstrated for the first time that a 21 day intervention of prebiotic galactooligosaccharide (B-GOS) can markedly reduce the severity of HIB in physically active asthmatics. The B-GOS intervention almost halved the post EVH decline in $\%\Delta FEV_1$ and this occurred in conjunction with reduced serum concentrations of TNF-α, CCL17, and CRP in the HIB asthmatics. After 21 days of B-GOS the change in the fall in FEV_1 in response to EVH from day 0 to day 21 was ~270 mL which is considerably greater than the smallest measurable change of 88 mL calculated from Chapter 4. Therefore we can be confident that the B-GOS intervention had a genuine meaningful impact upon the pulmonary function response to EVH to reduce the severity of HIB. This supports previous research whereby improvements in asthma symptoms and lung function have occurred in parallel to changes in immunological markers (Gutkowski, et al. 2010, Van de Pol, et al. 2010). The gut microbiota is instrumental in the development of the gut immune system and gut associated lymphoid tissue to establish immune tolerance (McLoughlin and Mills 2011), consequently it is likely to play a role in the regulation of allergic and asthmatic inflammatory responses. The microbial communities of both the gut and airways have been shown to be dysregulated in patients suffering with respiratory diseases (Madan, et al. 2012, Hilty, et al. 2010, Huang, et al. 2011). It is plausible that the bifidogenic properties of B-GOS were able to modify the gut microflora and influence the underlying inflammation of asthmatics and supress the subsequent inflammatory responses of HIB in response to EVH.

The dose of B-GOS chosen in the current study has previously been shown to significantly modify the faecal microflora profile and immune function in both young and elderly populations (Depeint, et al. 2008, Vulevic, et al. 2008). B-GOS is a non-

digestible carbohydrate, when consumed it is highly bifidogenic significantly increasing the numbers of beneficial bacteria, primarily bifidobacteria, at the expense of less beneficial bacteria, thereby improving the gut microflora (Depeint, et al. 2008). In conjunction with an improved microflora these initial studies found changes in markers of immune function showing suppression of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) and significant increases in phagocytosis and anti-inflammatory cytokine IL-10. Our current study extends the research knowledge of B-GOS further suggesting it can attenuate the bronchoconstriction associated with HIB and reduce chemokine and cytokine markers of airway inflammation in physically active asthmatics. Although not measured in the current study it is likely that 21 days treatment of B-GOS had a significant bifidogenic effect within the gut microflora similar to the previous research (Depeint, et al. 2008).

6.4.1. Pulmonary function response to B-GOS

The prebiotic B-GOS intervention attenuated the severity of HIB as measured by post EVH $AUC_{0\text{-}30}$%ΔFEV_1 by 38%. This level of improvement is similar to those found following both pharmacological and dietary interventions (Mickleborough, et al. 2003, Tecklenburg-Lund, et al. 2010, Villaran, et al. 1999). To our knowledge this is the first time a prebiotic intervention has been shown to improve the pulmonary function response of adult asthmatics to a bronchoprovocation challenge.

These findings are in broad agreement with the limited number of studies that have explored the effects of probiotics and synbiotics on lung function and immunological parameters in children with atopic asthma and atopic diseases (Gutkowski, et al. 2010, Van der Aa, et al. 2011, Van de Pol, et al. 2010). In 2010, Van

de Pol and colleagues found an increase in peak expiratory flow and inhibition of pro-inflammatory cytokine IL-5 (both measured at rest) following 4 weeks of synbiotic supplementation (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides: Immunofortis and *Bifidobacterium breve* M-16V) in asthmatic children. Direct comparisons between the current study and that of previous paediatric asthma research have to be made with caution. Adult asthmatics in the current study were likely to have more stable pulmonary function as it increases linearly with age up to 18 years (Wang, et al. 1993). The adult asthmatics in the current study had well controlled stable asthma, subsequently B-GOS is unlikely to offer any benefit on baseline pulmonary function in this cohort, but would be of benefit during HIB. The B-GOS supplement differed to the synbiotic used in the Van de Pol et al. (2010) study, it is possible that combined pre- and probiotics may have a greater therapeutic benefit in asthmatics who are likely to have reduced microbial diversity. Future research should look to combine B-GOS with bifidobacteria and lactobacilli. B-GOS is known to increase the quantity of bifidobacteria within the gastrointestinal tract, therefore a combined prebiotic and probiotic intervention to also include *lactobacillus* may confer a greater therapeutic effect than B-GOS alone.

A combined probiotic treatment (1.6 × 109 lactic acid bacteria cells: *Lactobacillus acidophilus* – 37.5%, *Bifidobacterium bifidum* – 37.5% and *Lactobacillus delbrueckii subsp. bulgaricus* – 25%) was shown to reduce asthma exacerbations and reliance upon medication in children and improve FEV_1 (Gutkowski, et al. 2010). However other studies using probiotic treatment in asthmatic children have been less clear, with weak effects in 131 asthmatic children (Rose, et al. 2010), or limited only to pregnant women with atopic dermatitis treated with 3 strains of *Lactobacillus* (Dotterud, et al. 2010). Attempting to establish the efficacy of probiotics is complicated

by the fact that some probiotic strains have varying immunomodulatory capabilities (De Roock, et al. 2010). This variability has been attributed to contrasting capabilities of probiotic bacteria to produce T-regulatory cells (Treg cells) and cytokines (Smits, et al. 2005). It is currently not fully understood if a global gut increase in a specific bacterial strain over a specific length of time will be sufficient to induce long-term sustainable immunologic effects to influence allergic asthma in all patients (McLoughlin and Mills 2011). Consequently the use of prebiotics as in the current study can enhance and promote the growth of the hosts commensal bacteria and this may represent a more applicable approach.

6.4.2. Serum TNF-α concentration response to B-GOS

Although not measured directly in the current study, previous research suggests that B-GOS is able to promote the growth of beneficial commensal bacteria within the gut (Depeint, et al. 2008, Vulevic, et al. 2008, Vulevic, et al. 2013). These increases may well have occurred within the asthmatics to subsequently impact on immunological and inflammatory function associated with asthma and HIB. In addition to improving pulmonary function in the HIB asthmatics, B-GOS attenuated inflammation, as evident by reductions in serum TNF-α.

Serum TNF-α concentration at Day 21 of the B-GOS intervention was significantly less when compared to day 21 of the placebo intervention in the HIB asthmatics. B-GOS has previously been shown to reduce TNF-α in isolated peripheral blood mononuclear cells (Vulevic, et al. 2008) from elderly participants after 5 weeks of intervention. This study shows for the first time that circulating serum TNF-α can also be modulated by B-GOS supplementation. The suppression of TNF-α by B-GOS

is an important indicator of immunological changes occurring within the HIB participants. TNF-α is a potent pro-inflammatory cytokine produced by a number of inflammatory and epithelial cells that have been implicated in the pathogenesis of asthma and HIB (macrophages, mast cells, eoisnophils, airway epithelial cells, $CD4^+$ lymphocytes) (Chung and Barnes 1999, Berry, et al. 2007). Furthermore TNF-α is associated with the contraction of airway smooth muscle and development of airway hyperresponsiveness (Amrani 2014).

At rest the HIB asthmatics had greater concentrations of TNF-α compared to the control participants, which supports previous reports of raised TNF-α in cross country skiers and elite athletes with EIB compared with non-asthmatic counterparts (Mickleborough, et al. 2003, Sue-Chu, et al. 1999). Chronic airway inflammation associated with asthma is in part maintained by the suppressive action of TNF-α on Treg cell function. Treg cells contribute to the maintenance of immune homeostasis in the airways by reducing inflammation and controlling immune tolerance. Commonly studied Treg cells in the context of pulmonary health and asthma are the natural thymic-derived CD4+Foxp3+ Treg cells and peripherally antigen-induced adaptive CD4+ Treg cells, which comprise both Foxp3-positive and -negative populations (Lloyd and Hawrylowicz 2009). Natural Treg cells suppress allergic responses to inhaled antigens such as grass or pollen (Ling, et al. 2004, Grindebacke, et al. 2004), and may respond to changes in the airway environment associated with dry-gas hyperpnoea. TNF-α plays a crucial role in bridging innate and adaptive immunity in chronic inflammation such as asthma and crucially it impairs the regulatory activity of natural Treg cells via the TNF-α receptor 2 (TNFR2) signalling pathway to down modulate Foxp3 expression (Lin, Shieh and Wang 2008). The transcription factor Foxp3 is a crucial regulator for Treg cell development and function and is currently the most specific Treg marker (Hori,

Nomura and Sakaguchi 2003, Fontenot, Gavin and Rudensky 2003). The B-GOS mediated reduction in serum TNF-α pre- and post-EVH suggests Treg cell expression may be altered (see section 6.5 for more details).

Future research to investigate the mechanisms by which B-GOS is effective in treating HIB should look to measure Foxp3 expression in isolated PBMCs from asthmatics. Although previous research suggests there are no differences in the number of peripheral blood $CD4^{+}CD25^{high}$ cells between asthmatic participants and control participants, importantly Foxp3 protein expression within the $CD4^{+}CD25^{high}$ Treg cells is significantly decreased in asthmatics (Provoost, et al. 2009). This reduction in Foxp3 protein expression may be linked to an increased TNF-α expression (Valencia, et al. 2006). Therefore it is attractive to speculate the B-GOS mediated reduction in TNF-α resulted in increased Foxp3 expression in this study. This is further supported by anti-TNF-α treatment (to inhibit TNF receptor signalling) resulting in priming naïve $CD4^{+}$ T cells towards regulatory IL-10^{+} T cells to improved immune regulation and function (Boks, et al. 2014).

The concentratrion of TNF-α is increased in response to exercise in asthmatics in both serum and sputum (Mickleborough, et al. 2003, Mickleborough, et al. 2006). In support of this we have shown increases in serum TNF-α following the EVH challenge suggesting similar pathophysiology in EIB and HIB. These increases were subsequently supressed by 36 ± 18% following the 21 days intervention of B-GOS this may indicate improved expression of Foxp3 within $CD4^{+}$CD25 Tcells to enhance immune regulation in the HIB asthmatics so dampening the bronchoconstrictive response to EVH. Previous research has shown Foxp3 mRNA delivered therapeutically can rebalance pulmonary T helper cell responses and protect murine models of asthma from allergen-induced inflammation and airway hyperresponsiveness (Mays, et al. 2013). Mechanistically

Foxp3 induction controls Th2 and Th17 inflammation by regulating innate immune cell recruitment through IL-10 dependent pathways. The B-GOS suppression in TNF-α in the current study might suggest alterations in Treg cell expression bought about by enhanced Foxp3 activity in the HIB asthmatics.

Although an exact cause and effect relationship cannot be made, the B-GOS treatment was able to reduce serum TNF-α concentration. Consequently this may indicate less suppression of Foxp3 protein that is commonly seen in asthmatics (Provoost, et al. 2009, Valencia, et al. 2006). This in turn will favourably alter the priming of naïve Tcells to support $CD25^{+}$ Foxp3 Treg cells to enhance immune function and reduce the inflammatory responses to EVH associated with HIB. Future research should look to measure serum levels of IL-10, and soluble CD4 as markers of altered Treg cell functioning and Foxp3 expression in PBMCs in HIB-asthmatics following B-GOS treatment. Commensal bacteria within the gut can regulate Foxp3 expression and subsequent Treg cell development to impact on immune function (Round and Mazmanian 2010) this could be a novel therapeutic mechanism for the use of B-GOSs in HIB. The proposed mechanistic relationship between prebiotic treatment and improved immune function to alter the inflammatory response to EVH is discussed further in section 6.5.

6.4.3. F_ENO response to B-GOS

In addition to altered Treg cell functioning, HIB in asthmatic individuals is associated with increased eosinophilic airway inflammation and F_ENO (Lee, et al. 2006). F_ENO is commonly used as marker of eosinophilic airway inflammation and levels in the HIB asthmatics in the current study are similar to those previously reported

in physically active asthmatics (Tecklenburg-Lund, et al. 2010). However, B-GOS did not confer any reduction in the F_ENO levels unlike the omega-3 PUFA intervention as seen in Chapter 5 and previous pharmacological interventions of montelukast (Tecklenburg-Lund, et al. 2010). It is possible that a longer duration of B-GOS supplementation is required to see a significant reduction in the raised F_ENO levels. The increased eosinophilic airway inflammation in HIB asthmatics highlights the role of eosinophil chemoattractant chemokines in the manifestation of the condition. Research suggests that local and systemic expression of the CC ligand chemokines such as CCL11 (eotaxin), and CCL17 (TARC), are increased in patients with asthma (Lilly, et al. 1999, Kalayci, et al. 2004, Zietkowski, et al. 2009, Zietkowski, et al. 2011).

6.4.4. Serum chemokine CCL11 concentration response to B-GOS

Chemokines can be produced in substantial amounts from structural cells including airway epithelial and endothelial cells, not only in response to their immunological environment but also as a response to microbial and viral stimuli. There was a trend for the HIB asthmatics to have greater serum CCL11 concentrations than the control groups, but due to high heterogeneity in this marker the increase did not reach significance. Future research should look to include a larger population of asthmatics with HIB when assessing CCL11 concentrations. Furthermore the asthmatics in the current study had well controlled stable asthma which may indicate no change in this chemokine. Despite this previous research shows that the expression of CCL11 is increased in the airways of asthmatics and CCL11 is an important attractant for eoisnophils which are upregulated in epithelial cells of asthmatics (Smit and Lukacs 2006). The expression of CCL11 has been found to be increased in the airways of asthmatics and the level of CCL11 is proportional to eosinophil infiltration and the

severity of bronchial hyperreactivity (Ying, et al. 1997, Lamkhioued, et al. 1997). Further to this human airway smooth muscle cells are capable of producing CCL11 upon stimulation of cytokines including IL-4, IL-5, and TNF-α. CCL11 is then involved in further recruitment of inflammatory cells into the airways to maintain an increased level of airway hyper-responsiveness which would further exacerbate HIB. As such treatments to reduce the activity of chemokines are warranted in asthma. To our knowledge this is the first time serum CCL11 values have been reported in a group of physically active asthmatics with documented HIB. The CCL11 serum concentrations of ~200 $pg \cdot mL^{-1}$ are similar to those reported in plasma of stable asthmatics (232 ± 188 $pg \cdot mL^{-1}$) (Tateno, et al. 2004). Despite the trend for raised serum CCL11 at day 0 in the HIB asthmatics, the B-GOS intervention did not reduce the concentration at day 21. The pathogenesis of HIB and asthma is known to be multifactorial with a number of inflammatory cells and mediators involved in its development. It is possible that although the B-GOS intervention significantly reduced the level of HIB in the asthmatic group this was independent of changes to CCL11. This further supports the finding of no reductions in F_ENO. It is possible that although CCL11 is known to be associated with airway inflammation the mechanisms of prebiotic action to induce a clinical improvement in HIB are independent of this chemokine. Further to this a limitation of the current study was that inflammatory markers were studied from the collection of serum, and as such only give an indication of systemic levels. It is possible the chemokines are likely to be in higher concentrations at the site of airway inflammation and may show more responses to treatment through the collection of sputum or exhaled breath condensate (Zietkowski, et al. 2009, Zietkowski, et al. 2011).

6.4.5. Serum chemokine CCL17 concentration response to B-GOS

The HIB asthmatics had significantly higher concentrations of serum CCL17 at day 0 of the interventions compared with the control group (408.69 ± 182.53 vs 239.73 ± 80.28 $pg \cdot mL^{-1}$). CCL17 is a key chemokine for attracting Th2 lymphocytes into the sites of inflammation (Imai, et al. 1999) it is also preferentially produced in the presence of Th2 cytokines to further increase the recruitment of inflammatory T cells such as $CD4^{+}$Th2 cells thereby generating a self-sustaining pro-inflammatory loop. Consequently chemokines and their receptors are important therapeutic targets in asthma because of this central role in cell recruitment and activation during inflammation. Plasma and serum CCL17 concentrations are shown to be raised in adults and children with asthma (Sekiya, et al. 2002, Leung, et al. 2002). To our knowledge this is the first study to report serum CCL17 concentrations in HIB asthmatics and they are similar to those found previously in allergic asthmatics (Sekiya, et al. 2002). The increased serum CCL17 in the asthmatics supports the notion of increased Th2 lymphocyte infiltration in the pathogenesis of the condition (Hallstrand, et al. 1998).

B-GOS supplementation reduced the pre-EVH concentration of serum CCL17 in the HIB participants which may be due to the bifidogenic properties of B-GOS. Manipulating the commensal bacteria through B-GOS supplementation will subsequently affect the production and expression of cytokines and chemokines associated with inflammatory responses (Wells, et al. 2011). A suppression of CCL17 concentration may have subsequently reduced the Th2 lymphocyte infiltration into the airways of the HIB asthmatics so reducing the inflammatory and bronchoconstrictive response to EVH. It should be noted that direct cause and effect relationship of B-GOS supplementation and CCL17 cannot be established from the current study and the exact mechanisms of how prebiotics influence the inflammatory cascade associated with

chemokine expression still remains to be elucidated. Research into the direct effects of prebiotic supplementation and probiotic bacteria on chemokine function is limited, but probiotic supplementation of *Lactobacillus sakei* has been shown to reduce serum levels of CCL17 in children with atopic eczema-dermatitis syndrome and improve severity (Woo, et al. 2010). Atopic dermatitis is similar to asthma in that both are Th2 driven suggesting that favourable manipulation of the gut microflora either through probiotics (Woo, et al. 2010) or B-GOS as in the current study can influence Th2 infiltration by suppression of CCL17 activity. CCL17 is known to drive Th2 lymphocyte recruitment into the airways so favourable manipulation of the commensal bacteria may supress systemic chemokine expression to reduce this infiltration of pro-inflammatory Th2 lymphocytes in the airways of HIB asthmatics. With the findings of the current study showing for the first time that B-GOS can reduce levels of serum CCL17 in asthmatics, more research is warranted to establish the clinical benefit and mechanism of action of prebiotic supplementation on chemokine function.

6.4.6. Serum CRP concentration response to B-GOS

Further to a reduction in serum concentration of TNF-α and CCL17 in the asthmatic group, the current study also demonstrates a reduction in serum CRP following the 21 day B-GOS intervention. Circulating CRP is an acute phase protein that is produced by hepatocytes primarily under the action of inflammatory cytokine IL-6. Serum CRP is known to be a marker of systemic inflammation in asthmatics and is associated with airway inflammation, obstruction, and bronchial hyperresponsiveness (Takemura, et al. 2006, Kony, et al. 2004). Increasing levels of CRP correlate with worsening FEV_1 values (Shaaban, et al. 2006) which supports the greater concentration of CRP in the HIB participants compared to the controls in the current study. The

reductions in serum-CRP at day 21 in the B-GOS intervention in both the controls and HIB participants supports previous observations of reduced plasma CRP following 12 weeks of B-GOS in obese participants (Vulevic, et al. 2013). The reductions in CRP in response to B-GOS highlight the interventions effect on improving immune function, and suppression of low-grade inflammation. With prebiotic B-GOS increasing the number of beneficial bifidobacteria in parallel with reduced CRP concentrations (Vulevic, et al. 2013) it is plausible that changes in the microbiota may significantly alter the immune response in the current asthmatic group to reduce the subsequent inflammatory response to HIB. Serum CRP is negatively correlated with indices of pulmonary function, and positively correlates with the number of sputum eosinophils (Takemura, et al. 2006). Following B-GOS supplementation the opposite was true in the current study serum CRP levels were reduced in conjunction with improvements in pulmonary function.

6.4.7. Serum IgE concentration response to B-GOS

The current study showed no alterations in serum IgE following B-GOS intervention, although IgE was significantly greater in HIB asthmatics compared to the control group. This supports previous research showing serum IgE concentrations are related to airway hyperresponsiveness and severity of allergy in children and adults with asthma (Sears, et al. 1991, Sunyer, et al. 1996). The improvement in HIB is likely to be independent of IgE levels and the current study supports previous human intervention studies that found no benefit of symbiotic treatment (*Bifidobacterium breve* M-16V and a galacto/fructooligosaccharide) on IgE levels despite improvements in atopic dermatitis (Van der Aa, et al. 2011). In contrast *Lactobacillus reuteri* interventions in infants with atopic diseases have been shown to suppress IgE levels

(Abrahamsson, et al. 2007). B-GOS is known to increase the numbers of bifidobacteria (Depeint, et al. 2008) to a greater extent than *lactobacillus*. It is likely that different commensal bacteria strains will influence IgE differently and therefore may not respond to B-GOS treatment. Future research should look to investigate the use of synbiotics that could include B-GOS in combination and *lactobacillus reuteri* as this may confer a greater immunomodulatory benefit and influence IgE levels in asthmatics to further improve severity.

6.4.8. Limitations of analysis

A limitation of the current study was that faecal samples were not obtained to analyse the bifidogenic properties of B-GOS and potential alterations in the microbiota of the HIB asthmatics as a result of the intervention. Consequently, an exact cause and effect relationship of the B-GOS intervention improving the bronchoconstriction and reducing airway inflammation has to be made with caution. However, previous research has shown B-GOS to have substantial bifidogenic properties (Depeint, et al. 2008) and compliance to the current intervention was greater than 88%. There is growing evidence that beneficial manipulation of the gut microbiota can influence immune and inflammatory responses beyond the gut (Noverr and Huffnagle 2004). The precise mechanisms of action for the beneficial effects of prebiotics on airway inflammation still remain to be elucidated and are discussed in more detail in section 6.5. In an attempt to understand these mechanisms through non-invasive means serum blood markers were measured. A limitation of this is relating systemic changes to direct changes within the lung, and therefore future research should assess sputum and cellular changes from within the airways. In the current study attempts were made to measure the inflammatory cytokine IL-33. IL-33 is known to be involved in the development of

allergic sensitization and the exacerbation of lung inflammation, to our knowledge this is the first study that has attempted to measure serum IL-33 in physically active asthmatics with HIB. Upon analysis using a multiplex assay, all samples were below the limit of detection. It may suggest that IL-33 is not raised in HIB asthmatics or, as some research suggests, IL-33 acts solely as an alarmin to signal further inflammatory responses (Besnard, et al. 2011, Sattler, et al. 2013). Therefore systemic measures of IL-33 are not an applicable means to establish its activity. IL-33 activity occurs via its ST2 receptor in an ST2-dependent manner, consequently, serum soluble ST2 may be a more appropriate measure of IL-33 activity (Mok, et al. 2010, Ho, et al. 2013) and should be investigated in future research.

For the first time, this study has shown that a 3 week intervention of prebiotic B-GOS can successfully attenuate the bronchoconstriction and airway inflammation associated with HIB in physically active asthmatic. As such it may be a novel treatment option for asthmatics who suffer with exercise induced asthma. The results add to the growing body of evidence that manipulation of the commensal bacteria can influence inflammatory responses beyond the gastrointestinal tract. The precise mechanisms and exact cause and effect relationships are yet to be fully determined but proposed mechanisms and current understandings are discussed in section 6.5.

6.5. Proposed mechanism of action of prebiotic bacteria on asthma and exercise induced bronchoconstriction

Asthma was once considered as a single disease, but its high level of heterogeneity has given rise to an understanding of numerous phenotypes (Wenzel 2013). Once such phenotype is exercise induced bronchoconstriction and as outlined by Wenzel (2013) it is primarily Th2 derived and overlaps with allergic asthma (Figure 6. 8).

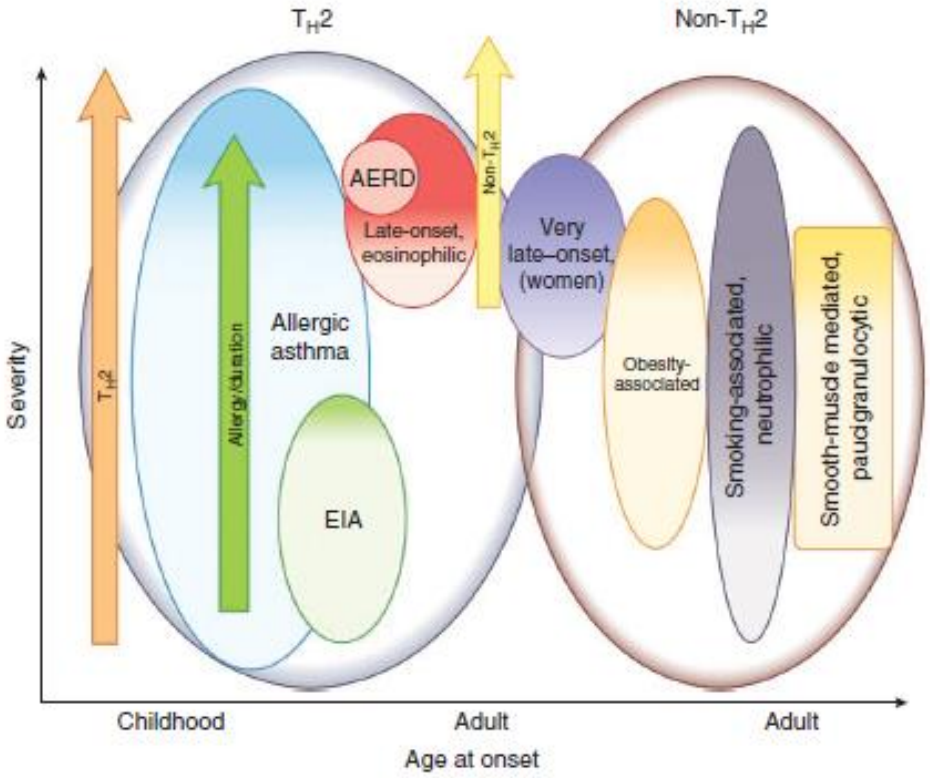

Figure 6. 8 Theoretical grouping of asthma phenotypes based on distinction between Th2 high asthma and non-Th2 asthma. Th2 asthma consists of both early and late onset disease over a range of severities. It is likely that majority of early onset allergic asthma is mild but increasing complexity of immune responses leads to greater severity and EIA is a milder form of Th2 asthma (Wenzel 2013).

The overlapping phenotypes of allergic asthma and EIB suggest that treatment to target underlying immune function and inflammation associated with Th2 type diseases is warranted. It is now known that altered gut microbiota composition is thought to contribute to various inflammatory diseases including asthma (Noverr and

Huffnagle 2004, Kranich, Maslowski and Mackay 2011). We have now shown for the first time that potential manipulation of the gut microflora through B-GOS supplementation can reduce the severity of HIB and supress markers of airway inflammation in physically active asthmatics. This supports previous research into allergic asthma, where different species of probiotic *lactobacillus* can inhibit the allergic airway hyperresponsiveness and preferentially alter the Th1/Th2 balance to prevent asthma in murine models (Forsythe, Inman and Bienenstock 2007, Yu, et al. 2010, Jan, et al. 2011). Supplementation with probiotic or B-GOS in the current study can favourable manipulate the gut microbiome which then interacts with the immune system via toll-like receptors, specifically toll-like receptor 2, toll-like receptor 9, human dendritic cells, and intestinal epithelial cells. These have all been reported to be involved in the induction of regulatory T cells in response to the intestinal microbiota and this may be responsible for the protective effects of pre/probiotics associated with asthma and allergy (McLoughlin and Mills 2011, Sagar 2014).

As described in section 2.2 EIB is characterised by airway hyperresponsiveness, bronchoconstriction, and excess mucous production to exercise or dry-gas hyperpnoea. The pathogenesis of these symptoms is likely due to the thermal and osmotic consequences of water loss and evaporation of the airway surface liquid in an attempt to humidify and condition large volumes of air in a short time (Anderson and Daviskas 2000). In response to changes in airway osmolality there is an influx of inflammatory cells to the airways of EIB sufferers (Hallstrand, et al. 2005, Hallstrand 2012). The current study used an experimental model of EIB through the use of EVH to cause hyperpnoea induced bronchoconstriction. This study has shown similar increases in inflammatory cytokine TNF-α and similar severity of bronchoconstriction as caused by exercise (Mickleborough, et al. 2003) supporting the use of EVH in nutritional

intervention studies. Subsequently the pathogenesis of HIB is identical to that of EIB and the inflammatory cell infiltration associated with asthmatic airways is depicted in Figure 6. 9, with focus on Tcells (Lloyd and Hessel 2010). It highlights that inflammatory cells (mast cells, dendritic cells, eosinophils, and neutrophils) infiltrate the airways along with T-cells which can promote exacerbations and Th2 can specifically promote further mast cell and eosinophil recruitment, mucous production and airway hyperresponsiveness. Regulatory T-cells oppose the Th2 cells and are important for a maintaining and modulating a balanced immune response (Lloyd and Hessel 2010).

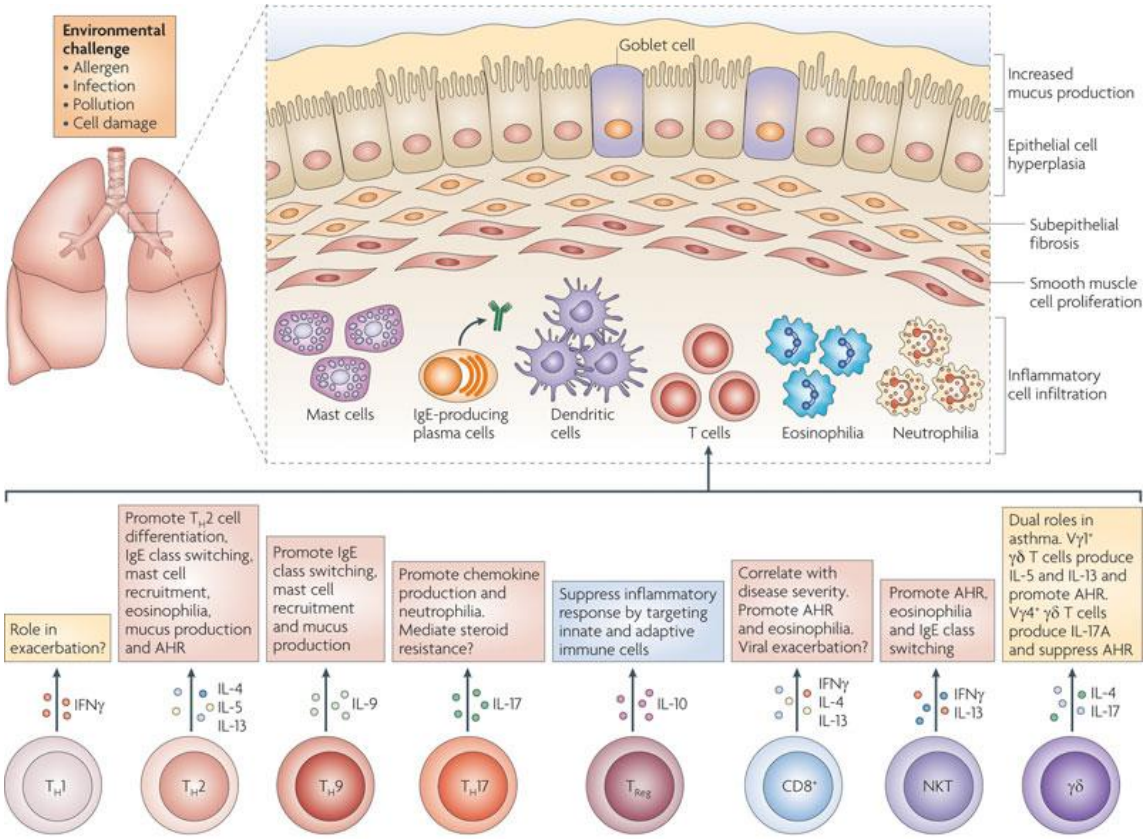

Figure 6. 9. A number of different T cell subsets are thought to influence the nature and magnitude of the allergic immune response by the cytokines that they secrete. T helper 2 (TH2) cells are thought to promote eosinophil recruitment, in conjunction with nature killer T (NKT) cells and CD8+ T cells. By contrast, TH1 cells and TH17 cells are thought to be associated with severe, steroid-resistant asthma, which is often marked by neutrophilic infiltrates. Regulatory T (TReg) cells and subtypes of γδ T cells are able to downregulate pulmonary immune responses and are thought to be important for maintenance of immune homeostasis in the lungs. The nature and magnitude of allergic inflammation in the lung is influenced by external environmental stimuli, such as exposure to allergens and pollution as well as infection with pathogens. IFNγ, interferon-γ; IL, interleukin. (Lloyd and Hessel 2010)

T-regulatory cells are important in balancing immune responses, and moderating inflammatory response. Traditionally the Th2 characterization of asthma was viewed as a skewed Th1/Th2 ratio in favour of Th2 inflammation. However, it is now accepted that in addition to this a reduced expression of Treg cells is commonly associated with asthma (Provoost, et al. 2009, Lloyd and Hawrylowicz 2009, Lloyd and Hessel 2010), and murine models of asthma (Sagar, et al. 2014, Feleszko, et al. 2007). Manipulation of Treg cell expression through their interaction with the intestinal mucosal immune system may be a key therapeutic target for pre- and probiotic treatment. Following the B-GOS intervention the serum concentration of TNF-α was reduced at rest, and completely attenuated following EVH. TNF-α suppresses Treg cell function to maintain chronic inflammation associate with asthma, a reduction in TNF-α concentration suggests that B-GOS may have enhanced Treg cell functioning which is essential for the maintenance and balance of immune responses supporting the improvement in post-EVH pulmonary function. Furthermore, in support of the use of pre- and probiotics in the treatment of asthma and EIB, beneficial commensal bacteria are known to drive the differentiation of naive T-cells in the gut towards Treg cells via the action of TGF-β (McLoughlin and Mills 2011). Increasing Treg cell activation will further improve the immune response in asthmatic airways suppressing the post-EVH decline in FEV_1 in the asthmatic participants.

Prebiotic B-GOS has been shown to increase the numbers of commensal bifidobacteria within the human gut (Depeint, et al. 2008). This increase in beneficial bifidobacteria will interact with the intestinal mucosal immune system and have subsequent beneficial immunomodulatory effects (Figure 6. 10). With B-GOS increasing the growth of beneficial bifidobacteria these probiotic bacteria contain specific toll-like receptor ligands and can therefore modulate toll-like receptor-driven

responses and attempt to modulate immune responses in favour of Th1 and Treg cells (Kalliomaki, et al. 2010). Improving immune responses in favour of Treg cells will suppress the inflammation associated with asthma supporting the reductions in TNF-α and CRP in the current study this further suggests there may have been reductions in Th2 cell infiltration into the airways in response to EVH suppressing the subsequent decline in FEV_1. Increasing Treg cell function and reducing Th2 infiltration is likely to be a key mechanism of B-GOS in modulating HIB in asthmatic individuals. Future research should look to investigate this in more detail and this is discussed in section 7.4. Furthermore it should be noted that the intestinal mucosal immune system is multifaceted and includes the intestinal epithelial cells (IECs), Peyers patches, intestinal lamina propria, intraepithelial lymphocytes and microfold cells. Some of these areas and antigen presenting cells such as dendritic cells will respond to changes in the diversity of the commensal bacteria in response to B-GOS interventions (Figure 6. 10).

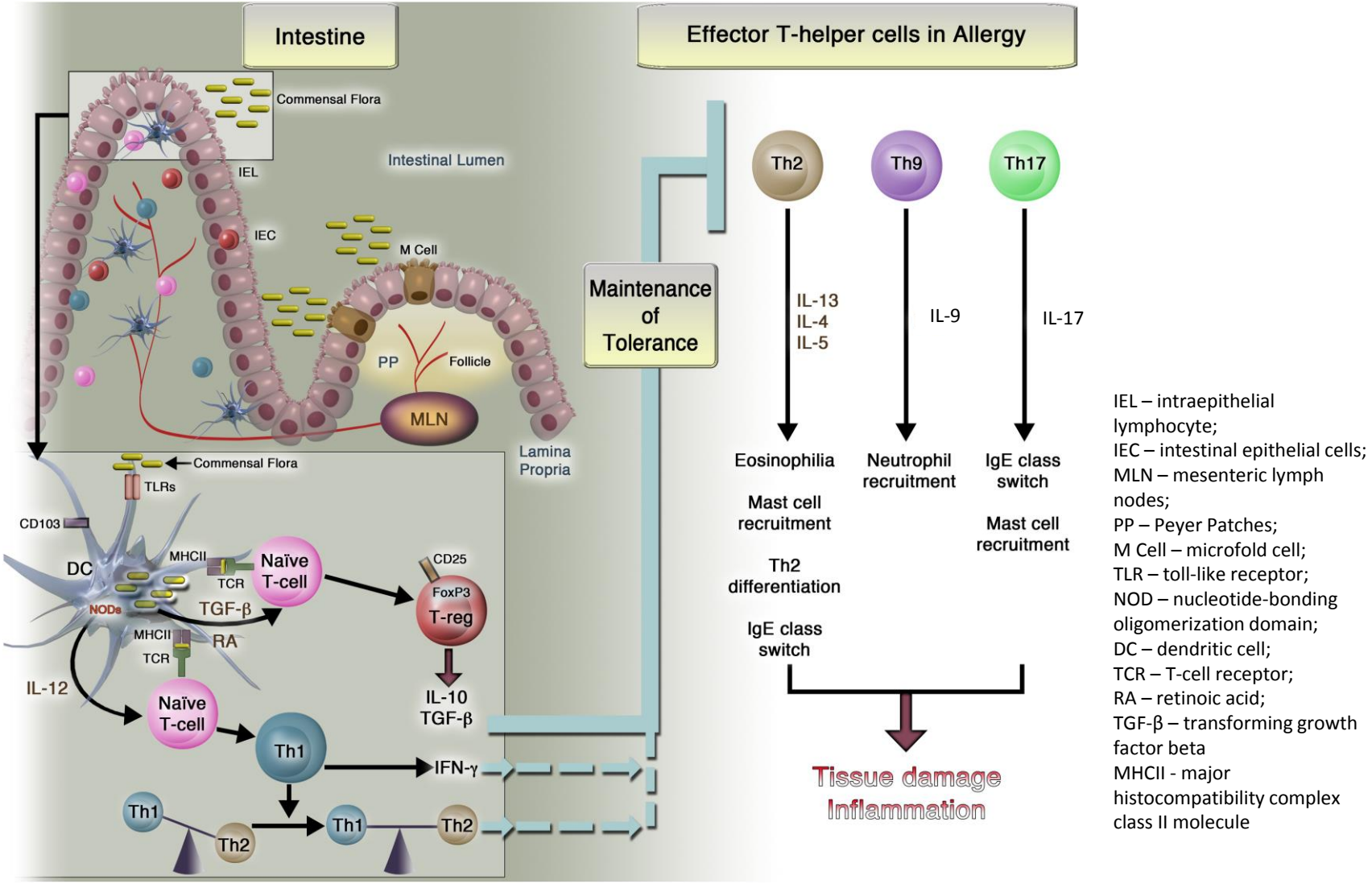

Figure 6. 10 Immunomodulatory effects of the gut microbiota and subsequent effects on decreasing inflammatory responses. (McLoughlin and Mills 2011).

Intestinal epithelial cells are the first part of the intestinal mucosal immune system to encounter the increase in beneficial commensal bacteria (as a result of B-GOS interventions) (Figure 6. 10 and Figure 6. 11). The intestinal epithelial cells create a barrier between the lumen of the intestine and the rest of the body. Despite this physical barrier the contents of the intestinal lumen are continually sampled by specialized areas of the gut associated lymphoid tissue. These areas include Peyers patches in the small intestine and lymphoid follicles embedded in the lamina propria, and microfold cells (m cells) throughout the intestinal tract (Figure 6. 10 and Figure 6. 11). The intestinal epithelial cells play a critical role in distinguishing between pathogenic and beneficial commensal bacteria to regulate immune responses (McLoughlin and Mills 2011). B-GOS increases the quantity of beneficial probiotic gut bacteria with which the intestinal epithelial cells are in direct contact. Intestinal epithelial cells then can express various toll-like receptors and nucleotide-binding oligomerization domain-like receptors to interact with the probiotic bacteria and activate immune defences. Activation of toll-like receptors on the intestinal epithelial cells from interaction with beneficial probiotic bacteria can induce synthesis of growth factors and cytokines that exert protective and reparative effects on the intestinal epithelial cells to help maintain intestinal homeostasis and immune function (Abreu 2010, Rakoff-Nahoum, et al. 2004)

In response to their interaction with probiotic bacteria, intestinal epithelial cells can also influence the function of antigen presenting cells including dendritic cells. Intestinal epithelial cells can activate toll-like receptors on dendritic cells and secretion of immunomodulatory molecules such as thymic stromal lymphopotein and TGF-β (Iliev, et al. 2009). Thymic stromal lymphopotein can limit the expression of IL-12 by dendritic cells and promote the production of IL-10 to enhance T-reg cell differentiation

from naïve T-cells (Figure 6. 10), this helps to create a regulatory as opposed to a pro-inflammatory cytokine response (Iliev, et al. 2009, Rimoldi, et al. 2005). This supports the finding of a reduction in TNF-α following B-GOS intervention in asthmatics. Furthermore suppression of other Th2 derived pro-inflammatory cytokines (IL-4, IL-5, and IL-13) (Figure 6. 10) may explain the improvements in post-EVH $\%\Delta FEV_1$ in the asthmatics following B-GOS supplementation via reduced eosinophil and mast cell recruitment to the airways . Dendritic cell pro-inflammatory cytokine production is also limited by TGF-β (McLoughlin and Mills 2011). TGF-β in incubated PBMCs increases favourably following probiotic supplementation with *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Bifidobacterium bifidum* in healthy participants (Hepburn, et al. 2013). Therefore B-GOS mediated increases in gut bifidobacteria may increase TGF-β in the asthmatics which enabled an improved immune response to the EVH challenge. Future research may wish to explore levels of TGF-β in PBMCs of asthmatics pre and post B-GOS interventions as this is likely to be involved in improved maintenance of immune function to supress the bronchoconstrictive response to EVH. In addition to crosstalk between intestinal epithelial cells and dendritic cells; dendritic cells are actively involved in immune regulation in the intestinal immune system in their own right and will directly interact with probiotic bacteria within the gut lumen (Figure 6. 11) (Lebeer, Vanderleyden and De Keersmaecker 2010).

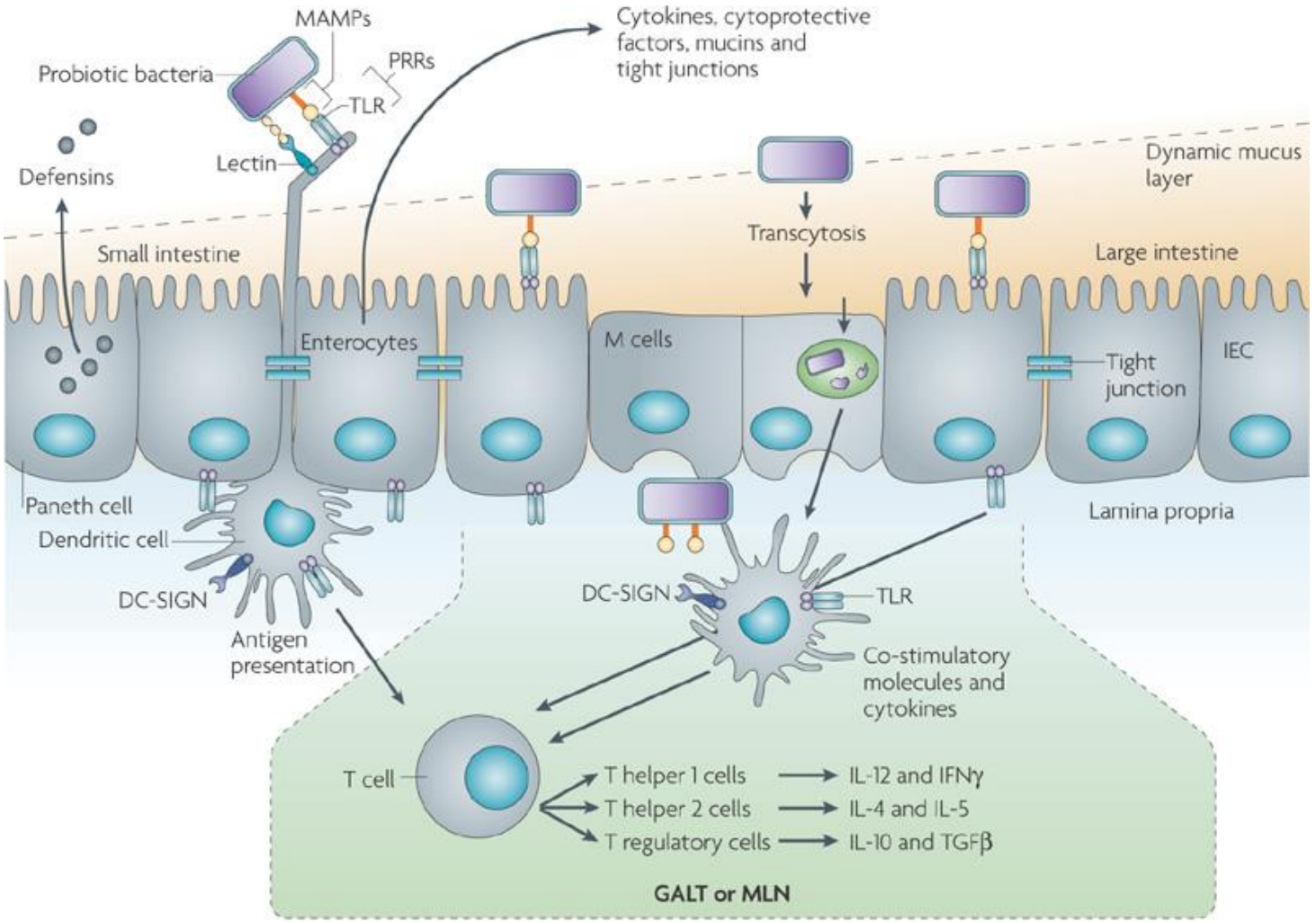

Figure 6. 11 probiotic bacteria interaction with intestinal epithelial cells (IECs) and dendritic cells (DCs) (Lebeer, Vanderleyden and De Keersmaecker 2010).

Dendritic cells are able to continually sample the gut lumen content by extending their dendrites between tight junctions of IECs (Figure 6. 11). Dendritic cells have pathogen recognition receptors on their surface which allows them to respond to pathogen and microorganism associated molecular patterns such as toll-like receptors and nucleotide-bonding oligomerization like receptors expressed on pathogens and commensal bacteria (Lebeer, Vanderleyden and De Keersmaecker 2010). Subsequently dendritic cells will positively respond to increases in beneficial probiotic bacteria as a result of B-GOS supplementation. Simultaneously they will present antigens to naïve Tcells (Figure 6. 10 and Figure 6. 11). Antigen presentation in conjunction with immunomodulatory cytokines directs the development of specific T cell subsets in the gut associated lymphoid tissue or mesenteric lymph nodes (Figure 6. 11). In response to antigen presentation following increased interaction with beneficial probiotic bacteria (as a consequence of B-GOS supplementation), dendritic cells will drive Treg cell differentiation (from naïve Tcells) in the gut this is critical in improving or maintaining a balanced immune response in asthmatics (McLoughlin and Mills 2011). Improving Treg cell differentiation is essential to enhance immune tolerance in asthmatics and thereby suppress the inflammatory response in asthmatics to exercise and EVH. Future research should look to elucidate this mechanism in more detail in asthmatics with EIB/HIB.

A specific subset of DCs ($CD103^{+}$) is capable of converting naive Tcells to inducible $FoxP3^{+}$ Treg cells through a mechanism that is dependent upon TGF-β (Round and Mazmanian 2010, Hooper, Littman and Macpherson 2012). It is known that asthmatic individuals typically have reduced expression of FoxP3 within $CD4^{+}CD25^{high}$ T cells (Provoost, et al. 2009, Lin, Shieh and Wang 2008). Specific probiotic strains (*Bifidobacterium infantis, Lactobacillus species, Bacteroides fragilis*) have been shown

to increase the numbers of $CD4^{+}CD25^{+}FoxP3^{+}$ cells in the spleen (O'Mahony, et al. 2008), upregulate $FoxP3^{+}$ expression in $CD25^{-}$ cells *in vitro* (De Roock, et al. 2010) and induce expansion of CD4+ CD25+FoxP3+ T Reg cells (Round and Mazmanian 2010), all contributing to improved moderation of immune responses. It is possible that B-GOS mediated increases in bifidobacteria may have increased numbers of CD4+ CD25+FoxP3+ T Reg cells thereby improving immune tolerance and reducing inflammation associated with HIB. These proposed mechanisms are supported by work showing that a synbiotic supplement (*Bifidobacterium breve* and non-digestible oligosaccharide) had strong anti-inflammatory effects in a murine model of severe asthma (Sagar, et al. 2014). The supplement resulted in reduced airway remodelling and chronic inflammation which were associated with altered toll-like receptor activity, increased expression of anti-inflammatory cytokine IL-10, and increased expression of Treg transcription factor FoxP3 (Sagar, et al. 2014). Increased expression of Treg cells in the gut associated lymphoid tissue and mesenteric lymph nodes result in greater Treg cell counts throughout the lymphatic system. Therefore, beneficial alterations in gut bacteria affect, amongst other tissues, the airway mucosal immune system response. In this way the balance of Th1, Th2, and Treg cell arriving in the airways upon stimulation is positively affected by B-GOS supplementation. Furthermore recent research now highlights that the airways are home to their own unique microbiome that is altered in individuals suffering from airway diseases (Madan, et al. 2012, Han, et al. 2012). This unique lung microbiome can also respond in diversity to dietary interventions (Madan, et al. 2012). Consequently it is plausible that in addition to altered immune tolerance through the gut microbiota, B-GOS supplementation may be having a direct impact on the microbial communities of the asthmatic airways however this remains to be elucidated.

Consequently feeding pre or probiotic to increase the beneficial commensal bacteria being sampled by intestinal epithelial cells and dendritic cells in the gut are likely to have a subsequent positive effect on Treg cell differentiation to improve immune tolerance and inflammatory function in asthmatics which would support the improved pulmonary function responses and reduced inflammatory markers in the current study.

6.6. Conclusion

For the first time this study shows that a 21-day supplementation of prebiotic B-GOS can significantly reduce the severity of HIB in asthmatics and supress markers of airway inflammation. Favourable manipulation of the commensal bacteria by B-GOS will influence immune regulation and inflammatory responses via intestinal epithelial cells, dendritic cells, Treg cells, and Thelper cells. Favourable increases in beneficial commensal bacteria through B-GOS treatment will help to improve the immune response in asthmatics towards greater Treg activity to maintain immune modulation and suppress inflammatory responses to triggers such as exercise and hyperpnoea.

7. Chapter 7 – General Discussion

7.1. Introduction

Asthma has been an increasing public health issue over the past 40 years. In the UK, 5.4 million people are currently receiving treatment for asthma, 1.1 million children (1 in 11) and 4.3 million adults (1 in 12). The disease has a considerable financial burden associated with it costing the NHS £1 billion a year (Asthma UK Accessed on 12.06.2013). Eighty percent of clinically recognised asthmatics experience exercise induced bronchoconstriction (EIB) (Anderson and Holzer 2000), with asthma and airway hyperresponsiveness being amongst the most common chronic medical conditions reported by elite athletes with a prevalence of 7-8% (Kippelen, et al. 2012) although large variations exist between sports (Fitch 2012). EIB is a phenotype of asthma that is characterized by a transient airway narrowing during or on the cessation of exercise (McFadden Jr and Gilbert 1994, Anderson and Holzer 2000, Suman, et al. 1999), and it is generally accepted that airway drying and changes in airway osmolality trigger the response (Rundell and Jenkinson 2002).

Asthma and EIB are traditionally treated with β2-agonists which typically target symptoms alone, and long-term use of inhaled corticosteroids can have systemic side effects. Furthermore, over 50% of asthmatics are documented to have poorly controlled asthma largely due to poor adherence to medication (Barnes 2010). An estimated 75% of hospital admissions for asthma are avoidable through improved management techniques and as many as 90% of deaths from asthma are preventable (Asthma UK Accessed on 12.06.2013). These striking statistics and limitations with current medication provide a strong rationale to develop novel therapeutic strategies which encourage adherence and that target the underlying inflammation and immune response. In addition, to establish the efficacy of novel treatment options understanding the reproducibility of techniques used to measure the severity of EIB was needed.

Determining the reproducibility of techniques used to diagnose and monitor EIB allows for confidence when assessing changes in the severity of bronchoconstriction in response to interventions of ω3-PUFA and B-GOS. Changes in severity can be established as a genuine effect if above any inherent variability in the bronchoprovocation technique used.

These questions initiated the rationale for this research thesis. A summary of the key findings are detailed below.

Chapter 4 *confirmed the efficacy of a 6 min EVH protocol as a bronchoprovocation test. The study demonstrated excellent within-participant test-retest reproducibility for EVH and the evoked changes in pulmonary function in HIB participants, over both short- (21 days) and long-term (70 days) periods. Reporting reproducibility statistics highlighted that the EVH protocol used in the current thesis was a valuable tool for assessing the efficacy of interventions aimed at reducing HIB.*

Chapter 5 *showed for the first time that a lower dose of ω3-PUFA (3.1 $g \cdot d^{-1}$) was as effective as previously used high doses (6.2 $g \cdot d^{-1}$) in suppressing HIB in physically active asthmatics. This was in conjunction with equal suppression of F_ENO, and urinary 9α, 11β-PGF_2 concentration as markers of airway inflammation. It highlights that lower ω3-PUFA doses represent a potentially beneficial treatment for physically active asthmatics suffering with EIB whilst reducing the burden of cost, compliance and potential for GI distress.*

Chapter 6 *showed for the first time that 21 days of prebiotic B-GOS supplementation attenuated the post-EVH decline in pulmonary function in physically active HIB participants. This was in combination with a reduction in airway inflammatory markers including serum TNF-α, CCL17, and CRP. This suggests that potentially favourable*

manipulation of the commensal gut bacteria through B-GOS may alter systemic inflammation and immune function to reduce the bronchoconstriction following EVH.

7.2. Experimental findings and recommendations

Prior to investigating the use of novel dietary interventions in the treatment of HIB, a reproducible technique to induce and measure the severity of bronchoconstriction needed to be established. Self-presentation of symptoms by asthmatics and EIB sufferers lack specificity and sensitivity which makes diagnosis using this information alone extremely inaccurate with a risk of either false positive or false negative results (Rundell, et al. 2001a, Holzer, Anderson and Douglass 2002, Parsons 2009). Bronchial provocation techniques to induce bronchoconstriction have been developed to diagnose and monitor EIB. A number of bronchoprovocation techniques have been established but none are generally considered as a gold standard. The eucapnic voluntary hyperpnoea challenge is recommended by the International Olympic Committee-Medical Commission to diagnose EIB in elite athletes (Weiler, et al. 2007, Fitch, et al. 2008), but the reproducibility of the reduction in pulmonary function to EVH was not known. It is important to ascertain the reproducibility of such reductions and the smallest measurable change in these reductions as it will allow for greater confidence when assessing interventions that aim to reduce the severity of EIB. If changes in post-EVH FEV_1 exceed the smallest measurable change then it can be assumed that the intervention has a genuine effect beyond any inherent variability in the bronchoprovocation challenge. Understanding the long-term reproducibility of the 6 min EVH test may thus improve the monitoring and evaluation of HIB management and intervention strategies.

In Chapter 4, an EVH method that incorporated real-time visual feedback of $\dot{V}_E$ and end-tidal PCO_2 through the use of a breath-by-breath analyser was described. It was found that this method induced a highly reproducible Broncho constrictive response, over both short- term (21 days) and long-term (70 days) periods, in physically active asthmatics with HIB. To the author's knowledge this is the first time that the smallest measurable change in the fall in FEV_1 post-EVH has been calculated from repeated EVH challenges. The smallest measurable change of 88 mL suggests that alterations in the post-EVH fall in FEV_1 that are greater than this are likely to represent a genuine change. The average fall in FEV_1 in the HIB participants was >770 mL, this suggests that the fall in FEV_1 must change by more than 11% to exceed the smallest measurable change and this would allow for confidence in monitoring nutritional interventions. The low smallest measurable change for post-EVH change in FEV_1 supports previous research that suggests EVH is also more sensitive in diagnosing HIB than (non-dry-air) exercise challenges, which are more difficult to standardize due to potential variability in exercise intensity and duration, and inspirate moisture content (Rundell, et al. 2004, Rundell, et al. 2000). Furthermore the results in chapter 4 show consistent pre-EVH pulmonary function tests in asthmatic HIB sufferers; this allows for a highly reproducible target $\dot{V}_E$ for the EVH challenge to enhance the reproducibility of the bronchoconstrictive response to EVH.

The outcomes of Chapter 4 allow us to confidently recommend the use of EVH utilising real-time visual feedback through a breath-by-breath analyser as a simulation of exercise induced bronchoconstriction in the diagnoses and management of HIB. Further to this, Chapters 5 and 6 showed increases in pro-inflammatory mediators similar to those seen in exercise challenges. This suggests a similar pathogenic pathway in HIB as EIB and further supports the use of EVH as an experimental model of EIB.

Therapeutic strategies to target the underlying inflammation of EIB/HIB are warranted and one area that has received considerable interest is the use of high doses of ω3-PUFA (Mickleborough etc.). ω3-PUFA interventions aim to reduce the release of pro-inflammatory mediators associated with the metabolism of arachidonic acid and increase anti-inflammatory and pro-resolving mediators to suppress inflammation. Polyunsaturated fatty acids (PUFAs) are important constituents of the phospholipids of all cell membranes. Increasing the dietary intake of the ω3-PUFAs EPA and DHA can result in higher concentrations of these fatty acids in inflammatory cells including lymphocytes, macrophages, and neutrophils; this can subsequently impact upon inflammatory processes (Yaqoob, Newsholme and Calder 1995, Brouard and Pascaud 1990, Palombo, et al. 1999). Typically the increase in EPA and DHA in cell membranes is at the expense of ω6 arachidonic acid (Calder 2010) (Figure 7. 1).

Previous research (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010) used very large dosages of ω3-PUFA (5.2g ω3-PUFA; 3.2 g EPA and 2.0g DHA) equating to ~10 capsules a day. There is a large cost implication with such a high dose along with potential for problems with consumption adherence and the potential side effect of gastrointestinal discomfort (Van de Rest, et al. 2008). In Chapter 5 we have shown for the first time that a dose of 3.1g·d^{-1} (1.8 g·d^{-1} EPA and 1.3 g·d^{-1} DHA) is as effective in treating HIB in physically active asthmatics as a higher dose of 6.2 g·d^{-1} (3.7 g·d^{-1} EPA and 2.5 g·d^{-1} DHA). Changes in the severity of HIB assessed by $AUC_{0\text{-}30}$%ΔFEV_1 were similar in both the 6.2 g·d^{-1} and 3.1g·d^{-1} ω3-PUFA interventions. The magnitude of the improvement in HIB (30-40%) is comparable to previous ω3-PUFA research (Mickleborough, et al. 2003, Mickleborough, et al. 2006, Tecklenburg-Lund, et al. 2010) and that which can occur from pharmacological interventions (Villaran, et al. 1999). In addition this magnitude of

improvement was greater than the smallest measurable change recorded in chapter 4 highlighting the efficacy of the ω3-PUFA intervention. Furthermore, both 3.1g·d^{-1} and 6.2 g·d^{-1} interventions resulted in equal reductions in resting levels of exhaled nitric oxide, and equal suppression of urinary 9α, 11β-PGF_2 concentration following the EVH challenge. It is also important to note that two participants withdrew from the study due to gastrointestinal distress during the higher dose (6.2 g·d^{-1}) supporting the rationale for understanding the treatment effects of lower doses. Furthermore, on the back of this research sports physicians, medical officers, and nutritionists working for the English Institute of Sport have expressed an interest in the results. High dose ω3-PUFA supplement is widely used in elite sport (personal communication) and this study provides evidence to reduce dose levels to improve adherence and reduce costs in this cohort of athletes.

The findings of Chapter 5 suggest that lower doses of ω3-PUFA can significantly modulate the inflammatory process associated with HIB. Both ω3-PUFA doses resulted in significant increases in the content of EPA and the 6.2 g·d^{-1} dose increased the content of DHA in the phospholipid membranes of isolated neutrophils at day 21 when compared to day 0. This verified compliance to the supplementation regimes and provides evidence to support alterations in the inflammatory responses to EVH. The increases in EPA and DHA were in accordance with a reduction in the content of ω6-PUFA arachidonic acid following the 6.2 g·d^{-1} and 3.1 g·d^{-1} interventions in the HIB asthmatics. This is important because the arachidonic acid derived pro-inflammatory eicosanoids have consistently been associated with the pathogenesis of asthma and EIB (Hallstrand, et al. 2013). Both ω3-PUFA interventions significantly reduced the urinary concentration of the PGD_2 metabolite 9α, 11β-PGF_2 at Day 21 compared to Day 0 post EVH. The increases in urinary 9α, 11β-PGF_2 following EVH

on all interventions are similar to those shown previously in response to exercise challenges in asthmatics, rising from a baseline of ~30-40 ng·mmol^{-1} creatinine up to ~80 ng·mmol^{-1} creatinine (Mickleborough, et al. 2003). This highlights the utility of EVH to cause a hyperpnoea induced bronchoconstriction that is pathologically similar to EIB. The 6.2 g·d^{-1} and 3.1 g·d^{-1} ω3-PUFA interventions significantly attenuated the rise in post-EVH 9α, 11β-PGF_2 urinary concentration and the concentrations were subsequently similar to the control participants suggesting suppression of arachidonic acid derived inflammation. Both doses increased the content of EPA in the phospholipid membranes of neutrophil cells with a consequential improvement in HIB and suppression of 9α, 11β-PGF_2. This supports the notion that EPA is key in the competitive inhibition of arachidonic acid (Calder. 2010) (Figure 7. 1) and that this is a likely mechanism of action for the beneficial effect of ω3-PUFA on HIB.

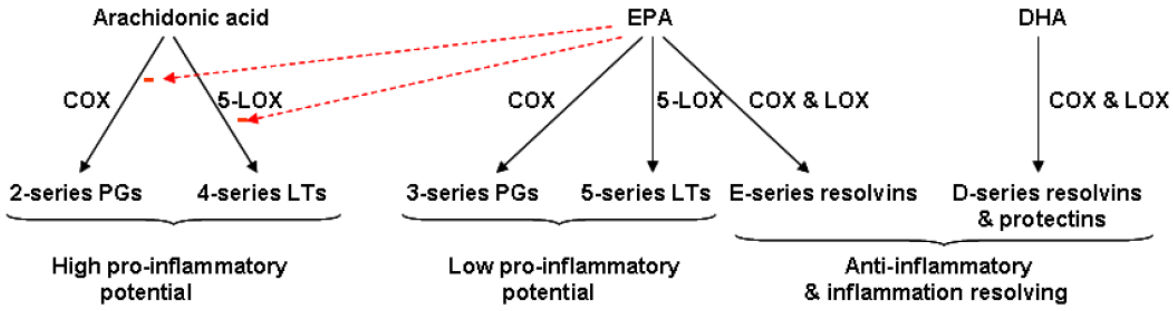

Figure 7. 1 Overview of synthesis and actions of lipid derived mediators (eicosanoids, resolvins and protectins) produced from arachidonic acid, EPA, and DHA. Red lines highlight competitive inhibition of arachidonic acid enzymatic pathways by EPA (Calder. 2010).

Both 6.2 g·d^{-1} and 3.1 g·d^{-1} ω3-PUFA doses reduced F_ENO levels after 21 days of intervention in the HIB positive participants. Fraction of exhaled nitric oxide is now commonly used as a marker of asthma control and an objective measure of airway

inflammation (Sandrini, et al. 2010). In asthma sufferers, F_ENO is an indicator of eosinophilic airway inflammation, raised IgE levels and allergic sensitisation (Strunk, et al. 2003). The results of the current study support previous findings that high dose ω3-PUFA alone, and in combination with montelukast, can reduce pre-hyperpnoea F_ENO in HIB sufferers from 42.5 ± 6.6 ppb to 25.5 ± 3.9 ppb; and 23.2 ± 3.4 ppb respectively (Tecklenburg-Lund, et al. 2010). To the author's knowledge, only one other study has directly investigated the response of F_ENO in asthmatics to ω3-PUFA interventions (Moreira, et al. 2007). This study found no benefit of daily ω3-PUFA intake on F_ENO, however the intervention dose was markedly lower than the $3.1g \cdot d^{-1}$ dose in the current study (0.91 g EPA and 0.65 g DHA vs 1.8 g EPA and 1.3 g DHA). It is likely that this dose was not sufficient to raise levels of EPA and DHA in the phospholipid membranes of inflammatory cells to influence F_ENO levels. The data suggest that there is likely to be a minimum effective dose of ω3-PUFA in treating HIB in physically active asthmatics, but to date no dose-response studies have elucidated the exact response.

Recommendations based on the findings of Chapter 5 are that lower ω3-PUFA doses are as effective as higher doses in attenuating HIB in physically active asthmatics. These data highlight that lower doses represent a potentially beneficial treatment for physically active asthmatics suffering with HIB whilst reducing the burden of cost, compliance and potential for GI distress. It is clear that the combination of EPA and DHA can provide anti-inflammatory effects to reduce airway inflammation as assessed by FeNO and urinary 9α, 11β-PGF_2.

The limitations of current pharmacological treatment for asthma and EIB provide a strong rationale to develop therapeutic strategies that target underlying immune function and inflammation that can be deregulated in asthmatics. Recent evidence suggests that the gastrointestinal commensal bacteria could be a therapeutic

target to influence the immune and inflammatory responses that mediate allergy and asthma (Noverr and Huffnagle 2004, McLoughlin and Mills 2011). Chapter 6 shows for the first time that intervention with a prebiotic supplement (trans-galactooligosaccharide [B-GOS]) can significantly attenuate the bronchoconstriction and serum markers of inflammation associated with HIB in physically active asthmatics. The use of B-GOS for the management of HIB in physically active asthmatics is a new treatment option that currently is not used within sporting environments. The efficacy of B-GOS to manage HIB is similar to that shown from both ω3-PUFA and pharmacological interventions subsequently sporting bodies such as the English Institute of Sport have expressed an interest in these results and are currently starting to implement B-GOS supplementation in their athletes suffering with asthma. Thus, Chapter 6 presents novel evidence that favourable manipulation of the gastrointestinal microflora can be a suitable treatment option for individuals suffering from HIB. Twenty-one days of B-GOS intervention significantly improved the $\%\Delta FEV_1$ after EVH at the 3, 6, 10 and 20 minute time points ($p < 0.05$) in the HIB asthmatics by 36 ± 17, 36 ± 19, 43 ± 25%, and 33 ± 24% respectively. This is similar to the improvements seen in Chapter 5 and those from previous nutritional intervention studies and pharmacological interventions (Mickleborough, et al. 2003, Tecklenburg, et al. 2007, Mickleborough 2005). As with chapter 5, the B-GOS supplement was able to induce a significant improvement in pulmonary function of the HIB participants. Furthermore this was greater than the smallest measureable change from chapter 4, indicating that B-GOS was an effective treatment strategy for HIB.

A limitation of the study in Chapter 6 is that no measurements of faecal microflora were taken to support the efficacy of the B-GOS intervention in manipulating the gut microflora. However, previous research using the same

supplement has shown B-GOS to significantly increase the numbers of beneficial bacteria, primarily bifidobacteria, at the expense of less beneficial bacteria to improve the microflora of the gut (Depeint, et al. 2008). Coinciding with improvements in the gut microflora, previous research showed suppression of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) (Depeint, et al. 2008). Our current study extends the research knowledge of B-GOS further suggesting it can attenuate the bronchoconstriction associated with HIB and reduce chemokine and cytokine markers of airway inflammation in physically active asthmatics.

The microbial communities of both the gut and airways have been shown to be dysregulated in patients suffering with respiratory diseases (Madan, et al. 2012, Hilty, et al. 2010, Huang, et al. 2011). It is plausible that the bifidogenic properties of B-GOS were able to modify the gut microflora and influence the underlying inflammation of asthmatics to supress the subsequent inflammatory responses of HIB in response to EVH. In conjunction with a significant improvement in the level of bronchoconstriction caused by EVH, the HIB asthmatics showed reductions in serum concentrations of TNF-α, CCL17, and CRP.

Chronic airway inflammation associated with asthma is in part maintained by the action of TNF-α in suppressing Treg cell function which is essential for the maintenance and balance of immune responses. The suppression of pre-EVH and post-EVH serum concentrations of TNF-α suggests that B-GOS intervention enhances Treg cell functioning in the HIB asthmatics thus diminishing the level of bronchoconstriction associated with HIB. Natural Treg cells are important suppressors of allergic responses to inhaled antigens such as grass or pollen (Ling, et al. 2004, Grindebacke, et al. 2004) and it may be hypothesised that they could supress the response of HIB asthmatics to dry gas hyperpnoea. Importantly, TNF-α impairs the regulatory activity of natural Treg

cells via the TNF-α receptor 2 (TNFR2) signalling pathway to down modulate Foxp3 expression (Lin, Shieh and Wang 2008). The transcription factor Foxp3 is a crucial regulator for Treg cell development and function, and asthmatics have a reduced Foxp3 expression (Provoost, et al. 2009). Twenty-one days intervention of B-GOS reduced serum TNF-α by 36 ± 18% in the HIB participants and this may have allowed for improved expression of Foxp3 within $CD4^{+}CD25$ Tcells to enhance immune regulation in the HIB participants thus dampening the bronchoconstrictive response to EVH. Further research is required to elucidate the mechanisms of TNF-α suppression and improvements in asthma following B-GOS interventions (see section 7.4 for more details).

Chapter 6 reports serum chemokine concentrations in HIB positive participants for the first time along with their subsequent responses to B-GOS intervention. It was found that B-GOS reduced the concentration of CCL17, but not CCL11. CCL11 is increased in the airways of asthma sufferers and is an important attractant for eosinophils which are up-regulated in epithelial cells (Smit and Lukacs 2006). B-GOS failed to reduce CCL11 concentration and F_ENO as markers of eosinophilic airway inflammation. Despite this there were still significant clinical improvements in the HIB participants. This supports the concept that the pathogenesis of EIB/HIB is multifaceted and despite no change in markers of eosinophil recruitment to the airways, a significant improvement in post-EVH lung function was still evident. To the authors knowledge this is the first study to report serum CCL17 concentrations in HIB participants and they are similar to those found previously in allergic asthma sufferers (Sekiya, et al. 2002). The increased serum CCL17 in the HIB positive participants supports the notion of increased Th2 lymphocyte infiltration in the pathogenesis of the condition (Hallstrand, et al. 1998). The HIB asthmatics had significantly higher concentrations of CCL17 at

day 0 of the interventions compared with the control group (408.69 ± 182.53 vs 239.73 ± 80.28 $pg \cdot mL^{-1}$). The gut associated lymphoid tissue develops immune responses to different stimuli which involve the production of cytokines and chemokines (Sanz and De Palma 2009). Manipulating the commensal bacteria may subsequently affect the production and expression of cytokines and chemokines associated with inflammatory responses (Wells, et al. 2011). The B-GOS intervention successfully reduced the serum concentration of CCL17 in the HIB participants. A suppression of CCL17 concentration may have subsequently reduced the Th2 lymphocyte infiltration into the airways of the HIB participants so reducing the inflammatory and bronchoconstrictive response to EVH.

In support of previous research (Vulevic, et al. 2013), Chapter 6 shows a reduction in serum CRP concentrations following the B-GOS intervention. The reductions in CRP in response to B-GOS highlight the beneficial effect on immune function, and the suppression of low-grade inflammation. With prebiotic B-GOS increasing the number of beneficial bifidobacteria in parallel to reduced CRP concentrations in Vulevic et al (2013) it is plausible that changes in the microbiota may significantly alter the immune response in the HIB positive participants to reduce the subsequent inflammatory response to HIB.

For the first time, Chapter 6 shows that a 3 week intervention of prebiotic B-GOS can successfully attenuate the bronchoconstriction and airway inflammation associated with HIB in physically active asthma sufferers. As such, it may be a novel treatment option for asthmatics who suffer with exercise induced asthma. The results add to the growing body of evidence that manipulation of the commensal bacteria influences inflammatory responses beyond the gastrointestinal tract. The precise

mechanisms and exact cause and effect relationships are yet to be fully determined; future research directions are discussed in section 7.4.

7.3. Limitations

The specific limitations of each study are discussed in the relevant experimental chapters. Globally the issues with assessing asthma severity are the high heterogeneity in the severity of disease and numerous phenotypes in its manifestation (Wenzel 2013). The current studies used relatively small cohorts, but in an attempt to keep the groups as homogenous as possible all participants had to be physically active (completing 4-6 hours of exercise a week), have a previous GP diagnosis of asthma with the severity well controlled and prior to the studies all participants were initially screened for HIB through EVH. Despite the potential heterogeneity in asthma severity Chapter 4 documents a high level of reproducibility in baseline FEV_1 and FVC values which subsequently allows for reproducible EVH tests to be performed. Ideally, a larger cohort of asthmatics would be sought to confirm the high level of reproducibility from a wider array of asthma phenotypes.

Chapter's 5 and 6 show the successful intervention of dietary supplements to improve the severity of HIB in a relatively small cohort of physically active asthmatics. Larger replication studies should be conducted to support or refute these novel findings. Due to the heterogeneity of asthma Chapter 6 found a number of inflammatory markers to not be normally distributed; a larger participant number will allow for this heterogeneity and develop a better understanding into the effectiveness of these interventions.

A limitation of the studies in Chapters 5 and 6 are that no measures of quality of life, and medication usage were taken. Previous research suggests that nutritional

interventions do not only result in improvements in clinical measures of pulmonary function and markers of inflammation but these coincide with reductions in medication usage (Mickleborough, et al. 2003). Future research should look to include questionnaires assessing medication usage, asthma severity, and quality of life as outcome measure in response to nutritional interventions.

7.4. Significance of findings and future research direction

Due to the novelty of the findings in Chapter 5 and 6, there is significant scope for the implementation of these protocols to alleviate HIB in physically active asthmatics. Well-controlled asthma should not be considered a barrier to physical activity and nutritional interventions at the doses currently studied are easy to implement and have limited, if any, adverse side effects. This thesis expands the current knowledge of effective dose levels of ω3-PUFA in HIB, and shows for the first time that treatment with prebiotic B-GOS can significantly attenuate the bronchoconstriction associated with HIB in physically active asthmatics. On the back of this novel research there are a wealth of future research directions to gain a better understanding into the therapeutic and mechanistic benefits of both ω3-PUFA and B-GOS in the treatment of asthma and EIB:

- The $3.1 g \cdot d^{-1}$ ω3-PUFA dose significantly improved the severity of HIB, and reduced urinary markers of airway inflammation and F_ENO. However 3 weeks of supplementation at $3.1 g \cdot d^{-1}$ did not elevate the phospholipid content of DHA. Therefore a longer duration of supplementation at $3.1\ g \cdot d^{-1}$ should be investigated. This may elicit a significant increase in phospholipid DHA content and thus attenuate the severity of HIB further.

- Further to this, investigations into an initial high dose similar to 6.2 $g \cdot d^{-1}$ to rapidly increase saturation of the phospholipid membranes followed by a lower maintenance dose of 3.1 $g \cdot d^{-1}$ may well be warranted. This would have the potential of improving long-term adherence to ω3-PUFA interventions, but also allow for an initial increase in EPA and DHA content. Furthermore an important development would be to gain an understanding of an exact dose-response relationship between ω3-PUFA and HIB/EIB.
- A greater understanding into the isolated and combined effects of EPA and DHA is needed in both *in vivo*, and *in vitro* research protocols. To support the use of certain dose levels of ω3-PUFA a better understanding into the mechanisms by which EPA and DHA regulate inflammation and produce pro-resolvins mediators is needed.
- Site specific markers of airway inflammation such as sputum and bronchoalveolar lavage fluid in response to EPA and DHA doses should be measured.
- Furthermore, elite level athletes are known to undergo airway epithelial damage and airway injury therefore measures of this through urinary clara cell 16 in response to ω3-PUFA interventions are warranted.

We have shown for the first time that a prebiotic B-GOS intervention can significantly improve HIB and markers of inflammation in physically active asthmatics, however the exact mechanisms of action remain to be elucidated. Future research studies should look to focus on:

- Future research studies should collect faecal samples from asthmatics receiving B-GOS to confirm its bifidogenic properties in this population. It

would also confirm if this population has a reduced diversity of gut microflora that could contribute to the onset of the disease.

- There is a wealth of potential research studies to follow the initial proof of concept described in Chapter 6. A larger scale research study to confirm or refute the main outcome of reduced severity of HIB is needed. A larger population will also allow for a better understanding into the underlying mechanisms.
- There are a number of methods to try and elucidate the mechanisms of action of prebiotic B-GOS on asthma further. The collection of sputum for the assessment of site specific inflammation markers would allow for direct changes within the airways to be understood.
- Recent research has also shown that the airways of patients with respiratory diseases have a dysregulated microflora which can be manipulated through dietary interventions (Madan, et al. 2012). Through the use of epithelial brushing technique the lung micro biome could be analysed to establish differences in diversity between asthmatics and non-asthmatics and its subsequent response to B-GOS treatment.
- Chapter 6 showed reductions in the serum concentration of TNF-α and this may be a marker of, or, as a consequence of alterations in T cell differentiation and Foxp3 expression. Peripheral blood mononuclear cells (PBMC) from control and asthmatic patients' pre and post B-GOS intervention should be collected in future research. These cells can be labelled for CD4, CD25, and intracellular Foxp3 and analysed using flow cytometry to establish changes in protein expression in response to B-GOS intervention.

- Since the mechanisms of action may be different it would be pertinent to establish if there is a combination effect of ω3-PUFA and B-GOS on asthma and EIB.
- Furthermore, a combination of B-GOS with strains of lactobacilli and bifidobacteria in the form of a synbiotic may enhance the immune regulation in asthmatics and suppress markers of inflammation further
- There is scope for nutritional intervention strategies to be used in other phenotypes of asthma such as allergic asthma which is driven by Th2 cell which could be suppressed by B-GOS supplementation
- There is also scope for other populations to be studied such as asthma in the elderly and children, and in other respiratory diseases associated with airway inflammation such as cystic fibrosis and COPD.

7.5. Conclusion

In conclusion, this thesis provides novel evidence of the efficacy of two nutritional interventions as treatment options for hyperpnoea induced bronchoconstriction in physically active asthmatics. We have shown for the first time that a lower dose of ω3-PUFA is effective in attenuating the post EVH decline in FEV_1 and suppression of inflammation markers. This reduces the financial and adherence burdens of trying to maintain a high dose, and reduces the risk of gastrointestinal side effects. Furthermore, our novel findings show for the first time that potential manipulation of the gut microflora through prebiotic B-GOS interventions can significantly alter the immune and inflammatory function of asthmatics and reduce the severity of bronchoconstriction following EVH. This data adds to the growing body of

evidence that the gut microbiome can have a significant impact on immune function and inflammation beyond the gastrointestinal tract and as such is a potential therapeutic target for future research.

Reference List

Abrahamsson, T.R., et al., 2007. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *Journal of Allergy and Clinical Immunology,* 119 (5), 1174-1180.

Abrahamsson, T., et al., 2014. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clinical & Experimental Allergy,* 44 (6), 842-850.

Abreu, M.T., 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature Reviews Immunology,* 10 (2), 131-144.

Adams, F., 1856. *The extant works of Aretaeus the Cappadocian.* London: Sydenham Society.

Adlerberth, I. and Wold, A.E., 2009. Establishment of the gut microbiota in Western infants. *Acta Paediatrica,* 98 (2), 229-238.

Agostoni, P., et al., 1990. Inspired gas relative humidity affects systemic to pulmonary bronchial blood flow in humans. *Chest,* 97 (6), 1377-1380.

Ahrens, R., et al., 1984. A method for comparing the peak intensity and duration of action of aerosolized bronchodilators using bronchoprovocation with methacholine. *The American Review of Respiratory Disease,* 129 (6), 903-906.

Aitken, M. and Marini, J., 1985. Effect of heat delivery and extraction on airway conductance in normal and in asthmatic subjects. *The American Review of Respiratory Disease,* 131 (3), 357.

Allan, K. and Devereux, G., 2011. Diet and asthma: nutrition implications from prevention to treatment. *Journal of the American Dietetic Association,* 111 (2), 258-268.

Alm, J.S., et al., 2002. An anthroposophic lifestyle and intestinal microflora in infancy. *Pediatric Allergy and Immunology,* 13 (6), 402-411.

Alm, J.S., et al., 1999. Atopy in children of families with an anthroposophic lifestyle. *The Lancet,* 353 (9163), 1485-1488.

American Thoracic Society, 2000. Guidelines for methacholine and exercise challenge testing. *American Journal of Respiratory and Critical Care Medicine,* 161 (1), 309.

Amrani, Y., 2014. Modulation of Airway Smooth Muscle Contractile Function by TNFα and IL-13 and Airway Hyperresponsiveness in Asthma. *In:* Modulation of Airway Smooth Muscle Contractile Function by TNFα and IL-13 and Airway

Hyperresponsiveness in Asthma. *Calcium Signaling In Airway Smooth Muscle Cells.* Springer, 2014, pp. 423-439.

Anderson, S.D., 1984. Is there a unifying hypothesis for exercise-induced asthma? *Journal of Allergy and Clinical Immunology,* 73 (5), 660-665.

Anderson, S.D., et al., 1997. A new method for bronchial-provocation testing in asthmatic subjects using a dry powder of mannitol. *American Journal of Respiratory and Critical Care Medicine,* 156 (3), 758.

Anderson, S.D. and Brannan, J.D., 2011. Bronchial provocation testing: the future. *Current Opinion in Allergy and Clinical Immunology,* 11 (1), 46-52.

Anderson, S.D. and Daviskas, E., 2000. The mechanism of exercise-induced asthma is... *Journal of Allergy and Clinical Immunology,* 106 (3), 453-459.

Anderson, S.D. and Daviskas, E., 1997. Pathophysiology of exercise-induced asthma: the role of respiratory water loss. *In:* J.M. Weiler, ed., *Allergic and respiratory disease in sports medicine.* New York: Marcel Dekker, 1997, pp. 87-114.

Anderson, S.D. and Daviskas, E., 1992. The airway microvasculature and exercise induced asthma. *Thorax,* 47 (9), 748.

Anderson, S.D., Daviskas, E. and Smith, C.M., 1989. Exercise-induced asthma: a difference in opinion regarding the stimulus. *In: Allergy and Asthma Proceedings,* OceanSide Publications, Inc, pp. 215-226.

Anderson, S.D., et al., 2003. Responses to bronchial challenge submitted for approval to use inhaled β2-agonists before an event at the 2002 winter Olympics. *Journal of Allergy and Clinical Immunology,* 111 (1), 45-50.

Anderson, S.D. and Holzer, K., 2000. Exercise-induced asthma: Is it the right diagnosis in elite athletes? *Journal of Allergy and Clinical Immunology,* 106 (3), 419-428.

Anderson, S.D. and Kippelen, P., 2008. Airway injury as a mechanism for exercise-induced bronchoconstriction in elite athletes. *Journal of Allergy and Clinical Immunology,* 122 (2), 225-235.

Anderson, S.D. and Kippelen, P., 2005a. Exercise-induced bronchoconstriction: pathogenesis. *Current Allergy and Asthma Reports,* 5 (2), 116-122.

Anderson, S.D. and Kippelen, P., 2005b. Exercise-induced bronchoconstriction: pathogenesis. *Current Allergy and Asthma Reports,* 5 (2), 116-122.

Anderson, S.D., et al., 2006. Bronchial challenges in athletes applying to inhale a [beta] 2-agonist at the 2004 Summer Olympics. *Journal of Allergy and Clinical Immunology,* 117 (4), 767-773.

Anderson, S.D., et al., 2001. Provocation by eucapnic voluntary hyperpnoea to identify exercise induced bronchoconstriction. *British Journal of Sports Medicine,* 35 (5), 344-347.

Anderson, S.D., et al., 2010. Reproducibility of the airway response to an exercise protocol standardized for intensity, duration, and inspired air conditions, in subjects with symptoms suggestive of asthma. *Respir Res,* 11, 120.

Anderson, S.D., et al., 1995. The use of non-isotonic aerosols for evaluating bronchial hyperresponsiveness. *Provocation Challenge Procedures,* , 249-278.

Anderson, S., et al., 1979. An evaluation of pharmacotherapy for exercise-induced asthma. *Journal of Allergy and Clinical Immunology,* 64 (6), 612-624.

Anderson, S., 2011. Bronchial challenge tests: usefulness, availability and limitations. *Breathe,* 8 (1), 53-60.

Anderson, S. and Henriksen, J., 1999. Management of exercise-induced asthma. *Exercise-Induced Asthma and Sports in Asthma,* , 99-108.

Anderson, S. and Kippelen, P., 2010. Stimulus and mechanisms of exercise-induced bronchoconstriction. *Breathe,* 7 (1), 25-33.

Anderson, S., et al., 1982. Sensitivity to heat and water loss at rest and during exercise in asthmatic patients. *European Journal of Respiratory Diseases,* 63 (5), 459.

Anderson, S.D., et al., 2001. Laboratory protocol for exercise asthma to evaluate salbutamol given by two devices. *Medicine and Science in Sports and Exercise,* 33 (6), 893-900.

Anderson, S.D., et al., 1985. Airway cooling as the stimulus to exercise-induced asthma--a re-evaluation. *European Journal of Respiratory Diseases,* 67 (1), 20-30.

Anderton, R., et al., 1979. Bronchial responsiveness to inhaled histamine and exercise. *Journal of Allergy and Clinical Immunology,* 63 (5), 315-320.

Andrew, D.P., et al., 1998. STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. *The Journal of Immunology,* 161 (9), 5027-5038.

Angus, F., Smart, S. and Shortt, C., 2005. Prebiotic ingredients with emphasis on galacto-oligosaccharides and fructo-oligosaccharides. *Probiotic Dairy Products.Blackwell Publishing, Marcel Dekker, Oxford,* , 120-137.

Aoki, H., et al., 2008. Resolvin E1 dampens airway inflammation and hyperresponsiveness in a murine model of asthma. *Biochemical and Biophysical Research Communications,* 367 (2), 509-515.

Aoki, H., et al., 2010. Protective effect of resolvin E1 on the development of asthmatic airway inflammation. *Biochemical and Biophysical Research Communications,* 400 (1), 128-133.

Argyros, G.J., et al., 1996. Eucapnic voluntary hyperventilation as a bronchoprovocation technique. *Chest,* 109 (6), 1520.

Argyros, G.J., et al., 1995. The refractory period after eucapnic voluntary hyperventilation challenge and its effect on challenge technique. *Chest,* 108 (2), 419.

Arm, J., et al., 1988. Effect of dietary supplementation with fish oil lipids on mild asthma. *Thorax,* 43 (2), 84-92.

Artis, D., 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nature Reviews Immunology,* 8 (6), 411-420.

Asthma UK, Accessed on 12.06.2013.

Bach, J., 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *New England Journal of Medicine,* 347 (12), 911-920.

Baile, E.M., et al., 1987. Effect of cold and warm dry air hyperventilation on canine airway blood flow. *Journal of Applied Physiology,* 62 (2), 526.

Baldwin, A.S., 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual Review of Immunology,* (12), 649-683.

Bandyopadhyay, B. and Mandal, N.C., 2014. Probiotics, Prebiotics and Synbiotics-In Health Improvement by Modulating Gut Microbiota: The Concept Revisited. *Int.J.Curr.Microbiol.App.Sci,* 3 (3), 410-420.

Barnes, P.J., 2010. New therapies for asthma: is there any progress? *Trends in Pharmacological Sciences,* 31 (7), 335-343.

Barnes, P.J., 2001. Th2 cytokines and asthma: an introduction. *Respir Res,* 2 (2), 64-65.

Barnes, P.J., 1996. Pathophysiology of asthma. *British Journal of Clinical Pharmacology,* 42 (1), 3-10.

Barnes, P.J., 1995. Nitric oxide and airway disease. *Annals of Medicine,* 27 (3), 389-393.

Barrett, N.A., et al., 2009. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *Journal of Immunology (Baltimore, Md.: 1950),* 182 (2), 1119-1128.

Barton, G.M., 2008. A calculated response: control of inflammation by the innate immune system. *The Journal of Clinical Investigation,* 118 (2), 413.

Beasley, R., et al., 2000. Prevalence and etiology of asthma. *Journal of Allergy and Clinical Immunology,* 105 (2), S466-S472.

Berin, M.C., 2002. The role of TARC in the pathogenesis of allergic asthma. *Drug News Perspect,* 15 (1), 10-16.

Berry, M., et al., 2007. TNF-α in asthma. *Current Opinion in Pharmacology,* 7 (3), 279-282.

Besnard, A., et al., 2011. IL-33-activated dendritic cells are critical for allergic airway inflammation. *European Journal of Immunology,* 41 (6), 1675-1686.

Bisgaard, H., et al., 2011. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *Journal of Allergy and Clinical Immunology,* 128 (3), 646-652. e5.

Bjermer, L. and Larsson, L., 1996. Obstructive symptoms in athletes: is it asthma and what to do about it? *Respiratory Medicine,* 90 (1), 1-3.

Black, P. and Sharpe, S., 1997. Dietary fat and asthma: is there a connection? *European Respiratory Journal,* 10 (1), 6-12.

Bland, J.M. and Altman, D.G., 1996. Measurement error. *BMJ (Clinical Research Ed.),* 312 (7047), 1654.

Blasbalg, T.L., et al., 2011. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *The American Journal of Clinical Nutrition,* 93 (5), 950-962.

Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology,* 37 (8), 911-917.

Bloemen, K., et al., 2007. The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunology Letters,* 113 (1), 6-18.

Bochenek, G., et al., 2004. Plasma 9α, 11β-PGF2, a PGD2 metabolite, as a sensitive marker of mast cell activation by allergen in bronchial asthma. *Thorax,* 59 (6), 459.

Boks, M.A., et al., 2014. Inhibition of TNF receptor signaling by anti-TNFα biologicals primes naïve $CD4^+$ T cells towards $IL\text{-}10^+$ T cells with a regulatory phenotype and function. *Clinical Immunology,* 151 (2), 136-145.

Bolger, C., et al., 2011. Effect of inspired air conditions on exercise-induced bronchoconstriction and urinary CC16 levels in athletes. *Journal of Applied Physiology (Bethesda, Md.: 1985),* 111 (4), 1059-1065.

Borsboom, G.J.J.M., et al., 1999. Diurnal variation in lung function in subgroups from two Dutch populations. *American Journal of Respiratory and Critical Care Medicine,* 159 (4), 1163-1171.

Böttcher, M., et al., 2000. Microflora-associated characteristics in faeces from allergic and nonallergic infants. *Clinical & Experimental Allergy,* 30 (11), 1591-1596.

Bouhnik, Y., et al., 2004. The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. *The American Journal of Clinical Nutrition,* 80 (6), 1658-1664.

Boulet, L., et al., 1994. Effects of long-term use of high-dose inhaled steroids on bone density and calcium metabolism. *Journal of Allergy and Clinical Immunology,* 94 (5), 796-803.

Boulet, L., Turcotte, H. and Tennina, S., 1989. Comparative efficacy of salbutamol, ipratropium, and cromoglycate in the prevention of bronchospasm induced by exercise and hyperosmolar challenges. *Journal of Allergy and Clinical Immunology,* 83 (5), 882-887.

Boyce, J.A., 2008. Eicosanoids in asthma, allergic inflammation, and host defense. *Current Molecular Medicine,* 8 (5), 335-349.

Bradding, P., et al., 1994. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *American Journal of Respiratory Cell and Molecular Biology,* 10 (5), 471-480.

Brannan, J., et al., 2003. Evidence of mast cell activation and leukotriene release after mannitol inhalation. *European Respiratory Journal,* 22 (3), 491-496.

Brannan, J.D., et al., 2014. The effect of omega-3 fatty acids on bronchial hyperresponsiveness, sputum eosinophilia and mast cell mediators in asthma. *CHEST Journal,* .

Brannan, J.D., et al., 1998. Responsiveness to mannitol in asthmatic subjects with exercise-and hyperventilation-induced asthma. *American Journal of Respiratory and Critical Care Medicine,* 158 (4), 1120-1126.

Brehm, J.M., et al., 2009. Serum vitamin D levels and markers of severity of childhood asthma in Costa Rica. *American Journal of Respiratory and Critical Care Medicine,* 179 (9), 765-771.

British Nutrition Foundation, 2000. *Briefing paper: n-3 fatty acids and health.* [online]. British Nutrition Foundation. Available at: http://www.nutrition.org.uk/publications/briefingpapers/n-3-fatty-acids-and-health [Accessed 12/21 2013].

British Thoracic Society Scottish Intercollegiate Guidelines Network, 2008. British Guideline on the Management of Asthma. *Thorax,* 63 Suppl 4, iv1-121.

Broide, D.H., et al., 1992. Cytokines in symptomatic asthma airways. *Journal of Allergy and Clinical Immunology,* 89 (5), 958-967.

Brouard, C. and Pascaud, M., 1990. Effects of moderate dietary supplementations with omega-3 fatty acids on macrophage and lymphocyte phospholipids and macrophage eicosanoid synthesis in the rat. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,* 1047 (1), 19-28.

Broughton, K.S., et al., 1997. Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. *The American Journal of Clinical Nutrition,* 65 (4), 1011.

Buchvald, F., et al., 2005. Exhaled nitric oxide predicts exercise-induced bronchoconstriction in asthmatic school children. *CHEST Journal,* 128 (4), 1964-1967.

Burdge, G.C. and Calder, P.C., 2006. Dietary a-linolenic acid and health-related outcomes: a metabolic perspective. *Nutrition Research Reviews,* 19 (1), 26-52.

Burney, P.G., et al., 1989. Effect of changing dietary sodium on the airway response to histamine. *Thorax,* 44 (1), 36-41.

Cabado, A.G., Vieytes, M.R. and Botana, L.M., 1998. Inhibition of Na+/K+ ATPase under hypertonic conditions in rat mast cells. *Life Sciences,* 63 (14), 1227-1237.

Calder, P.C., 2012. Mechanisms of Action of (n-3) Fatty Acids. *The Journal of Nutrition,* .

Calder, P.C., 2010. Omega-3 Fatty Acids and Inflammatory Processes. *Nutrients,* 2 (3), 355-374.

Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 77 (5), 327-335.

Camargo, C.A., et al., 2007. Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *The American Journal of Clinical Nutrition,* 85 (3), 788-795.

Carlsen, K., et al., 2008. Treatment of exercise-induced asthma, respiratory and allergic disorders in sports and the relationship to doping: Part II of the report from the Joint Task Force of European Respiratory Society (ERS) and European Academy of Allergy and Clinical Immunology (EAACI) in cooperation with GA2LEN*. *Allergy,* 63 (5), 492-505.

Carson, R.T. and Vignali, D.A., 1999. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *Journal of Immunological Methods,* 227 (1), 41-52.

Charlson, E.S., et al., 2011. Topographical continuity of bacterial populations in the healthy human respiratory tract. *American Journal of Respiratory and Critical Care Medicine,* 184 (8), 957-963.

Chinellato, I., et al., 2011. Serum vitamin D levels and exercise-induced bronchoconstriction in children with asthma. *European Respiratory Journal,* 37 (6), 1366.

Chung, K. and Barnes, P., 1999. Cytokines in asthma. *Thorax,* 54 (9), 825-857.

Clough, J.B., et al., 1991. Airway response to exercise and methacholine in children with respiratory symptoms. *Archives of Disease in Childhood,* 66 (5), 579-583.

Cockcroft, D. and Swystun, V., 1997. Effect of single doses of S-salbutamol, R-salbutamol, racemic salbutamol, and placebo on the airway response to methacholine. *Thorax,* 52 (10), 845-848.

Cohen, J.W., 1988. *Statistical power analysis for the behavioral sciences.* 2nd ed. Hillsdale, NJ, USA: Lawrence Erlbaum Associates.

Cone, E.J., et al., 2009. Normalization of urinary drug concentrations with specific gravity and creatinine. *Journal of Analytical Toxicology,* 33 (1), 1-7.

Coombes, J.L. and Powrie, F., 2008. Dendritic cells in intestinal immune regulation. *Nature Reviews Immunology,* 8 (6), 435-446.

Corrigan, C. and Kay, A., 1990. CD4 T-lymphocyte activation in acute severe asthma: relationship to disease severity and atopic status. *American Review of Respiratory Disease,* 141 (4_pt_1), 970-977.

Dahl, R., 2006. Systemic side effects of inhaled corticosteroids in patients with asthma. *Respiratory Medicine,* 100 (8), 1307-1317.

Dahlen, B., et al., 2001. The reproducibility and sample size requirements of exercise-induced bronchoconstriction measurements. *European Respiratory Journal,* 17 (4), 581-588.

Dahlen, S.E. and Kumlin, M., 2004. Monitoring mast cell activation by prostaglandin D2 in vivo. *Thorax,* 59 (6), 453-455.

Daley, D., 2014. The evolution of the hygiene hypothesis: the role of early-life exposures to viruses and microbes and their relationship to asthma and allergic diseases. *Current Opinion in Allergy and Clinical Immunology,* 14 (5), 390-396.

Daviskas, E., Gonda, I. and Anderson, S.D., 1991. Local airway heat and water vapour losses. *Respiration Physiology,* 84 (1), 115-132.

Daviskas, E., Gonda, I. and Anderson, S.D., 1990. Mathematical modeling of heat and water transport in human respiratory tract. *Journal of Applied Physiology,* 69 (1), 362-372.

De Filippo, C., et al., 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences of the United States of America,* 107 (33), 14691-14696.

De Roock, S., et al., 2010. Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans. *Clinical & Experimental Allergy,* 40 (1), 103-110.

Deal Jr, E.C., et al., 1979. Role of respiratory heat exchange in production of exercise-induced asthma. *Journal of Applied Physiology,* 46 (3), 467-475.

Deffebach, M., et al., 1989. Cold and hyperosmolar fluids in canine trachea: vascular and smooth muscle tone and albumin flux. *Journal of Applied Physiology,* 66 (3), 1309-1315.

Demirel, G., et al., 2013. Impact of probiotics on the course of indirect hyperbilirubinemia and phototherapy duration in very low birth weight infants. *Journal of Maternal-Fetal and Neonatal Medicine,* 26 (2), 215-218.

Denning, T.L., et al., 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17–producing T cell responses. *Nature Immunology,* 8 (10), 1086-1094.

Depeint, F., et al., 2008. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. *The American Journal of Clinical Nutrition,* 87 (3), 785-791.

Devereux, G. and Seaton, A., 2005. Diet as a risk factor for atopy and asthma. *Journal of Allergy and Clinical Immunology,* 115 (6), 1109-1117.

Devereux, G., et al., 2007. Maternal vitamin D intake during pregnancy and early childhood wheezing. *The American Journal of Clinical Nutrition,* 85 (3), 853-859.

Dickinson, J., McConnell, A. and Whyte, G., 2011. Diagnosis of exercise-induced bronchoconstriction: eucapnic voluntary hyperpnoea challenges identify previously undiagnosed elite athletes with exercise-induced bronchoconstriction. *British Journal of Sports Medicine,* 45 (14), 1126-1131.

Dickinson, J., et al., 2006. Screening elite winter athletes for exercise induced asthma: a comparison of three challenge methods. *British Journal of Sports Medicine,* 40 (2), 179.

Dickinson, J., et al., 2005. Impact of changes in the IOC-MC asthma criteria: a British perspective. *Thorax,* 60 (8), 629-632.

Dotterud, C., et al., 2010. Probiotics in pregnant women to prevent allergic disease: a randomized, double-blind trial. *British Journal of Dermatology,* 163 (3), 616-623.

Doucet, C., et al., 1998. Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. *Journal of Clinical Investigation,* 101 (10), 2129.

Douglas, G., et al., 2008. British guideline on the management of asthma: a national clinical guideline. *Thorax,* 63 (Suppl. 4), iv1-iv121.

Drazen, J.M., 2002. Anti-leukotrienes as novel anti-inflammatory treatments in asthma. *In:* Anti-leukotrienes as novel anti-inflammatory treatments in asthma. *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury, 5.* Springer, 2002, pp. 217-221.

Drazen, J.M., 1998. Leukotrienes as mediators of airway obstruction. *American Journal of Respiratory and Critical Care Medicine,* 158 (supplement_2), S193-S200.

Droste, J., et al., 2000. Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? *Clinical & Experimental Allergy,* 30 (11), 1548-1553.

Dry, J. and Vincent, D., 1991. Effect of a fish oil diet on asthma: results of a 1-year double-blind study. *International Archives of Allergy and Immunology,* 95 (2-3), 156-157.

Dweik, R.A., et al., 2011. An Official ATS Clinical Practice Guideline: Interpretation of Exhaled Nitric Oxide Levels (FENO) for Clinical Applications. *American Journal of Respiratory and Critical Care Medicine,* 184 (5), 602.

Ege, M.J., et al., 2011. Exposure to environmental microorganisms and childhood asthma. *New England Journal of Medicine,* 364 (8), 701-709.

Eggleston, P.A., 1979. A comparison of the asthmatic response to methacholine and exercise. *Journal of Allergy and Clinical Immunology,* 63 (2), 104-110.

Eggleston, P.A., Kagey-Sobotka, A. and Lichtenstein, L.M., 1987. A comparison of the osmotic activation of basophils and human lung mast cells. *The American Review of Respiratory Disease,* 135 (5), 1043-1048.

Eggleton, P., Gargan, R. and Fisher, D., 1989. Rapid method for the isolation of neutrophils in high yield without the use of dextran or density gradient polymers. *Journal of Immunological Methods,* 121 (1), 105-113.

ElHalawani, S.M., et al., 2003. Exhaled nitric oxide as a predictor of exercise-induced bronchoconstriction. *CHEST Journal,* 124 (2), 639-643.

Eliasson, A.H., et al., 1992. Sensitivity and specificity of bronchial provocation testing. An evaluation of four techniques in exercise-induced bronchospasm. *Chest,* 102 (2), 347-355.

Ellis, A., 1908. The pathological anatomy of bronchial asthma. *The American Journal of the Medical Sciences,* 136 (3), 407-428.

Elson, C.O. and Cong, Y., 2002. Understanding immune-microbial homeostasis in intestine. *Immunologic Research,* 26 (1-3), 87-94.

Emelyanov, A., et al., 2002. Treatment of asthma with lipid extract of New Zealand green-lipped mussel: a randomised clinical trial. *The European Respiratory Journal,* 20 (3), 596-600.

Endres, S., et al., 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *The New England Journal of Medicine,* 320 (5), 265.

Erb-Downward, J.R., et al., 2011. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PloS One,* 6 (2), e16384.

Erjefält, J., et al., 2004. Rapid and efficient clearance of airway tissue granulocytes through transepithelial migration. *Thorax,* 59 (2), 136-143.

Erkkola, M., et al., 2009. Maternal vitamin D intake during pregnancy is inversely associated with asthma and allergic rhinitis in 5-year-old children. *Clinical & Experimental Allergy,* 39 (6), 875-882.

Eschenbacher, W., Boushey, H. and Sheppard, D., 1984. Alteration in osmolarity of inhaled aerosols cause bronchoconstriction and cough, but absence of a permeant anion causes cough alone. *The American Review of Respiratory Disease,* 129 (2), 211-215.

Eschenbacher, W. and Sheppard, D., 1985. Respiratory heat loss is not the sole stimulus for bronchocontriction induced by isocapnic hyperpnea with dry air. *The American Review of Respiratory Disease,* 131 (6), 894-901.

Evans, T.M., et al., 2005. Cold air inhalation does not affect the severity of EIB after exercise or eucapnic voluntary hyperventilation. *Medicine & Science in Sports & Exercise,* 37 (4), 544.

Feldberg, W. and Kellaway, C.H., 1938. Liberation of histamine and formation of lysocithin-like substances by cobra venom. *The Journal of Physiology,* 94 (2), 187-226.

Feleszko, W., et al., 2007. Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clinical & Experimental Allergy,* 37 (4), 498-505.

Fitch, K.D., 2006. β 2-Agonists at the Olympic Games. *Clinical Reviews in Allergy and Immunology,* 31 (2), 259-268.

Fitch, K.D., et al., 2008. Asthma and the elite athlete: summary of the International Olympic Committee's consensus conference, Lausanne, Switzerland, January 22-24, 2008. *Journal of Allergy and Clinical Immunology,* 122 (2), 254-260. e7.

Fitch, K.D., 2012. An overview of asthma and airway hyper-responsiveness in Olympic athletes. *British Journal of Sports Medicine,* 46 (6), 413-416.

Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y., 2003. Foxp3 programs the development and function of CD4 CD25 regulatory T cells. *Nature Immunology,* 4 (4), 330-336.

Forsythe, P., Inman, M.D. and Bienenstock, J., 2007. Oral treatment with live Lactobacillus reuteri inhibits the allergic airway response in mice. *American Journal of Respiratory and Critical Care Medicine,* 175 (6), 561-569.

Franklin, P.J., et al., 2003. Exhaled nitric oxide and asthma: complex interactions between atopy, airway responsiveness, and symptoms in a community population of children. *Thorax,* 58 (12), 1048-1052.

Franzius, D., Hoth, M. and Penner, R., 1994. Non-specific effects of calcium entry antagonists in mast cells. *Pflügers Archiv,* 428 (5-6), 433-438.

Freed, A.N., et al., 2000. Thermally induced asthma and airway drying. *American Journal of Respiratory and Critical Care Medicine,* 161 (6), 2112.

Freedman, S.D., et al., 2004. Association of cystic fibrosis with abnormalities in fatty acid metabolism. *New England Journal of Medicine,* 350 (6), 560-569.

Frey, U., Maksym, G. and Suki, B., 2011. Temporal complexity in clinical manifestations of lung disease. *Journal of Applied Physiology,* 110 (6), 1723-1731.

Galli, C. and Calder, P.C., 2009. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. *Ann Nutr Metab,* 55 (1-3), 123-139.

Garcia, G., Godot, V. and Humbert, M., 2005. New chemokine targets for asthma therapy. *Current Allergy and Asthma Reports,* 5 (2), 155-160.

GeurtsvanKessel, C. and Lambrecht, B., 2008. Division of labor between dendritic cell subsets of the lung. *Mucosal Immunology,* 1 (6), 442-450.

Gibson, G.R., et al., 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev,* 17 (2), 259-275.

Gilbert, I.A., Fouke, J.M. and McFadden Jr, E., 1987. Heat and water flux in the intrathoracic airways and exercise-induced asthma. *Journal of Applied Physiology,* 63 (4), 1681-1691.

Gilbert, I.A. and McFadden Jr, E., 1992. Airway cooling and rewarming. The second reaction sequence in exercise-induced asthma. *Journal of Clinical Investigation,* 90 (3), 699.

Gill, H. and Prasad, J., 2008. Probiotics, immunomodulation, and health benefits. *In:* Probiotics, immunomodulation, and health benefits. *Bioactive Components of Milk.* Springer, 2008, pp. 423-454.

Ginde, A.A., Mansbach, J.M. and Camargo, C.A., 2009. Vitamin D, respiratory infections, and asthma. *Current Allergy and Asthma Reports,* 9 (1), 81-87.

Glover, W., et al., 2006. Lung deposition of mannitol powder aerosol in healthy subjects. *Journal of Aerosol Medicine,* 19 (4), 522-532.

Goldblatt, M., 1935. Properties of human seminal plasma. *The Journal of Physiology,* 84 (2), 208-218.

Gollwitzer, E.S. and Marsland, B.J., 2014. Microbiota abnormalities in inflammatory airway diseases—Potential for therapy. *Pharmacology & Therapeutics,* 141 (1), 32-39.

Gon, Y., et al., 1998. Cooling and rewarming-induced IL-8 expression in human bronchial epithelial cells through p38 MAP kinase-dependent pathway. *Biochemical and Biophysical Research Communications,* 249 (1), 156-160.

Greenspon, L.W. and Morrissey, W.L., 1986. Factors that contribute to inhibition of methacholine-induced bronchoconstriction. *The American Review of Respiratory Disease,* 133 (5), 735-739.

Grindebacke, H., et al., 2004. Defective suppression of Th2 cytokines by CD4 CD25 regulatory T cells in birch allergics during birch pollen season. *Clinical & Experimental Allergy,* 34 (9), 1364-1372.

Gulliksson, M., et al., 2006. Release of prostaglandin D2 and leukotriene C4 in response to hyperosmolar stimulation of mast cells. *Allergy,* 61 (12), 1473-1479.

Gustafsson, D., Sjöberg, O. and Foucard, T., 2000. Development of allergies and asthma in infants and young children with atopic dermatitis–a prospective follow-up to 7 years of age. *Allergy,* 55 (3), 240-245.

Gutkowski, P., et al., 2010. Effect of orally administered probiotic strains Lactobacillus and Bifidobacterium in children with atopic asthma. *Centr Eur J Immunol,* 35 (4), 233-238.

Haby, M.M., et al., 1994. An exercise challenge protocol for epidemiological studies of asthma in children: comparison with histamine challenge. *The European Respiratory Journal,* 7 (1), 43-49.

Hahn, A., et al., 1984. A reinterpretation of the effect of temperature and water content of the inspired air in exercise-induced asthma. *The American Review of Respiratory Disease,* 130 (4), 575.

Hallstrand, T.S., 2012. New insights into pathogenesis of exercise-induced bronchoconstriction. *Current Opinion in Allergy and Clinical Immunology,* 12 (1), 42.

Hallstrand, T.S. and Henderson, W.R., 2009. Role of leukotrienes in exercise-induced bronchoconstriction. *Current Allergy and Asthma Reports,* 9 (1), 18-25.

Hallstrand, T.S., et al., 2005. Inflammatory basis of exercise-induced bronchoconstriction. *American Journal of Respiratory and Critical Care Medicine,* 172 (6), 679.

Hallstrand, T.S., et al., 2013. Role of cells and mediators in exercise-induced bronchoconstriction. *Immunology and Allergy Clinics of North America,* 33 (3), 313-328.

Hallstrand, T.S., et al., 1998. Peripheral blood manifestations of Th2 lymphocyte activation in stable atopic asthma and during exercise-induced bronchospasm. *Annals of Allergy, Asthma & Immunology,* 80 (5), 424-432.

Hallstrand, T.S., et al., 2005. Airway immunopathology of asthma with exercise-induced bronchoconstriction. *Journal of Allergy and Clinical Immunology,* 116 (3), 586-593.

Hamid, Q., et al., 1993. Induction of nitric oxide synthase in asthma. *The Lancet,* 342 (8886), 1510-1513.

Hammad, H. and Lambrecht, B.N., 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nature Reviews Immunology,* 8 (3), 193-204.

Han, M.K., et al., 2012. Significance of the microbiome in obstructive lung disease. *Thorax,* 67 (5), 456-463.

Hanania, N.A., et al., 1995. Dose-related decrease in bone density among asthmatic patients treated with inhaled corticosteroids. *The Journal of Allergy and Clinical Immunology,* 96 (5 Pt 1), 571.

Hansen, R., et al., 2012. Microbiota of de-novo pediatric IBD: increased Faecalibacterium prausnitzii and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *The American Journal of Gastroenterology,* 107 (12), 1913-1922.

Harizi, H., Corcuff, J. and Gualde, N., 2008. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in Molecular Medicine,* 14 (10), 461-469.

Harris, S.G., et al., 2002. Prostaglandins as modulators of immunity. *Trends in Immunology,* 23 (3), 144-150.

Hashimoto, S., et al., 1999. Hyperosmolarity-induced interleukin-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase. *American Journal of Respiratory and Critical Care Medicine,* 159 (2), 634-640.

Haworth, O., et al., 2008. Resolvin E1 regulates interleukin 23, interferon-γ and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nature Immunology,* 9 (8), 873-879.

Head, S. and Mickleborough, T., 2013. Randomized Cross-Over Controlled Pilot Study of Docosahexaenoic Acid Supplementation on Airway Inflammation and Hyperpnea-Induced Bronchoconstriction in Adults with Asthma. *The Internet Journal of Asthma, Allergy and Immunology,* 9 (1).

Heir, T. and Oseid, S., 1994. Self-reported asthma and exercise-induced asthma symptoms in high-level competitive cross-country skiers. *Scandinavian Journal of Medicine & Science in Sports,* 4 (2), 128-133.

Helenius, I. and Haahtela, T., 2000. Allergy and asthma in elite summer sport athletes. *Journal of Allergy and Clinical Immunology,* 106 (3), 444-452.

Helenius, I.J., Tikkanen, H.O. and Haahtela, T., 1998. Occurrence of exercise induced bronchospasm in elite runners: dependence on atopy and exposure to cold air and pollen. *British Journal of Sports Medicine,* 32 (2), 125.

Helenius, I.J., Tikkanen, H.O. and Haahtela, T., 1997. Association between type of training and risk of asthma in elite athletes. *Thorax,* 52 (2), 157-160.

Helenius, I.J., et al., 1998. Asthma and increased bronchial responsiveness in elite athletes: atopy and sport event as risk factors. *Journal of Allergy and Clinical Immunology,* 101 (5), 646-652.

Henderson Jr, W.R., et al., 2002. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *American Journal of Respiratory and Critical Care Medicine,* 165 (1), 108-116.

Hepburn, N., et al., 2013. Probiotic supplement consumption alters cytokine production from peripheral blood mononuclear cells: a preliminary study using healthy individuals. *Beneficial Microbes,* 4 (4), 313-317.

Hilty, M., et al., 2010. Disordered microbial communities in asthmatic airways. *PloS One,* 5 (1), e8578.

Ho, J.E., et al., 2013. Soluble ST2 predicts elevated SBP in the community. *Journal of Hypertension,* 31 (7), 1431-6; discussion 1436.

Hodge, L., et al., 1998. Effect of dietary intake of omega-3 and omega-6 fatty acids on severity of asthma in children. *European Respiratory Journal,* 11 (2), 361.

Hofstra, W., et al., 1997. Sample size estimation in studies monitoring exercise-induced bronchoconstriction in asthmatic children. *Thorax,* 52 (8), 739-741.

Holley, A.B., et al., 2012. Eucapnic voluntary hyperventilation is superior to methacholine challenge testing for detecting airway hyperreactivity in nonathletes. *Journal of Asthma,* 49 (6), 614-619.

Holzer, K., et al., 2003. Mannitol as a challenge test to identify exercise-induced bronchoconstriction in elite athletes. *American Journal of Respiratory and Critical Care Medicine,* 167 (4), 534.

Holzer, K., Anderson, S.D. and Douglass, J., 2002. Exercise in elite summer athletes: challenges for diagnosis. *Journal of Allergy and Clinical Immunology,* 110 (3), 374-380.

Hooper, L.V., Littman, D.R. and Macpherson, A.J., 2012. Interactions between the microbiota and the immune system. *Science (New York, N.Y.),* 336 (6086), 1268-1273.

Hopkins, W.G., 2000. Measures of reliability in sports medicine and science. *Sports Medicine,* 30 (1), 1-15.

Hori, S., Nomura, T. and Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science (New York, N.Y.),* 299 (5609), 1057-1061.

Horrobin, D. and Manku, M., 1987. Genetically low arachidonic acid and high dihomogammalinolenic acid levels in Eskimos may contribute to low incidence of coronary heart disease, psoriasis, arthritis and asthma. *Polyunsaturated Fatty Acids and Eicosanoids.Champaign, Illinois, USA: American Oil Chemists Society,* , 413-415.

Horwitz, R.J. and Busse, W.W., 1995. Inflammation and asthma. *Clinics in Chest Medicine,* 16 (4), 583.

Horwitz, R.J., McGILL, K.A. and Busse, W.W., 1998. The role of leukotriene modifiers in the treatment of asthma. *American Journal of Respiratory and Critical Care Medicine,* 157 (5), 1363-1371.

Howarth, G.S. and Wang, H., 2013. Role of endogenous microbiota, probiotics and their biological products in human health. *Nutrients,* 5 (1), 58-81.

Hoyles, L. and Vulevic, J., 2008. Diet, immunity and functional foods. *In:* G.B. Huffnagle and M.C. Noverr, eds., *GI Microbiota and Regulation of the Immune System.* Austin, TX, USA: Landes Bioscience, 2008, pp. 79-92.

Hruby, J. and Butler, J., 1975. Variability of routine pulmonary function tests. *Thorax,* 30 (5), 548-553.

Huang, Y.J., et al., 2011. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *Journal of Allergy and Clinical Immunology,* 127 (2), 372-381. e3.

Huber, H.L. and Koessler, K.K., 1922. THE PATHOLOGY OF BRONCHIAL ASTHMA. *Archives of Internal Medicine,* 30 (6), 689-760.

Human Microbiome Project Consortium, 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486 (7402), 207-214.

Hurwitz, K.M., et al., 1995. Interpretation of eucapnic voluntary hyperventilation in the diagnosis of asthma. *Chest,* 108 (5), 1240-1245.

Iliev, I.D., et al., 2009. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut,* 58 (11), 1481-1489.

Imai, T., et al., 1999. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *International Immunology,* 11 (1), 81-88.

Isolauri, E., et al., 2000. Probiotics in the management of atopic eczema. *Clinical & Experimental Allergy,* 30 (11), 1605-1610.

James, M.J. and Cleland, L.G., 1997. Dietary n-3 fatty acids and therapy for rheumatoid arthritis. *In: Seminars in arthritis and rheumatism,* Elsevier, pp. 85-97.

Jan, R.L., et al., 2011. Lactobacillus gasseri suppresses Th17 pro-inflammatory response and attenuates allergen-induced airway inflammation in a mouse model of allergic asthma. *The British Journal of Nutrition,* , 1.

Janeway, C.A., et al., 2001. *Immunobiology: the immune system in health and disease.* Churchill Livingstone.

Johnson, M., 2001. Beta$_2$-adrenoceptors: mechanisms of action of beta$_2$-agonists. *Paediatric Respiratory Reviews,* 2 (1), 57-62.

Joos, G. and O'Connor, B., 2003. Indirect airway challenges. *European Respiratory Journal,* 21 (6), 1050-1068.

Jouaville, L., et al., 2003. Interrelationships among asthma, atopy, rhinitis and exhaled nitric oxide in a population-based sample of children. *Clinical & Experimental Allergy,* 33 (11), 1506-1511.

Junge, W., et al., 2004. Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffe method. *Clinica Chimica Acta,* 344 (1), 137-148.

Kalayci, O., et al., 2004. Monocyte chemotactic protein-4 (MCP-4; CCL-13): a biomarker of asthma. *Journal of Asthma,* 41 (1), 27-33.

Kalliomäki, M., et al., 2001. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *Journal of Allergy and Clinical Immunology,* 107 (1), 129-134.

Kalliomaki, M., et al., 2010. Guidance for substantiating the evidence for beneficial effects of probiotics: prevention and management of allergic diseases by probiotics. *The Journal of Nutrition,* 140 (3), 713S-21S.

Karjalainen, E.M., et al., 2000. Evidence of airway inflammation and remodeling in ski athletes with and without bronchial hyperresponsiveness to methacholine. *American Journal of Respiratory and Critical Care Medicine,* 161 (6), 2086-2091.

Khalfoun, B., et al., 1996. Docosahexaenoic and eicosapentaenoic acids inhibit in vitro human endothelial cell production of interleukin-6. *Advances in Experimental Medicine and Biology,* 400, 589-597.

Khalili, B., Boggs, P. and Bahna, S., 2007. Reliability of a new hand-held device for the measurement of exhaled nitric oxide. *Allergy,* 62 (10), 1171-1174.

Kharitonov, S., et al., 1994. Increased nitric oxide in exhaled air of asthmatic patients. *The Lancet,* 343 (8890), 133-135.

Kidd, P., 2003. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Alternative Medicine Review,* 8 (3), 223-246.

Kim, H.H., et al., 1996. Effect of hyperventilation on airway mucosal blood flow in normal subjects. *American Journal of Respiratory and Critical Care Medicine,* 154 (5), 1563-1566.

Kippelen, P., et al., 2012. Respiratory health of elite athletes–preventing airway injury: a critical review. *British Journal of Sports Medicine,* 46 (7), 471-476.

Kippelen, P. and Anderson, S.D., 2012. Airway injury during high-level exercise. *British Journal of Sports Medicine,* 46 (6), 385-390.

Kippelen, P., et al., 2010a. Effect of sodium cromoglycate on mast cell mediators during hyperpnea in athletes. *Medicine and Science in Sports and Exercise,* 42 (10), 1853-1860.

Kippelen, P., et al., 2010b. Acute effects of beclomethasone on hyperpnea-induced bronchoconstriction. *Medicine and Science in Sports and Exercise,* 42 (2), 273-280.

Kirjavainen, P.V., et al., 2001. Characterizing the composition of intestinal microflora as a prospective treatment target in infant allergic disease. *FEMS Immunology & Medical Microbiology,* 32 (1), 1-7.

Knudson, R.J., et al., 1983. Changes in the normal maximal expiratory flow-volume curve with growth and aging. *The American Review of Respiratory Disease,* 127 (6), 725-734.

Kolida, S. and Gibson, G.R., 2011. Synbiotics in health and disease. *Annual Review of Food Science and Technology,* 2, 373-393.

Kondo, Y., et al., 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *International Immunology,* 20 (6), 791-800.

Kony, S., et al., 2004. Association of bronchial hyperresponsiveness and lung function with C-reactive protein (CRP): a population based study. *Thorax,* 59 (10), 892-896.

Kranich, J., Maslowski, K.M. and Mackay, C.R., 2011. Commensal flora and the regulation of inflammatory and autoimmune responses. *In: Seminars in immunology,* Elsevier, pp. 139-145.

Kremer, J., Lawrence, D. and Jubiz, W., 1989. Different doses of fish-oil fatty acid ingestion in active rheumatoid arthritis: a prospective study of clinical and immunological parameters. *NATO ASI Series: Series A: Life Sciences,* 171.

Kumlin, M., et al., 1992. Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B2 in response to bronchial provocations with allergen, aspirin, leukotriene D4, and histamine in asthmatics. *American Review of Respiratory Disease,* 146 (1), 96-103.

Kwon, H.K., et al., 2010. Generation of regulatory dendritic cells and CD4+Foxp3+ T cells by probiotics administration suppresses immune disorders. *Proceedings of the National Academy of Sciences of the United States of America,* 107 (5), 2159-2164.

Laitinen, L.A. and Laitinen, A., 1995. Inhaled corticosteroid treatment for asthma. *In: Allergy and Asthma Proceedings,* OceanSide Publications, Inc, pp. 63-66.

Lambrecht, B.N. and Hammad, H., 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity,* 31 (3), 412-424.

Lamkhioued, B., et al., 1997. Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatics contributes to the chemotaxis of eosinophils to the site of inflammation. *Journal of Immunology (Baltimore, Md.: 1950),* 159 (9), 4593-4601.

Langdeau, J.B. and Boulet, L.P., 2001. Prevalence and mechanisms of development of asthma and airway hyperresponsiveness in athletes. *Sports Medicine,* 31 (8), 601-616.

Langdeau, J.B., et al., 2000. Airway hyperresponsiveness in elite athletes. *American Journal of Respiratory and Critical Care Medicine,* 161 (5), 1479.

Larsson, K., et al., 1993. High prevalence of asthma in cross country skiers. *British Medical Journal,* 307 (6915), 1326.

Lattka, E., et al., 2010. Genetic variants of the FADS1 FADS2 gene cluster as related to essential fatty acid metabolism. *Current Opinion in Lipidology,* 21 (1), 64-69.

Lebeer, S., Vanderleyden, J. and De Keersmaecker, S.C., 2010. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nature Reviews Microbiology,* 8 (3), 171-184.

Lee, S., et al., 2006. Eosinophils play a major role in the severity of exercise-induced bronchoconstriction in children with asthma. *Pediatric Pulmonology,* 41 (12), 1161-1166.

Lee, T.H., et al., 1985. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *New England Journal of Medicine,* 312 (19), 1217-1224.

Lee, J.Y., et al., 2003. Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. *Journal of Lipid Research,* 44 (3), 479-486.

Lee, J.Y., et al., 2004. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. *The Journal of Biological Chemistry,* 279 (17), 16971-16979.

Lee, T.H., et al., 1984. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene B5 and the double lipoxygenase product. *The Journal of Biological Chemistry,* 259 (4), 2383-2389.

Leung, T., et al., 2002. Plasma concentration of thymus and activation-regulated chemokine is elevated in childhood asthma. *Journal of Allergy and Clinical Immunology,* 110 (3), 404-409.

Levy, B.D., et al., 2007. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *Journal of Immunology (Baltimore, Md.: 1950),* 178 (1), 496-502.

Lilly, C.M., et al., 1999. Elevated plasma eotaxin levels in patients with acute asthma. *Journal of Allergy and Clinical Immunology,* 104 (4), 786-790.

Lin, Y., Shieh, C. and Wang, J., 2008. The functional insufficiency of human CD4 CD25high T-regulatory cells in allergic asthma is subjected to TNF-α modulation. *Allergy,* 63 (1), 67-74.

Ling, E.M., et al., 2004. Relation of CD4 CD25 regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *The Lancet,* 363 (9409), 608-615.

Lloyd, C.M. and Hawrylowicz, C.M., 2009. Regulatory T cells in asthma. *Immunity,* 31 (3), 438-449.

Lloyd, C.M. and Hessel, E.M., 2010. Functions of T cells in asthma: more than just TH2 cells. *Nature Reviews Immunology,* 10 (12), 838-848.

Lomax, A.R. and Calder, P.C., 2009. Prebiotics, immune function, infection and inflammation: a review of the evidence. *Br J Nutr,* 101 (5), 633-658.

Lukacs, N.W., 2001. Role of chemokines in the pathogenesis of asthma. *Nature Reviews Immunology,* 1 (2), 108-116.

Lukacs, N.W., Oliveira, S.H. and Hogaboam, C.M., 1999. Chemokines and asthma: redundancy of function or a coordinated effort? *Journal of Clinical Investigation,* 104 (8), 995-999.

Ma, J. and Xiao, L., 2011. Use of dietary supplements among US adults with asthma. *Nutrition and Dietary Supplements; 2011; 3: 9,* 18.

Macfarlane, G., Steed, H. and Macfarlane, S., 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology,* 104 (2), 305-344.

Macfarlane, S. and Macfarlane, G., 2003. Food and the large intestine. *Gut Flora, Nutrition, Immunity and Health,* , 24-51.

Macfarlane, S., Macfarlane, G. and Cummings, J.t., 2006. Review article: prebiotics in the gastrointestinal tract. *Alimentary Pharmacology & Therapeutics,* 24 (5), 701-714.

Macpherson, A.J. and Harris, N.L., 2004. Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology,* 4 (6), 478-485.

Madan, J., et al., 2012. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. *MBio,* 3 (4).

Mahler, D.A., 1993. Exercise-induced asthma. *Medicine and Science in Sports and Exercise,* 25 (5), 554-561.

Manning, P.J., et al., 1990. Inhibition of exercise-induced bronchoconstriction by MK-571, a potent leukotriene D4–receptor antagonist. *New England Journal of Medicine,* 323 (25), 1736-1739.

Manning, P.J., Watson, R.M. and O'Byrne, P.M., 1993. Exercise-induced refractoriness in asthmatic subjects involves leukotriene and prostaglandin interdependent mechanisms. *The American Review of Respiratory Disease,* 148 (4 Pt 1), 950-954.

Mannix, E.T., Manfredi, F. and Farber, M.O., 1999. A Comparison of Two Challenge Tests for Identifying Exercise-Induced Bronchospasm in Figure Skaters. *Chest,* 115 (3), 649-653.

Marco, M.L., Pavan, S. and Kleerebezem, M., 2006. Towards understanding molecular modes of probiotic action. *Current Opinion in Biotechnology,* 17 (2), 204-210.

Marin, M.L., et al., 1998. Stimulation of cytokine production in clonal macrophage and T-cell models by Streptococcus thermophilus: comparison with Bifidobacterium sp. and Lactobacillus bulgaricus. *Journal of Food Protection®,* 61 (7), 859-864.

Martin, R.E., 1998. Docosahexaenoic acid decreases phospholipase A2 activity in the neurites/nerve growth cones of PC12 cells. *Journal of Neuroscience Research,* 54 (6), 805-813.

Maslowski, K.M. and Mackay, C.R., 2011. Diet, gut microbiota and immune responses. *Nature Immunology,* 12 (1), 5-9.

Matsui, H., et al., 2000. Osmotic water permeabilities of cultured, well-differentiated normal and cystic fibrosis airway epithelia. *Journal of Clinical Investigation,* 105 (10), 1419-1427.

Mays, L.E., et al., 2013. Modified Foxp3 mRNA protects against asthma through an IL-10–dependent mechanism. *The Journal of Clinical Investigation,* 123 (3), 1216-1228.

McCracken, V.J. and Lorenz, R.G., 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cellular Microbiology,* 3 (1), 1-11.

McFadden Jr, E., 1990. Hypothesis: exercise-induced asthma as a vascular phenomenon. *Lancet,* 335 (8694), 880.

McFadden Jr, E. and Gilbert, I.A., 1994. Exercise-induced asthma. *New England Journal of Medicine,* 330 (19), 1362-1367.

McFadden Jr, E., Lenner, K. and Strohl, K., 1986. Postexertional airway rewarming and thermally induced asthma. New insights into pathophysiology and possible pathogenesis. *Journal of Clinical Investigation,* 78 (1), 18.

McFadden Jr, E., et al., 1999. Thermally induced asthma and airway drying. *American Journal of Respiratory and Critical Care Medicine,* 160 (1), 221.

McFadden, E., 1983. Respiratory heat and water exchange: physiological and clinical implications. *Journal of Applied Physiology,* 54 (2), 331-336.

McKeever, T.M. and Britton, J., 2004. Diet and asthma. *American Journal of Respiratory and Critical Care Medicine,* 170 (7), 725-729.

McLoughlin, R.M. and Mills, K.H., 2011. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. *Journal of Allergy and Clinical Immunology,* 127 (5), 1097-1107.

McWilliam, A.S., et al., 1996. Dendritic cells are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. *The Journal of Experimental Medicine,* 184 (6), 2429-2432.

Medzhitov, R., 2008. Origin and physiological roles of inflammation. *Nature,* 454 (7203), 428-435.

Messer, J.W., Peters, G.A. and Bennett, W.A., 1960. Causes of death and pathologic findings in 304 cases of bronchial asthma. *CHEST Journal,* 38 (6), 616-624.

Mickleborough, T.D., et al., 2006. Protective Effect of Fish Oil Supplementation on Exercise-Induced Bronchoconstriction in Asthma*. *Chest,* 129 (1), 39.

Mickleborough, T.D., Lindley, M.R. and Ray, S., 2005. Dietary salt, airway inflammation, and diffusion capacity in exercise-induced asthma. *Medicine & Science in Sports & Exercise,* 37 (6), 904.

Mickleborough, T.D., et al., 2003. Fish oil supplementation reduces severity of exercise-induced bronchoconstriction in elite athletes. *American Journal of Respiratory and Critical Care Medicine,* 168 (10), 1181.

Mickleborough, T.D., et al., 2009. Eicosapentaenoic acid is more effective than docosahexaenoic acid in inhibiting proinflammatory mediator production and transcription from LPS-induced human asthmatic alveolar macrophage cells. *Clinical Nutrition,* 28 (1), 71-77.

Mickleborough, T. and Fogarty, A., 2006. Dietary sodium intake and asthma: an epidemiological and clinical review. *International Journal of Clinical Practice,* 60 (12), 1616-1624.

Mickleborough, T. and Rundell, K., 2005. Dietary polyunsaturated fatty acids in asthma-and exercise-induced bronchoconstriction. *European Journal of Clinical Nutrition,* 59 (12), 1335-1346.

Mickleborough, T.D., et al., 2013. Marine lipid fraction PCSO-524™(lyprinol® /omega XL®) of the New Zealand green lipped mussel attenuates hyperpnea-induced bronchoconstriction in asthma. *Respiratory Medicine,* .

Mickleborough, T.D., 2005. Dietary omega-3 polyunsaturated fatty acid supplementation and airway hyperresponsiveness in asthma. *J Asthma,* 42 (5), 305.

Miller, A.M., 2011. Role of IL-33 in inflammation and disease. *J Inflamm (Lond),* 8 (1), 22.

Miller, M., et al., 2005. Standardisation of spirometry. *Eur Respir J,* 26 (2), 319-338.

Mills, D.E., 2013. *Respiratory muscle work and inspiratory muscle training on cytokines, oxidative stress and diaphragm fatigue in younger and older populations.* Doctor of Philosophy., Nottingham Trent University.

Mok, M.Y., et al., 2010. Serum levels of IL-33 and soluble ST2 and their association with disease activity in systemic lupus erythematosus. *Rheumatology (Oxford, England),* 49 (3), 520-527.

Moloney, E., et al., 2003. Release of inflammatory mediators from eosinophils following a hyperosmolar stimulus. *Respiratory Medicine,* 97 (8), 928-932.

Molphy, J., et al., 2013. Prevalence of bronchoconstriction induced by eucapnic voluntary hyperpnoea in recreationally active individuals. *Journal of Asthma,* (0), 1-25.

Moreira, A., et al., 2007. Pilot study of the effects of n-3 polyunsaturated fatty acids on exhaled nitric oxide in patients with stable asthma.

Moro-García, M.A., et al., 2013. Oral supplementation with Lactobacillus delbrueckii subsp. bulgaricus 8481 enhances systemic immunity in elderly subjects. *Age,* 35 (4), 1311-1326.

Mosmann, T.R., et al., 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology,* 136 (7), 2348-2357.

Mosmann, T. and Coffman, R., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology,* 7 (1), 145-173.

Mowat, A.M., 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews Immunology,* 3 (4), 331-341.

Nagakura, T., et al., 1998. GC/MS analysis of urinary excretion of 9α, 11β-PGF2 in acute and exercise-induced asthma in children. *Clinical & Experimental Allergy,* 28 (2), 181-186.

Nagalingam, N.A., Cope, E.K. and Lynch, S.V., 2013. Probiotic strategies for treatment of respiratory diseases. *Trends in Microbiology,* 21 (9), 485-492.

Naguwa, S., Afrasiabi, R. and Chang, C., 2012. Exercise-induced asthma. *Bronchial Asthma,* , 251-266.

Nathan, C. and Xie, Q., 1994. Nitric oxide synthases: roles, tolls, and controls. *Cell,* 78 (6), 915-918.

National Asthma Education and Prevention Program, 2007. Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. *The Journal of Allergy and Clinical Immunology,* 120 (5 Suppl), S94-138.

Nauseef, W.M., 2007. Isolation of human neutrophils from venous blood. *In:* Isolation of human neutrophils from venous blood. *Neutrophil Methods and Protocols.* Springer, 2007, pp. 15-20.

NHLBI, *Asthma Attack Illustration - NIH - National Heart, Lung, and Blood Institute* [online]. . Available at: http://www.nhlbi.nih.gov/health/health-topics/topics/asthma/ [Accessed 08/14 2014].

Nord, C., 1993. The effect of antimicrobial agents on the ecology of the human intestinal microflora. *Veterinary Microbiology,* 35 (3), 193-197.

Noverr, M.C. and Huffnagle, G.B., 2004. Does the microbiota regulate immune responses outside the gut? *Trends in Microbiology,* 12 (12), 562-568.

O'byrne, P., 2000. Leukotriene bronchoconstriction induced by allergen and exercise. *American Journal of Respiratory and Critical Care Medicine,* 161 (supplement_1), S68-S72.

Oddy, W.H., et al., 2004. Ratio of Omega-6 to Omega-3 Fatty Acids and Childhood Asthma. *Journal of Asthma,* 41 (3), 319-326.

Okada, H., et al., 2010. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clinical & Experimental Immunology,* 160 (1), 1-9.

Olszak, T., et al., 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science (New York, N.Y.),* 336 (6080), 489-493.

O'Mahony, C., et al., 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-κB activation. *PLoS Pathogens,* 4 (8), e1000112.

OpenStax College, 07/08/2013. Normal and Bronchial Asthma Tissue Diagram. *Anatomy & Physiology* [online], , 13/08/2014.

Oshikawa, K., et al., 2001. Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *American Journal of Respiratory and Critical Care Medicine,* 164 (2), 277-281.

Ostrowski, K., et al., 2001. Chemokines are elevated in plasma after strenuous exercise in humans. *European Journal of Applied Physiology,* 84 (3), 244-245.

O'Sullivan, S., et al., 1996. Increased urinary excretion of the prostaglandin D2 metabolite 9 [alpha], 11 [beta]-prostaglandin F2 after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *Journal of Allergy and Clinical Immunology,* 98 (2), 421-432.

O'sullivan, S., et al., 1998. Evidence for mast cell activation during exercise-induced bronchoconstriction. *European Respiratory Journal,* 12 (2), 345-350.

Palombo, J.D., et al., 1999. Effect of short-term enteral feeding with eicosapentaenoic and γ-linolenic acids on alveolar macrophage eicosanoid synthesis and bactericidal function in rats. *Critical Care Medicine,* 27 (9), 1908-1915.

Pang, L., et al., 1998. The COX-1/COX-2 balance in asthma. *Clinical and Experimental Allergy,* 28 (9), 1050-1058.

Parsons, J.P., et al., 2007. Prevalence of exercise-induced bronchospasm in a cohort of varsity college athletes. *Medicine & Science in Sports & Exercise,* 39 (9), 1487.

Parsons, J.P., 2009. Exercise-induced bronchospasm: symptoms are not enough. *Expert Review of Clinical Immunology,* 5 (4), 357-359.

Parsons, J.P., et al., 2013. An Official American Thoracic Society Clinical Practice Guideline: Exercise-induced Bronchoconstriction. *American Journal of Respiratory and Critical Care Medicine,* 187 (9), 1016-1027.

Pavord, I. and Tattersfield, A., 1995. Bronchoprotective role for endogenous prostaglandin E_2. *The Lancet,* 345 (8947), 436-438.

Pawankar, R., et al., 2011. WAO white book on allergy. *Milwaukee, WI: World Allergy Organization,* , 1-216.

Platts-Mills, T., et al., 2005. Is the hygiene hypothesis still a viable explanation for the increased prevalence of asthma? *Allergy,* 60 (s79), 25-31.

Pober, J.S. and Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology,* 7 (10), 803-815.

Potts, J., 1996. Factors associated with respiratory problems in swimmers. *Sports Medicine (Auckland, NZ),* 21 (4), 256.

Powell, N., et al., 1996. Increased expression of mRNA encoding RANTES and MCP-3 in the bronchial mucosa in atopic asthma. *European Respiratory Journal,* 9 (12), 2454-2460.

Prefontaine, D., et al., 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *Journal of Immunology (Baltimore, Md.: 1950),* 183 (8), 5094-5103.

Prenosil, J., Stuker, E. and Bourne, J., 1987. Formation of oligosaccharides during enzymatic lactose: Part I: State of art. *Biotechnology and Bioengineering,* 30 (9), 1019-1025.

Provoost, S., et al., 2009. Decreased FOXP3 protein expression in patients with asthma. *Allergy,* 64 (10), 1539-1546.

Rajilić-Stojanović, M. and Vos, W.M., 2014. The First 1,000 Cultured Species of the Human Gastrointestinal Microbiota. *FEMS Microbiology Reviews,* .

Rakoff-Nahoum, S., et al., 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell,* 118 (2), 229-241.

Reid, G., 2005. The importance of guidelines in the development and application of probiotics. *Current Pharmaceutical Design,* 11 (1), 11-16.

Rescigno, M., et al., 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature Immunology,* 2 (4), 361-367.

Riedler, J., et al., 2001. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *The Lancet,* 358 (9288), 1129-1133.

Riedler, J., et al., 1994. Hypertonic saline challenge in an epidemiologic survey of asthma in children. *American Journal of Respiratory and Critical Care Medicine,* 150 (6 Pt 1), 1632-1639.

Rimoldi, M., et al., 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nature Immunology,* 6 (5), 507-514.

Robbins, R., et al., 1994. Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. *Biochemical and Biophysical Research Communications,* 198 (3), 835-843.

Robbins, S.L., Kumar, V. and Cotran, R.S., 2003. *Robbins basic pathology.* Saunders.

Roberfroid, M., et al., 2010. Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition,* 104 (S2), S1-S63.

Roger, E., Eston, R.G. and Reilly, T., 2009. *Kinanthropometry and exercise physiology laboratory manual: tests, procedures and data.* Taylor & Francis.

Romberg, K., Bjermer, L. and Tufvesson, E., 2011. Exercise but not mannitol provocation increases urinary Clara cell protein (CC16) in elite swimmers. *Respiratory Medicine,* 105 (1), 31-36.

Rose, M., et al., 2010. Efficacy of probiotic Lactobacillus GG on allergic sensitization and asthma in infants at risk. *Clinical & Experimental Allergy,* 40 (9), 1398-1405.

Rossi, D. and Zlotnik, A., 2000. The biology of chemokines and their receptors. *Annual Review of Immunology,* 18 (1), 217-242.

Round, J.L. and Mazmanian, S.K., 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America,* 107 (27), 12204-12209.

Rundell, K.W., et al., 2004. Field Exercise vs Laboratory Eucapnic Voluntary Hyperventilation To Identify Airway Hyperresponsiveness in Elite Cold Weather Athletes*. *Chest,* 125 (3), 909.

Rundell, K.W., et al., 2001a. Self-reported symptoms and exercise-induced asthma in the elite athlete. *Medicine & Science in Sports & Exercise,* 33 (2), 208.

Rundell, K.W., et al., 2001b. Mid expiratory flow rates of cold weather athletes with exercise-induced asthma. *Medicine & Science in Sports & Exercise,* 33 (5), S12.

Rundell, K.W. and Jenkinson, D.M., 2002. Exercise-induced bronchospasm in the elite athlete. *Sports Medicine,* 32 (9), 583-600.

Rundell, K.W. and Slee, J.B., 2008. Exercise and other indirect challenges to demonstrate asthma or exercise-induced bronchoconstriction in athletes. *Journal of Allergy and Clinical Immunology,* 122 (2), 238-246.

Rundell, K.W., et al., 2000. Exercise-induced asthma screening of elite athletes: field versus laboratory exercise challenge. *Medicine and Science in Sports and Exercise,* 32 (2), 309.

Rundell, K.W. and Spiering, B.A., 2003. Inspiratory stridor in elite athletes. *CHEST Journal,* 123 (2), 468-474.

Rundell, K., Wilber, R. and Lemanske Jr, R., 2002. Exercise-Induced Asthma: Pathophysiology and Treatment. *Human Kinetics, IL, USA,* .

Rundell, K.W., et al., 2005. Effects of montelukast on airway narrowing from eucapnic voluntary hyperventilation and cold air exercise. *British Journal of Sports Medicine,* 39 (4), 232-236.

Rupp, N.T., Guill, M.F. and Brudno, D.S., 1992. Unrecognized exercise-induced bronchospasm in adolescent athletes. *Archives of Pediatrics & Adolescent Medicine,* 146 (8), 941.

Ryan, G., et al., 1982. Bronchial responsiveness to histamine: relationship to diurnal variation of peak flow rate, improvement after bronchodilator, and airway calibre. *Thorax,* 37 (6), 423-429.

Sagar, S., 2014. Beneficial bacteria as a treatment for allergic asthma. *Inflammation and Cell Signaling,* 1 (4), 10-14800/ics. 173.

Sagar, S., et al., 2014. The combination of *Bifidobacterium breve* with non-digestible oligosaccharides suppresses airway inflammation in a murine model for chronic asthma. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1842 (4), 573-583.

Sakula, A., 1984. Sir John Floyer's A Treatise of the Asthma (1698). *Thorax,* 39 (4), 248-254.

Salvi, S.S., Suresh Babu, K. and Holgate, S.T., 2001. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *American Journal of Respiratory and Critical Care Medicine,* 164 (8), 1343-1346.

Sandrini, A., et al., 2010. Fractional exhaled nitric oxide in asthma: an update. *Respirology,* 15 (1), 57-70.

Sanz, Y. and De Palma, G., 2009. Gut microbiota and probiotics in modulation of epithelium and gut-associated lymphoid tissue function. *International Reviews of Immunology,* 28 (6), 397-413.

Sattler, S., et al., 2013. The evolutionary role of the IL-33/ST2 system in host immune defence. *Archivum Immunologiae Et Therapiae Experimentalis,* 61 (2), 107-117.

Schmitz, G. and Ecker, J., 2008. The opposing effects of omega 3 and omega 6 fatty acids. *Progress in Lipid Research,* 47 (2), 147-155.

Schmitz, J., et al., 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity,* 23 (5), 479-490.

Schoeffel, R.E., Anderson, S.D. and Altounyan, R., 1981. Bronchial hyperreactivity in response to inhalation of ultrasonically nebulised solutions of distilled water and saline. *British Medical Journal (Clinical Research Ed.),* 283 (6302), 1285-1287.

Schrezenmeir, J. and de Vrese, M., 2001. Probiotics, prebiotics, and synbiotics--approaching a definition. *The American Journal of Clinical Nutrition,* 73 (2 Suppl), 361S-364S.

Scientific Advisory Committee on Nutrition/Committee on Nutrition/Committee on Toxicity, 2004. *Scientific Advisory Committee on Nutrition/Committee on Nutrition/Committee on Toxicity. Advice on Fish Consumption: Benefits and Risks.* London: The Stationery Office.

Scollo, M., et al., 2000. Exhaled nitric oxide and exercise-induced bronchoconstriction in asthmatic children. *American Journal of Respiratory and Critical Care Medicine,* 161 (3), 1047-1050.

Sears, M., 2008. The definition and diagnosis of asthma. *Allergy,* 48 (s17), 12-16.

Sears, M., et al., 1991. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *New England Journal of Medicine,* 325 (15), 1067-1071.

Seki, H., Tani, Y. and Arita, M., 2009. Omega-3 PUFA derived anti-inflammatory lipid mediator resolvin E1. *Prostaglandins &Other Lipid Mediators,* 89 (3-4), 126-130.

Sekiya, T., et al., 2000. Inducible expression of a Th2-type CC chemokine thymus-and activation-regulated chemokine by human bronchial epithelial cells. *The Journal of Immunology,* 165 (4), 2205-2213.

Sekiya, T., et al., 2002. Increased levels of a TH2-type CC chemokine thymus and activation-regulated chemokine (TARC) in serum and induced sputum of asthmatics. *Allergy,* 57 (2), 173-177.

Serhan, C.N., Yacoubian, S. and Yang, R., 2008. Anti-inflammatory and pro-resolving lipid mediators. *Annual Review of Pathology,* 3, 279.

Serhan, C.N. and Chiang, N., 2008. Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. *British Journal of Pharmacology,* 153 (S1), S200-S215.

Serhan, C.N., 2007. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu.Rev.Immunol.,* 25, 101-137.

Serhan, C.N., et al., 2000. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2–nonsteroidal antiinflammatory drugs and transcellular processing. *The Journal of Experimental Medicine,* 192 (8), 1197-1204.

Serhan, C.N. and Savill, J., 2005. Resolution of inflammation: the beginning programs the end. *Nature Immunology,* 6 (12), 1191-1197.

Serhan, C.N., et al., 2002. Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *The Journal of Experimental Medicine,* 196 (8), 1025-1037.

Serhan, C.N., et al., 2011. Novel anti-inflammatory--pro-resolving mediators and their receptors. *Current Topics in Medicinal Chemistry,* 11 (6), 629-647.

Shaaban, R., et al., 2006. Change in C-reactive protein levels and FEV_1 decline: A longitudinal population-based study. *Respiratory Medicine,* 100 (12), 2112-2120.

Shaheen, S.O., et al., 2001. Dietary Antioxidants and Asthma in Adults Population-based Case–Control Study. *American Journal of Respiratory and Critical Care Medicine,* 164 (10), 1823-1828.

Sheppard, D. and Eschenbacher, W.L., 1984. Respiratory water loss as a stimulus to exercise-induced bronchoconstriction. *Journal of Allergy and Clinical Immunology,* 73 (5), 640-642.

Shi, H., et al., 1998. Effect of inhaled interleukin-5 on airway hyperreactivity and eosinophilia in asthmatics. *American Journal of Respiratory and Critical Care Medicine,* 157 (1), 204-209.

Simons, F.E.R., Gerstner, T.V. and Cheang, M.S., 1997. Tolerance to the bronchoprotective effect of salmeterol in adolescents with exercise-induced asthma using concurrent inhaled glucocorticoid treatment. *Pediatrics,* 99 (5), 655-659.

Simopoulos, A.P., 2002a. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedecine & Pharmacotherapy,* 56 (8), 365-379.

Simopoulos, A.P., 2002b. Omega-3 fatty acids in inflammation and autoimmune diseases. *Journal of the American College of Nutrition,* 21 (6), 495.

Simopoulos, A., 2006. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomedicine & Pharmacotherapy,* 60 (9), 502-507.

Simopoulos, A.P., 1994. Is insulin resistance influenced by dietary linoleic acid and trans fatty acids? *Free Radical Biology and Medicine,* 17 (4), 367-372.

Sjögren, Y.M., et al., 2009. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clinical & Experimental Allergy,* 39 (4), 518-526.

Smit, H.A., Grievink, L. and Tabak, C., 1999. Dietary influences on chronic obstructive lung disease and asthma: a review of the epidemiological evidence. *Proceedings of the Nutrition Society,* 58 (2), 309-320.

Smit, J.J. and Lukacs, N.W., 2006. A closer look at chemokines and their role in asthmatic responses. *European Journal of Pharmacology,* 533 (1), 277-288.

Smith, C.M. and Anderson, S.D., 1986. Hyperosmolarity as the stimulus to asthma induced by hyperventilation? *Journal of Allergy and Clinical Immunology,* 77 (5), 729-736.

Smith, C. and Anderson, S., 1989. A comparison between the airway response to isocapnic hyperventilation and hypertonic saline in subjects with asthma. *European Respiratory Journal,* 2 (1), 36-43.

Smith, D., 2010. IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clinical & Experimental Allergy,* 40 (2), 200-208.

Smits, H.H., et al., 2005. Selective probiotic bacteria induce IL-10–producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell–specific

intercellular adhesion molecule 3–grabbing nonintegrin. *Journal of Allergy and Clinical Immunology,* 115 (6), 1260-1267.

Solway, J., 1990. Airway heat and water fluxes and the tracheobronchial circulation. *The European Respiratory Journal.Supplement,* 12, 608s-617s.

Sperling, R.I., 1995. Eicosanoids in rheumatoid arthritis. *Rheumatic Diseases Clinics of North America,* 21 (3), 741-758.

Stadelmann, K., Stensrud, T. and Carlsen, K., 2011. Respiratory symptoms and bronchial responsiveness in competitive swimmers. *Med Sci Sports Exerc,* 43 (3), 375-381.

Stellato, C., et al., 1997. Production of the novel CC chemokine MCP-4 by airway cells and comparison of its biological activity to other CC chemokines. *Journal of Clinical Investigation,* 99 (5), 926.

Sterk, P.J., et al., 1993. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *The European Respiratory Journal.Supplement,* 16, 53-83.

Sterling, G.M., 1968. The mechanism of bronchoconstriction due to hypocapnia in man. *Clinical Science,* 34 (2), 277-285.

Stickland, M.K., et al., 2012. Effect of warm-up exercise on exercise-induced bronchoconstriction. *Med Sci Sports Exerc,* 44 (3), 383-391.

Stolarski, B., et al., 2010. IL-33 exacerbates eosinophil-mediated airway inflammation. *Journal of Immunology (Baltimore, Md.: 1950),* 185 (6), 3472-3480.

Storms, W.W., 2003. Review of exercise-induced asthma. *Medicine and Science in Sports and Exercise,* 35 (9), 1464-1470.

Strachan, D., 1989. Damp housing and ill health. *BMJ (Clinical Research Ed.),* 299 (6694), 325.

Strauss, R.H., et al., 1978. Influence of heat and humidity on the airway obstruction induced by exercise in asthma. *Journal of Clinical Investigation,* 61 (2), 433.

Strunk, R.C., et al., 2003. Relationship of exhaled nitric oxide to clinical and inflammatory markers of persistent asthma in children. *Journal of Allergy and Clinical Immunology,* 112 (5), 883-892.

Sue-Chu, M., et al., 2000. Placebo-controlled study of inhaled budesonide on indices of airway inflammation in bronchoalveolar lavage fluid and bronchial biopsies in cross-country skiers. *Respiration,* 67 (4), 417-425.

Sue-Chu, M., et al., 1999. Bronchoscopy and bronchoalveolar lavage findings in cross-country skiers with and without" ski asthma". *European Respiratory Journal,* 13 (3), 626-632.

Sue-Chu, M., Larsson, L. and Bjermer, L., 1996. Prevalence of asthma in young cross-country skiers in central Scandinavia: differences between Norway and Sweden. *Respiratory Medicine,* 90 (2), 99-105.

Sue-Chu, M., et al., 2010. Airway hyperresponsiveness to methacholine, adenosine 5-monophosphate, mannitol, eucapnic voluntary hyperpnoea and field exercise challenge in elite cross-country skiers. *British Journal of Sports Medicine,* 44 (11), 827-832.

Sullivan, Å., Edlund, C. and Nord, C.E., 2001. Effect of antimicrobial agents on the ecological balance of human microflora. *The Lancet Infectious Diseases,* 1 (2), 101-114.

Suman, O.E., et al., 1999. Airway obstruction during exercise and isocapnic hyperventilation in asthmatic subjects. *Journal of Applied Physiology,* 87 (3), 1107-1113.

Sundar, I.K. and Rahman, I., 2011. Vitamin d and susceptibility of chronic lung diseases: role of epigenetics. *Frontiers in Pharmacology,* 2, 50.

Sunyer, J., et al., 1996. Total serum IgE is associated with asthma independently of specific IgE levels. The Spanish Group of the European Study of Asthma. *The European Respiratory Journal,* 9 (9), 1880-1884.

Tabka, Z., et al., 1988. Effect of dry warm air on respiratory water loss in children with exercise-induced asthma. *Chest,* 94 (1), 81-86.

Taha, R.A., et al., 1999. Eotaxin and monocyte chemotactic protein-4 mRNA expression in small airways of asthmatic and nonasthmatic individuals. *Journal of Allergy and Clinical Immunology,* 103 (3), 476-483.

Tahan, F., et al., 2006. The role of chemokines in exercise-induced bronchoconstriction in asthma. *Annals of Allergy, Asthma & Immunology,* 96 (6), 819-825.

Takemura, M., et al., 2006. High sensitivity C-reactive protein in asthma. *The European Respiratory Journal,* 27 (5), 908-912.

Tateno, H., et al., 2004. Plasma eotaxin level and severity of asthma treated with corticosteroid. *Respiratory Medicine,* 98 (8), 782-790.

Tecklenburg, S.L., et al., 2007. Ascorbic acid supplementation attenuates exercise-induced bronchoconstriction in patients with asthma. *Respiratory Medicine,* 101 (8), 1770-1778.

Tecklenburg-Lund, S., et al., 2010. Randomized controlled trial of fish oil and montelukast and their combination on airway inflammation and hyperpnea-induced bronchoconstriction. *PLoS One,* 5 (10), e13487.

Teran, L.M., 2000. CCL chemokines and asthma. *Immunology Today,* 21 (5), 235-242.

Thiemermann, C., Mitchell, J.A. and Ferns, G.A., 1993. Eicosanoids and atherosclerosis. *Current Opinion in Lipidology,* 4 (5), 401-406.

Tikkanen, H.O. and Helenius, I., 1994. Asthma in runners. *Bmj,* 309 (6961), 1087.

To, T., et al., 2012. Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health,* 12 (1), 204.

To, T., et al., 2010. What is the lifetime risk of physician-diagnosed asthma in Ontario, Canada? *American Journal of Respiratory and Critical Care Medicine,* 181 (4), 337-343.

Togias, A.G., et al., 1988. The osmolality of nasal secretions increases when inflammatory mediators are released in response to inhalation of cold, dry air. *American Journal of Respiratory and Critical Care Medicine,* 137 (3), 625-629.

Togias, A., et al., 1985. Nasal challenge with cold, dry air results in release of inflammatory mediators. Possible mast cell involvement. *Journal of Clinical Investigation,* 76 (4), 1375.

Tribe, R.M., et al., 1994. Dietary sodium intake, airway responsiveness, and cellular sodium transport. *American Journal of Respiratory and Critical Care Medicine,* 149 (6), 1426-1433.

Turnbaugh, P.J., et al., 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine,* 1 (6), 6ra14.

Tzortzis, G., Goulas, A.K. and Gibson, G.R., 2005. Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, Bifidobacterium bifidum NCIMB 41171. *Applied Microbiology and Biotechnology,* 68 (3), 412-416.

Tzortzis, G., et al., 2005. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *The Journal of Nutrition,* 135 (7), 1726-1731.

Uddin, M. and Levy, B.D., 2010. Resolvins: Natural agonists for resolution of pulmonary inflammation. *Progress in Lipid Research,* .

Uematsu, S., et al., 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nature Immunology,* 9 (7), 769-776.

Umetsu, D.T., et al., 2002. Asthma: an epidemic of dysregulated immunity. *Nature Immunology,* 3 (8), 715-720.

Vachier, I., et al., 2002. Endogenous anti-inflammatory mediators from arachidonate in human neutrophils. *Biochemical and Biophysical Research Communications,* 290 (1), 219-224.

Valencia, X., et al., 2006. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood,* 108 (1), 253-261.

Van de Pol, M., et al., 2010. Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics. *Allergy,* 66 (1), 39-47.

Van de Rest, O., et al., 2008. Effect of fish oil on cognitive performance in older subjects A randomized, controlled trial. *Neurology,* 71 (6), 430-438.

Van der Aa, L., et al., 2011. Synbiotics prevent asthma-like symptoms in infants with atopic dermatitis. *Allergy,* 66 (2), 170-177.

Varol, C., et al., 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity,* 31 (3), 502-512.

Villaran, C., et al., 1999. Montelukast versus salmeterol in patients with asthma and exercise-induced bronchoconstriction. *Journal of Allergy and Clinical Immunology,* 104 (3), 547-553.

Vliagoftis, H., et al., 2008. Probiotics for the treatment of allergic rhinitis and asthma: systematic review of randomized controlled trials. *Annals of Allergy, Asthma, & Immunology,* 101 (6), 570.

von Hertzen, L.C. and Haahtela, T., 2000. Could the risk of asthma and atopy be reduced by a vaccine that induces a strong T-helper type 1 response? *American Journal of Respiratory Cell and Molecular Biology,* 22 (2), 139-142.

Vos, A., et al., 2007. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. *Critical Reviews™ in Immunology,* 27 (2).

Voy, R.O., 1986. The US Olympic Committee experience with exercise-induced bronchospasm, 1984. *Medicine and Science in Sports and Exercise,* 18 (3), 328.

Vulevic, J., et al., 2008. Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *The American Journal of Clinical Nutrition,* 88 (5), 1438-1446.

Vulevic, J., et al., 2013. A mixture of trans-galactooligosaccharides reduces markers of metabolic syndrome and modulates the fecal microbiota and immune function of overweight adults. *The Journal of Nutrition,* 143 (3), 324-331.

Wan, K. and Wu, W., 2007. Eicosanoids in asthma. *Acta Paediatrica Taiwan,* 48 (6), 299-304.

Wang, D. and DuBois, R.N., 2010. Eicosanoids and cancer. *Nature Reviews Cancer,* 10 (3), 181-193.

Wang, M., et al., 2008. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *Journal of Allergy and Clinical Immunology,* 121 (1), 129-134.

Wang, X., et al., 1993. Pulmonary function between 6 and 18 years of age. *Pediatric Pulmonology,* 15 (2), 75-88.

Wang, J.H., et al., 1996. Expression of RANTES by human bronchial epithelial cells in vitro and in vivo and the effect of corticosteroids. *American Journal of Respiratory Cell and Molecular Biology,* 14 (1), 27-35.

Wanger, J., et al., 2005. Standardisation of the measurement of lung volumes. *European Respiratory Journal,* 26 (3), 511-522.

Weiler, J.M., Layton, T. and Hunt, M., 1998. Asthma in United States Olympic athletes who participated in the 1996 Summer Games. *Journal of Allergy and Clinical Immunology,* 102 (5), 722-726.

Weiler, J.M., 1996. Exercise-induced asthma: a practical guide to definitions, diagnosis, prevalence, and treatment. *In: Allergy and Asthma Proceedings,* OceanSide Publications, Inc, pp. 315-325.

Weiler, J.M., et al., 2007. American Academy of Allergy, Asthma & Immunology work group report: exercise-induced asthma. *Journal of Allergy and Clinical Immunology,* 119 (6), 1349-1358.

Weiss, U., 2008. Inflammation. *Nature,* 454 (7203), 427-427.

Weldon, S.M., et al., 2007. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *The Journal of Nutritional Biochemistry,* 18 (4), 250-258.

Wells, J.M., et al., 2011. Epithelial crosstalk at the microbiota-mucosal interface. *Proceedings of the National Academy of Sciences of the United States of America,* 108 Suppl 1, 4607-4614.

Wenzel, S.E., 2013. Complex phenotypes in asthma: Current definitions. *Pulmonary Pharmacology & Therapeutics,* .

Wenzel, S.E., 1997. Arachidonic acid metabolites: mediators of inflammation in asthma. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy,* 17 (1P2), 3S-12S.

Weylandt, K.H., et al., 2012. Omega-3 fatty acids and their lipid mediators: towards an understanding of resolvin and protectin formation. *Prostaglandins & Other Lipid Mediators,* 97 (3), 73-82.

Whelan, K. and Quigley, E.M., 2013. Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease. *Current Opinion in Gastroenterology,* 29 (2), 184-189.

Wickens, K., et al., 1999. Antibiotic use in early childhood and the development of asthma. *Clinical and Experimental Allergy,* 29 (6), 766-771.

Willers, S.M., et al., 2011. Childhood diet and asthma and atopy at 8 years of age: the PIAMA birth cohort study. *The European Respiratory Journal,* 37 (5), 1060-1067.

Wills-Karp, M., Santeliz, J. and Karp, C.L., 2001. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nature Reviews Immunology,* 1 (1), 69-75.

Winkler, P., et al., 2007. Molecular and cellular basis of microflora-host interactions. *The Journal of Nutrition,* 137 (3 Suppl 2), 756S-72S.

Wong, C., et al., 2001. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clinical & Experimental Immunology,* 125 (2), 177-183.

Wong, C.A., et al., 2000. Inhaled corticosteroid use and bone-mineral density in patients with asthma. *The Lancet,* 355 (9213), 1399-1403.

Wong, K.W., 2005. Clinical efficacy of n-3 fatty acid supplementation in patients with asthma. *Journal of the American Dietetic Association,* 105 (1), 98-105.

Woo, S., et al., 2010. Effect of Lactobacillus sakei supplementation in children with atopic eczema–dermatitis syndrome. *Annals of Allergy, Asthma & Immunology,* 104 (4), 343-348.

Wood, A.J., et al., 1999. Treatment of asthma with drugs modifying the leukotriene pathway. *New England Journal of Medicine,* 340 (3), 197-206.

Woodruff, P.G., et al., 2009. T-helper type 2–driven inflammation defines major subphenotypes of asthma. *American Journal of Respiratory and Critical Care Medicine,* 180 (5), 388.

Woolley, M., Anderson, S.D. and Quigley, B., 1990. Duration of protective effect of terbutaline sulfate and cromolyn sodium alone and in combination on exercise-induced asthma. *CHEST Journal,* 97 (1), 39-45.

World Anti-Doping Agency, *2014 List of Prohibited Substances and Methods* [online]. . Available at: http://list.wada-ama.org/ [Accessed 09/12 2014].

Yagami, A., et al., 2010. IL-33 mediates inflammatory responses in human lung tissue cells. *Journal of Immunology (Baltimore, Md.: 1950),* 185 (10), 5743-5750.

Yaqoob, P., Newsholme, E.A. and Calder, P.C., 1995. Influence of cell culture conditions on diet-induced changes in lymphocyte fatty acid composition. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism,* 1255 (3), 333-340.

Ying, S., et al., 1997. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *European Journal of Immunology,* 27 (12), 3507-3516.

Yokoyama, A., et al., 2000. Effect of aerosolized docosahexaenoic acid in a mouse model of atopic asthma. *International Archives of Allergy and Immunology,* 123 (4), 327-332.

Yoshikawa, T., et al., 1998. Severity of exercise-induced bronchoconstriction is related to airway eosinophilic inflammation in patients with asthma. *European Respiratory Journal,* 12 (4), 879-884.

Yu, J., et al., 2010. The effects of Lactobacillus rhamnosus on the prevention of asthma in a murine model. *Allergy, Asthma & Immunology Research,* 2 (3), 199-205.

Zawadski, D., Lenner, K. and McFadden Jr, E., 1988. Comparison of intraairway temperatures in normal and asthmatic subjects after hyperpnea with hot, cold, and ambient air. *American Journal of Respiratory and Critical Care Medicine,* 138 (6), 1553.

Zhao, X., et al., 2003. CCL9 is secreted by the follicle-associated epithelium and recruits dome region Peyer's patch CD11b+ dendritic cells. *Journal of Immunology (Baltimore, Md.: 1950),* 171 (6), 2797-2803.

Zietkowski, Z., et al., 2009. RANTES in exhaled breath condensate of allergic asthma patients with exercise-induced bronchoconstriction. *Respiration,* 80 (6), 463-471.

Zietkowski, Z., et al., 2011. Eotaxin in exhaled breath condensate of allergic asthma patients with exercise-induced bronchoconstriction. *Respiration,* 82 (2), 169-176.

Zimmerman, M.P. and Pisarri, T.E., 2000. Bronchial vasodilation evoked by increased lower airway osmolarity in dogs. *Journal of Applied Physiology,* 88 (2), 425.

Zlotnik, A. and Yoshie, O., 2000. Chemokines: a new classification system and their role in immunity. *Immunity,* 12 (2), 121-127.

Appendix 1 - Self-reporting Health Screen

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

Name or Number_____

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc Yes ☐ No ☐

(q) Any problems affecting your nose e.g. recurrent nose bleeds

Yes ☐ No ☐

Any nasal fracture or deviated nasal septum Yes ☐ No ☐

(r) Are there any reasons why blood sampling may be difficult?

Yes ☐ No ☐

(s) Have you had a blood sample taken previously? Yes ☐ No ☐

(t) Have you had a cold, flu or any flu like symptoms in the last Month?

Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5 .**Asthma Specific questions**:

When was your last asthma exacerbation_____

Have you ever been admitted to hospital for your asthma Yes ☐ No ☐

Have you ever been prescribed a course of steroids for your asthma

Yes ☐ No ☐

Do you suffer from asthma symptoms post exercise Yes ☐ No ☐

Do you suffer from asthma symptoms when exposed to dry/cold air

Yes ☐ No ☐

Do you suffer from asthma symptoms when exposed to allergens (pollen, animal fur etc)

Yes ☐ No ☐

Appendix 2 - Chapter 4 Participant Information Sheet

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

A1 – Participant Information Sheet

"Reproducibility of diagnosing exercise induced asthma through eucapnic voluntary hyperventilation (EVH)"

Introduction

Exercise induced asthma or sometimes termed exercise induced bronchoconstriction (EIB) is a term used to describe a transient narrowing of the airways during or following exercise, that can occur in individuals with and without asthma. It is generally considered that exercise and high breathing rates are important initiating stimuli as they dehydrate and alter the airway surface lining. Exercise induced bronchoconstriction presents a significant problem for many elite and recreational athletes many of whom have to reduce training loads impacting both their performance, and quality of life.

Eucapnic Voluntary Hyperventilation is an indirect challenge used to identify and diagnose exercise induced bronchoconstriction (EIB) in individuals and athletes. It is based on the fact that increased ventilation rate causes bronchoconstriction in susceptible individuals. This occurs through drying of the airways and increasing its osmolality causing the airway smooth muscles to contract. EVH has demonstrated high sensitivity to identify those with EIB. It is currently the International Olympic Committee Medical Commissions (IOC-MC) recommended challenge to identify EIB among Olympic athletes. EVH requires participants to hyperventilate a dry gas (5% CO_2, 21% O_2, balance Nitrogen) for 6 minutes. Pre and post pulmonary function is assessed to diagnose EIB.

Inclusion Criteria

Aged 18-45

Body mass index 20-25 $kg \cdot m^{-2}$

Physically active (completing 3 or more exercise sessions a week lasting at least 45 minutes each)

Non-smoker

Asthmatics must have their own clinically prescribed medication (e.g. short acting β2-agonist)

Exclusion Criteria

Predicted forced expiratory volume over one second (FEV_1) of <65% – this will be measured during your assessment visit

Previously admitted to hospital for asthma or other breathing difficulties

Previously diagnosed with chronic obstructive pulmonary disease (COPD) emphysema, chronic bronchitis or similar respiratory illness

History of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease

History of recurrent chest infections

Smoker

Acute infection within the last four weeks

Major operation within the past four months

Currently taking long term asthma maintenance medications – corticosteroids, and leukotriene modifiers that you could not refrain from taking for 4 days prior to laboratory session

Requirements

All subjects will be allowed to continue to use their EIB medication as needed during the course of the studies. However, to identify treatment effects subjects will be required to refrain from:

Using short acting β_2-agonists for at least 12h before each testing session

Taking anti-histamines for 48h before testing sessions

Taking inhaled corticosteroids for 4 days before testing sessions

Ingesting caffeine for 8h prior to testing sessions

Physical exercise and alcohol for 24h prior to a testing sessions

Consuming food 2h prior to testing sessions

Female participants must be taking an oral contraceptive, or have a contraceptive implant

These are standardised requirements used in previous EIB, and supplementation studies. If your asthma symptoms are exacerbated due to the reduction in medication use prior to each testing session then you will be withdrawn from the study. This reduction in medication use may also have a very short term impact upon your exercise performance, that will be reversed following the testing session and when you take your medication again.

You will perform three initial baseline pulmonary function tests, used to assess the function of your lungs. Then you will perform the EVH protocol. The procedure will require you to breath dry (<5% relative humidity) hypercapnic air (4.5-5% CO_2) at a predetermined rate for 6 minutes. Breathing in through a mouthpiece with a nose clip on, your target breathing rate will be displayed on a screen to help you maintain this ventilation. Following the EVH protocol you will then perform pulmonary function tests at 3, 6, and 16 minutes. We then compare your post EVH lung function to your baseline, if there is a drop of more than 10% in your forced expiratory volume in 1 second then this is diagnostic of EIB.

EVH will induce a bronchoconstrictive response in asthmatic and EIB susceptible individuals. Asthmatic individuals may experience symptoms of wheezing, coughing, tightness in the chest, and slight shortness of breath. These symptoms although very unlikely may result in a slight detriment to exercise performance, immediately following the test. If you suffer from asthma or EIB you will not be allowed to complete the testing sessions unless you have your own bronchodilator medication to hand. There will also be a salbutamol reliever and spacer for treating asthma exacerbations. Pulmonary function will be measured pre and post EVH.

If you have further queries then please don't hesitate to get in touch.

Contact

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Appendix 3 - Chapter 4 Informed Consent Form

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

A2a – Subject Statement of Consent to Participate in the Investigation Entitled:

"*Reproducibility of diagnosing exercise induced asthma through eucapnic voluntary hyperventilation (EVH)"*

1) I, _____agree to partake as a subject in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with *Neil Williams* that this will involve me: *Attending the sport Science laboratories at NTU Clifton Campus on four occasions over a 21 day period or 3 occasions over a 70 day period, with each visit lasting up to 1 hour. I will be required to perform pulmonary function tests and undergo eucapnic voluntary hyperventilation (EVH).*

3) It has also been explained to me by *Neil Williams* that the risks and side effects which may result from my participation are as follows: *For asthmatic individuals mild bronchoconstriction (symptoms similar to mild asthma) will occur from completing the EVH protocol. This will be combated by regular measurements of pulmonary function and reversed by taking short acting β2 agonist medication.*

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the study at any time and for any reason, without having to explain my withdrawal.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have read the University's policy relating to the storage and subsequent destruction of sensitive information. I understand that sensitive information I have provided through my participation in this study, in the form of *health and asthma questionnaires, and data relating to the study* will be handled in accordance with this policy.

9) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists.

10) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Subject signature: ……
Date:_____

Independent witness signature: ……
Date:_____

Primary Researcher signature: …….
Date:_____

Appendix 4 - Chapter 5 Participant Information Sheet

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

A1 – Participant Information Sheet – Hand-out for participant recruitment

"A randomised, double blind, placebo controlled, cross-over study to compare the effectiveness of two dosages of omega-3 polyunsaturated fatty acid (ω3-PUFA) for the management of exercise induced bronchoconstriction in physically active healthy individuals."

Introduction

Exercise induced bronchoconstriction (EIB) more commonly known as exercise induced asthma is used to describe a narrowing of the airways during or following exercise. It presents a significant problem for many elite and recreational athletes many of whom have to reduce training loads and have it impact on their performance.

The common treatment for EIB involves the use of pharmacologic interventions. However, some have side effects, and are restricted by the International Olympic Committee. There is now evidence that dietary modification has the potential to be used in the management of EIB. Omega 3 polyunsaturated fatty acid (ω-3 PUFA) supplementation has shown to significantly improve pulmonary function in EIB sufferers, reduce markers of inflammation, and the need for bronchodilator medication. This study aims to assess the effects of 3 week supplementation periods of two different dose levels of ω-3 PUFA on exercise induced bronchoconstriction and markers of inflammation in healthy asthmatic and non-asthmatic individuals.

The protocol and procedures have been reviewed and approved by the Nottingham Trent Human Ethics Committee; the protocol and the safeguards that have been put in place have been done so in consultation with a respiratory consultant at Nottingham City Hospital.

Inclusion Criteria

- Male
- Aged 18-45
- Body mass index 20-25 $kg \cdot m^{-2}$
- Physically active (completing 3 or more exercise sessions a week lasting at least 45 minutes each)
- Non-smoker

- Non-vegan or vegetarian
- Asthmatic and EIB sufferers must have their own clinically prescribed medication (e.g. short acting β2-agonist)

Exclusion Criteria

- Predicted forced expiratory volume over one second (FEV_1) of <65% – this will be measured during visit 1
- Previously admitted to hospital for asthma or other breathing difficulties
- Asthma exacerbation within the last month (Course of steroids, or hospital visit)
- Previously diagnosed with chronic obstructive pulmonary disease (COPD) emphysema, chronic bronchitis or similar respiratory illness
- History of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease
- History of recurrent chest infections
- Smoker
- Acute infection within the last four weeks
- Major operation within the past four months
- Have a history of taking ω-3 PUFA supplements or supplements with antioxidants above recommended intake, or consume more than three fatty fish meals per week
- Take a daily dose of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs)
- Currently taking a daily dose of anti-histamine
- Currently taking long term asthma maintenance medications – corticosteroids, and leukotriene modifiers that you could not refrain from taking for 4 days prior to laboratory session
- Vegetarian or vegan diet

If you meet the inclusion criteria and consent to take part you will be required to attend Nottingham Trent University on 7 occasions You will undergo three different 3-week dietary supplementation conditions with a two week washout period between each (although the wash-out period between supplement conditions can be longer and can be worked around your own convenience). The three dietary supplementation interventions are:

• ω3-6.2 $g \cdot d^{-1}$– 8 ω-3 capsules equalling 6.2 g (3.7 g EPA and 2.5 g DHA) per day

• ω3-3.1 $g \cdot d^{-1}$ – 4 ω-3 capsules equalling 3.1 g (1.8 g EPA and 1.3 g DHA) + 4 placebo capsules per day

• Placebo (PLA) – 8 placebo capsules containing medium chain triglyceride GTCC per day

All three supplements will be identical in size and appearance. Supplements will be of pharmaceutical grade supplied by a reputable manufacturer.

Visit one will involve a briefing about the study and familiarisation of the measurement techniques to be used during the study. These include pulmonary function, fraction of exhaled nitric oxide, and eucapnic voluntary hyperventilation (a technique used to diagnose and assess EIB). – EVH is a 6 minute hyperventilation challenge that has been shown to elicit a mild but highly diagnostic EIB response and is currently the International Olympic Committee-Medical Commissions recommended challenge for diagnosing EIB.

EVH will induce a bronchoconstrictive response in asthmatic and EIB susceptible individuals. Asthmatic individuals may experience symptoms of wheezing, coughing, tightness in the chest, and slight shortness of breath. These symptoms although very unlikely may result in a slight detriment to exercise performance immediately following the challenge. Following the testing protocol, you will be free to use your own prescribed medication which should alleviate any remaining symptoms.

During visit two you will have baseline measurements taken, and will also provide a urine and blood sample. You will then be provided with your first dietary supplementation for three weeks.

Visits 3-7 will be the same measurements as above pre and post each 3-week supplementation period. You will also be required to complete 2 days of food diaries at the beginning of each supplementation period, and weekly asthma symptoms and control questionnaires.

You will be chaperoned at all times during all periods of testing and will be closely monitored following all tests. You will be allowed to leave when your pulmonary function has returned to baseline values.

Each visit will last up to 2 hours. Total blood taken per visit will be 40 mL which amounts to just over one sixth of a normal blood donation.

If you are an asthmatic and are eligible to participate in this study we will contact your GP to inform them of your participation. Your GP will be sent information including a copy of the medical questionnaire to inform them that you are taking part in a trial that is assessing your management of exercise induced bronchoconstriction. Your GP will be asked to contact Mr Neil Williams if they do not deem you fit to participate in the study. The GP will be given 2 weeks to respond.

In addition to this if you report to the study deeming yourself free from asthma and are subsequently diagnosed, you will be presented with your results and referred to your GP. You will be unable to volunteer for the study until you have visited your GP and received any subsequent medication to treat your asthma.

Contact

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Appendix 5 - Chapter 5 Informed Consent Form

Ethical Application

A2a – Participant Statement of Consent to Participate in the Investigation Entitled:

"A randomised, double-blind, placebo-controlled, cross-over study to compare the effectiveness of two dosages of omega-3 polyunsaturated fatty acid (ω3-PUFA) for the management of exercise induced bronchoconstriction (EIB) in physically active healthy individuals."

1) I, _____agree to partake as a participant in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with *Neil Williams* that this will involve me: *Attending the sport Science laboratories at NTU Clifton Campus on seven occasions over a 13 week period, with each visit lasting up to 90 minutes. I will undergo 3 different dietary supplementation conditions, and be required to undergo pre and post supplementation period measurements of – Pulmonary function, eucapnic voluntary hyperventilation, and fraction of expired nitric oxide. I will also be required to provide both urine and blood samples; and complete food diaries and asthma symptom questionnaires.*

3) It has also been explained to me by *Neil Williams* that the risks and side effects which may result from my participation are as follows: *EVH will induce a bronchoconstrictive response in EIB susceptible individuals. You may experience symptoms of wheezing, coughing, tightness in the chest, and slight shortness of breath. This will be combated by regular measurements of pulmonary function and reversed by taking short acting β2 agonist medication. Some discomfort can occur through cannulation although this will be carried out by trained members of staff. Some individuals may experience mild gastrointestinal discomfort when taken the ω-3 supplements.*

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the study at any time and for any reason, without having to explain my withdrawal.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by

individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have read the University's policy relating to the storage and subsequent destruction of sensitive information. I understand that sensitive information I have provided through my participation in this study, in the form of *health and asthma questionnaires, and data relating to the study* will be handled in accordance with this policy.

9) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists.

10) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Participant signature: ……
Date:_____

Independent witness signature: ……
Date:_____

Primary Researcher signature: …….
Date:_____

Appendix 6 - Chapter 6 Participant Information Sheet

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

A1 – Subject Information Sheet – Handout for subject recruitment

"A randomised, double blind, placebo controlled, study to establish the effect of 3 weeks of prebiotic supplementation on the management of exercise induced bronchoconstriction in physically active healthy individuals."

Introduction

Exercise induced bronchoconstriction (EIB) more commonly known as exercise induced asthma is used to describe a narrowing of the airways during or following exercise. It presents a significant problem for many elite and recreational athletes many of whom have to reduce training loads and have it impact on their performance.

The common treatment for EIB involves the use of pharmacologic interventions. However, some have side effects, and are restricted by the International Olympic Committee. There is now evidence that dietary modification has the potential to be used in the management of EIB. Omega 3 polyunsaturated fatty acid (ω-3 PUFA) supplementation has shown to significantly improve pulmonary function in EIB sufferers by reducing markers of inflammation, and the need for bronchodilator medication. Recent nutritional research into prebiotics (typically food derived carbohydrates) has highlighted their potential to improve gastrointestinal health, and reduce incidences of upper respiratory tract infections by increasing the numbers of positive microorganisms inside the gut. Research into prebiotics has highlighted their effects on the immune system, and the ability to fight infection, and inflammatory processes and conditions. In asthmatics and EIB susceptible individuals exercise is a trigger for airway inflammation. It could be hypothesised that with prebiotics having the potential to reduce airway inflammation they may influence the response to exercise in EIB susceptible individuals.

This study aims to assess the effects of a 3 week supplementation period of a prebiotic supplement on exercise induced bronchoconstriction and markers of inflammation in healthy asthmatics.

The protocol and procedures have been reviewed and approved by the Nottingham Trent Human Ethics Committee; the protocol and the safeguards that have been

put in place have been done so in consultation with a respiratory consultant at Nottingham City Hospital.

Inclusion Criteria

Aged 18-45

- Body mass index 20-25 $kg \cdot m^{-2}$
- Physically active (completing 3 or more exercise sessions a week lasting at least 45 minutes each)
- Non-smoker
- Female participants must be taking an oral contraceptive or have contraceptive implant
- Asthmatic and EIB sufferers must have their own clinically prescribed medication (e.g. short acting β2-agonist)

Exclusion Criteria

- Predicted forced expiratory volume over one second (FEV_1) of <65% – this will be measured during visit 1
- Previously admitted to hospital for asthma or other breathing difficulties
- Asthma exacerbation within the last month (Course of steroids, or hospital visit)
- Previously diagnosed with chronic obstructive pulmonary disease (COPD) emphysema, chronic bronchitis or similar respiratory illness
- History of heart failure, pulmonary hypertension, embolism, or other pulmonary heart disease
- Previously diagnosed with a bowel disease – ulcerative colitis, Crohn's disease, infectious colitis or similar illness
- History of irritable bowel syndrome
- History of recurrent chest infections
- Smoker
- Acute infection within the last four weeks
- Major operation within the past four months
- Have a history of taking ω-3 PUFA supplements or supplements with antioxidants above recommended intake, or consume more than three fatty fish meals per week
- History of taking probiotic or prebiotic supplements
- Recent history of taking antibiotics
- Take a daily dose of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs)
- Currently taking a daily dose of anti-histamine
- Currently taking long term asthma maintenance medications – corticosteroids, and leukotriene modifiers that you could not refrain from taking for 4 days prior to laboratory session

If you meet the inclusion criteria and consent to take part you will be required to attend Nottingham Trent University on 5 occasions over an 8 week period. You will undergo two, three week supplementation conditions with a two week washout. The two dietary supplementation interventions are:

The two supplementation conditions will consist of:

• Prebiotic (PRE) – A Daily dose of 5.5g of GOS, and participants will be required to consume this as one dose in the morning. This dose of prebiotic has been shown previously to be well tolerated by healthy individuals (Bouhnik, et al. 1999)

• Placebo (PLA) – The placebo will be identical to the prebiotic but will contain the equivalent dose of maltodextrin powder to be consumed in the morning. Maltodextrin is a food grade carbohydrate but is not selectively fermented by the gut bacteria. It looks and tastes identical to GOS

Both supplements will be identical in appearance and taste; and will be supplied by CLASADO Ltd healthcare. Consumption of other pre- and probiotic supplements or foods containing them will not be allowed during the study

Both supplements will be identical in size and appearance. Supplements will be of pharmaceutical grade supplied by a reputable manufacturer.

Laboratory Visits:

Visit one will involve a briefing about the study and familiarisation of the measurement techniques to be used during the study. These include pulmonary function, fraction of exhaled nitric oxide, and eucapnic voluntary hyperventilation (a technique used to diagnose and assess EIB). – EVH is a 6 minute hyperventilation challenge that has been shown to elicit a mild but highly diagnostic EIB response and is currently the International Olympic Committee-Medical Commissions recommended challenge for diagnosing EIB.

EVH will induce a bronchoconstrictive response in asthmatic and EIB susceptible individuals. Asthmatic individuals may experience symptoms of wheezing, coughing, tightness in the chest, and slight shortness of breath. These symptoms although very unlikely may result in a slight detriment to exercise performance. Following the testing protocol, you will be free to use your own prescribed medication which should alleviate any remaining symptoms.

Visit two will be your first pre supplementation baseline. You will have baseline lung function and responses to the EVH test measurements taken, and blood sample taken, a further blood sample will be taken 24hr after this visit. You be provided with your first dietary supplementation for three weeks.

Visits 3-5 will be the same measurements as above pre and post each 3-week supplementation period. You will also be required to complete 2 days of food diaries at the beginning of each supplementation period, and weekly asthma symptoms and control questionnaires.

You will be chaperoned at all times during all periods of testing and will be closely monitored following all tests. You will be allowed to leave when your pulmonary function has returned to baseline values.

Each visit will last up to 1.5 hours. Total blood taken per visit will be 80 mL which amounts to just over one sixth of a normal blood donation.

If you are an asthmatic and are eligible to participate in this study we will contact your GP to inform them of your participation. Your GP will be sent information including a copy of the medical questionnaire to inform them that you are taking part in a trial that is assessing your management of exercise induced bronchoconstriction Your GP will be asked to contact Mr Neil Williams if they do not deem you fit to participate in the study.

In addition to this, if you report to the study deeming yourself free of asthma and are subsequently diagnosed, you will be presented with your results and are strongly advised to see your GP and discuss with them what has been assessed, but ultimately, it remains a matter for you the patient to correspond and disclose this information with your GP. You would be unable to volunteer for the study until you have visited your GP and received any subsequent medication to treat your asthma.

Contact

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Appendix 7 - Chapter 6 Informed Consent Form

Ethical Application

NOTTINGHAM TRENT UNIVERSITY

A2a – Subject Statement of Consent to Participate in the Investigation Entitled:

"A randomised, double blind, placebo controlled, study to establish the effectiveness of 3 weeks of prebiotic supplementation on the management of exercise induced bronchoconstriction in physically active healthy individuals. ."

1) I, _____agree to partake as a subject in the above study.

2) I understand from the participant information sheet, which I have read in full, and from my discussion(s) with *Neil Williams* that this will involve me: *Attending the sport Science laboratories at NTU Clifton Campus on five occasions over a 8-11 week period, with each visit lasting up to 2 hours. I will undergo 2 different dietary supplementation conditions, and be required to undergo pre and post supplementation period measurements of – Pulmonary function, eucapnic voluntary hyperventilation, and fraction of expired nitric oxide. I will also be required to provide both urine and blood samples; and complete food diaries and asthma symptom questionnaires.*

3) It has also been explained to me by *Neil Williams* that the risks and side effects which may result from my participation are as follows: *For asthmatic individuals mild bronchoconstriction (symptoms similar to mild asthma) will occur from completing the EVH protocol. This will be combated by regular measurements of pulmonary function and reversed by taking short acting β2 agonist medication. Some discomfort can occur through cannulation although this will be carried out by trained members of staff. Some individuals may experience mild gastrointestinal discomfort when taken the prebiotic supplement.*

4) I confirm that I have had the opportunity to ask questions about the study and, where I have asked questions, these have been answered to my satisfaction.

5) I undertake to abide by University regulations and the advice of researchers regarding safety.

6) I am aware that I can withdraw my consent to participate in the study at any time and for any reason, without having to explain my withdrawal.

7) I understand that any personal information regarding me, gained through my participation in this study, will be treated as confidential and only handled by individuals relevant to the performance of the study and the storing of information thereafter. Where information concerning myself appears within published material, my identity will be kept anonymous.

8) I confirm that I have read the University's policy relating to the storage and subsequent destruction of sensitive information. I understand that sensitive information I have provided through my participation in this study, in the form of *health and asthma questionnaires, and data relating to the study* will be handled in accordance with this policy.

9) I understand that as part of this study I will be consuming a supplement. I am aware that elite sports people (i.e. international or national standard) may undergo either out-of or in-competition (or both) doping tests and appreciate that the supplement being studied could be contaminated with a substance that appears on the banned lists.

10) I confirm that I have completed the health questionnaire and know of no reason, medical or otherwise that would prevent me from partaking in this research.

Subject signature: ……
Date:_____

Independent witness signature: ……
Date:_____

Primary Researcher signature: …….
Date:_____