Airway inflammation and omega 3 PUFA in mild to moderate asthma

By

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Submitted in partial fulfilment of the requirements for the reward of

Doctor of Philosophy of Loughborough University

May 2013

Contents

Abstract

Asthma is a chronic inflammatory disease characterised by reversible airflow obstruction. Based on the relationship between a lack of exercise and chronic diseases, the latest guidelines from the Department of Health (DH) recommend physical activity across the whole population (DH, 2011). Exercise Induced Bronchoconstriction (EIB) is a 'sub-type' of asthma which affects approximately 90% of all individuals with asthma and an additional 10% of the healthy normal population (ATS/ACCP, 2003; Anderson & Kippelen, 2012); thus, EIB may be an important limiting factor for physical activity and an important 'barrier to exercise' for a number of individuals.

Asthma is identified primarily by the occurrence of symptoms (wheezing breathlessness and dyspnoea), peak expiratory flow rates (PEF) and spirometry (Pulmonary Function tests – PFT). The current spirometry guidelines for the characterisation of asthma include a fixed criteria for the ratio between forced expiratory volume in one second and forced vital capacity (FEV_1/FVC) (Miller et al., 2005b). This fixed criteria approach lacks specificity and is likely to misdiagnose approximately 20% of patients (Miller et al., 2011). The American Thoracic Society (ATS) and the European Respiratory Society (ERS) guidelines have acknowledged these concerns and have issued position statements for the use of a different approach using a 'lower limit of normality' (LLN) derived from a matched healthy population (Miller et al., 2009). Based on the fixed criteria, it has been shown that there is under diagnosis of participants with mild-moderate symptoms participants in the younger age group (Cerveri et al., 2009; Hansen et al., 2007; Miller et al., 2011; Roberts et al., 2006; Swanney et al., 2008).

The currently available pharmacological therapies for asthma and EIB are effective (corticosteroids and bronchodilators), however long-term usage of these medications is associated with issues of tachyphylaxis and negative side effects (Barnes, 2010; GINA, 2011). There is some evidence from observational and intervention studies to suggest a beneficial effect of fish oil (comprising of omega-3 (n-3) polyunsaturated fatty acids (PUFAs)) in inflammatory diseases, (specifically asthma). Marine based n-3 PUFA have therefore been proposed as a possible complimentary/alternative therapy for asthma. The proposed anti-inflammatory effects of fish oil may be linked to a change in cell membrane composition. This altered membrane composition following fish oil supplementation

(primarily EPA/DHA) can modify lipid mediator generation via the production of eicosanoids with a reduced inflammatory potential/impact (Calder, 2012). A recently identified group of lipid mediators derived from EPA include E series resolvins which are proposed to be important in the resolution of inflammation (Serhan, 2011). Reduced inflammation attenuates the severity of asthma including symptoms (dyspnoea) and exerts a bronchodilatory effect.

There is a lack of published data relating to fish oil supplementation in EIB prone individuals and most studies within the literature have been focussed predominantly on athletes. This thesis addresses issues related to disease severity, classification of EIB, the impact of EPA / DHA supplementation on post-exercise pulmonary function in EIB prone males and finally the impact of resolvins on EIB prone females.

The first aim of this thesis was to compare the approaches to characterisation of asthma in mild-moderate individuals based upon fixed criteria (80% $FEV_{1/}FVC$ ratio) and the LLN, using a control population. Sixteen male asthmatic and twenty one healthy normal controls were recruited for PFT, estimation of dyspnoea and measurement of venous blood based inflammatory mediators. Using the fixed criteria (80% ratio) the 16 asthmatics were demonstrated to have mild to moderate severity with all individuals showing a FEV_1/FVC ratio to be below the 80% ratio. When the control population (FEV_1/FVC ratio = 89%) were used to calculate the LNN then a value of 82% was indicated which meant that all 16 individuals previously characterised as asthmatics were confirmed as mild to moderate asthmatics with the newer approach to asthma characterisation. The asthmatics showed significantly elevated levels of serum IL-6 and TNF-α than controls and also exhibited worse dyspnoea score. The LLN when used alongside 80% ratio provides a comparable method for characterisation of mild-moderate asthma.

To assess the effect of EPA / DHA supplementation on post exercise FEV_1 in EIB prone males, a randomised crossover trial (RCT) with a three week intervention of n-3 supplementation (3.2 grams of EPA and 2.2 grams of DHA/ or placebo per day) was completed by 9 mild-moderate asthmatics with EIB. A significant improvement in post exercise FEV_1 for EIB was observed following supplementation and was mirrored by a significant reduction in bronchodilator usage and a drop in serum IL-6 levels. PBMC lipid composition showed a non-significant trend towards an increase in EPA/DHA content with

a concurrent reduction in AA levels. There was no significant improvement in the Fraction of Exhaled Breath Nitric Oxide (FeNO), or serum levels of TNF α and IL-10. The improvement in pulmonary function demonstrates a benefit to mild-moderate asthmatics of fish oil supplementation however, the lack of consistent inflammatory mediator data means that possible mechanisms are still not elucidated.

Finally, to investigate the effect of EPA-derived bioactive mediators (E series Resolvins) on post exercise FEV_1 in EIB-prone females, a RCT with 3 week supplementation with 100 mg of commercially available Resolvinol (Bionovex)/per day was completed by 8 females with EIB. There was a significant improvement in post exercise FEV_1, however no changes were observed for serum cytokine levels for IL-6 and TNF α and FeNO; while there was a significant reduction in serum IL-10 levels. The beneficial effects of E series resolvins on pulmonary function may suggest a different mechanism via which the n-3 PUFA exhibit anti-inflammatory effects.

In conclusion, both the fixed criteria and LLN are suitable for characterisation of young, mild-moderate asthmatics. There is a beneficial role of EPA/DHA supplementation in EIB prone males while resolvin supplementation appears to have a similar effect on pulmonary function of EIB prone females but an inconsistent impact upon systematic inflammation in EIB females. Further mechanistic approaches with a larger sample size are required to investigate the role of the n-3 and their lipid mediators on lung function at a population level.

Acknowledgements

This journey will not be complete without the mention of people who have walked with me through this long tunnel of knowledge and supported me all the way along. First of all, thank you God and my parents for giving me the strength and perseverance to complete this journey.

I thank Loughborough University for offering me this excellent opportunity to be associated with one of the top ranking Universities in the UK. I have gained invaluable experience that will remain with me always. My Loughborough journey will be incomplete without thanking my supervisor Dr Martin R. Lindley who has remarkably taught me concepts of physiology to help set in this research. Dr Lindley's exceptional scientific approach and rational thinking has always challenged my limits, helping me develop as an independent thinker. I extend my special thanks to my supervisor Dr Sarabjit S. Mastana for his guidance, support and eye for detail during this PhD. Thank you for being an extraordinary friend and an outstanding mentor, always guiding me towards excellence. Thank you once again to both my fascinating supervisors! Thanks to Dr Paula Griffiths, Prof. Barry Bogen, Dr. Katherine Brooke-Wavell, Prof. Noel Cameron and Dr Richard Ferguson for their expert guidance during the study. I also thank my examiners - Prof. P Yaqoob and Dr L Bishop for their guidance and suggestions for the improvement of my work.

I wish to thank all the support staff and chemistry technicians—David, Trevor and John for educating me with their experience. Thanks to Jayshree and Victoria for all the support in the biology labs. I wish to thank all my participants who have made an invaluable contribution to my research; without them this research would not have been successful. I also thank Biocare and Bionovate for providing free supplements to support the intervention studies.

Thanks to my dear friends - Dr Nicola Hawley and Dr Rachel Duckham for their critical evaluation of my work during the difficult phases. Special thanks to my family for constant mental support and patience. Last but not the least thank you my husband Vivek, for motivating me to continue this journey; without you, this was an impossible dream.

Thank you everyone.

Abbreviations

5-LOX - 5-Lipoxygenase
AA - Arachidonic Acid
ACCP - American College of Chest Physicians
AHR - Airway Hyperresponsiveness
ALA - Alpha Linoleic Acid
APCs - Antigen presenting cells
ASMs - Airway smooth muscles
ATLs - Aspirn triggered lipoxins
ATS - American Thoracic Society
BAL - Bronchoalveolar lavage
CAM - complementary and alternative medicine
BTS – British Thoracic Society
CD - Cluster of Differentiation
CKD - Chronic kidney disease
COX - 2 - Cycloxygenase-2
cNOS - Constitute isoform NOS
CR- Category ratio
CVD - Cardiovascular disease
CystLTs - cysteinyl leukotrienes
DHA - Docosahexaenoic acid
EFAs - Essential fatty acids
EIB - Exercise induced Bronchoconstriction
EPA - Eicosapentaenoic acid
eNOS - Endothelial NOS
ERS - European Respiratory Society
EBC - Exhaled breath condensate
FADS1- Fatty acid desaturase 1
FADS1- Fatty acid desaturase 2
FAME - Fatty acid methyl esters
FEV_1 – Forced Expiratory volume in 1 second
FVC - Forced vital capacity
FeNO - Fraction of exhaled breath nitric oxide
GA^2LEN - Global Allergy and Asthma European Network of Excellence
GC-FID - Gas chromatography – Flame ionization detection
GM-CSF - Granulocyte-macrophage colony stimulating factor
GST - Glutathione S-transferase
GWAS – Genome wide association study
ICAM-1 – Intercellular adhesion molecule1
IgE – Immunoglobulin G
IFN - Interferon
IL- Interleukin
iNOS - Inducible isoform of NOS
ISSFAL - International Society for the Study of Fatty Acids and Lipids
K-EDTA - Potassium Ethylene diamine tetraacetic acid
LA - Linoleic acid
LT - Leukotrienes
LX- Lipoxin
MMP - Matrix metalloprotein
mRNA - messenger RNA
n-3 - Omega 3
n-6 - Omega 6
n-9 - Omega 9
NK - natural killer
NFκB - Nuclear Factor Kappa B
NO - Nitric oxide
NOS - Nitric Oxide Synthase
PBMC - Peripheral Blood Mononuclear Cells
PG(s) – Prostaglandin(s)
PMN - Polymorphonuclear leukocytes
PPARs - Peroxisome proliferator activated receptors
PBS – Phosphate buffer saline
PUFAs - Polyunsaturated fatty acids
RNS - Reactive nitrogen species
ROS - Reactive oxygen species
SF - Saturated fat
SNPs - Single nucleotide polymorphism
TEa – Total analytical errot
Th1 - T helper 1 cells
Th2 - T helper 2 cells
TLRs - Toll like receptors
TNF-α - Tumour necrosis factor alpha
TLC – Total lung capacity
TX - Thromboxane
WHO - World Health Organisation
LABA - Long-acting β2-agonist
LTRA - Leukotriene receptor antagonist

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Chapter one

Introduction and literature review

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1.0. Introduction and Literature Review

Asthma is a chronic inflammatory disorder of the airways, which involves both cells and cellular elements. The chronic inflammation of asthma is also associated with airway hyperresponsiveness (AHR) which together lead to episodes of wheezing, breathlessness, chest tightness and coughing. These recurrent episodes are associated with widespread but variable obstruction in the lungs which is often reversible either spontaneously or with treatment (Bloemen et al., 2007; Wouters et al., 2009; GINA, 2011). Towards the end of a full exhalation an abnormally large amount of air may still be present in the lungs (Ryu & Scanlon, 2001). When an asthmatic encounters a trigger, the airway smooth muscles (ASMs) contract and the airways become narrower causing bronchoconstriction. An asthma trigger may be a substance or an event that sets off asthma symptoms; common triggers are cold air, dust, pollen and exercise. Asthma triggers can vary from person to person. Bronchoconstriction is accompanied by the airways becoming inflamed and thus starting to swell. In most cases, sticky mucus or phlegm develops which can further add to the narrowing of the airways. Thus, the three major characteristics associated with asthma are bronchoconstriction, inflammation and mucus secretion. These symptoms cause the airways to become narrower and irritated making it difficult to breathe, subsequently leading to characteristics of asthma (NHLBI, 2007; figure 1.1).

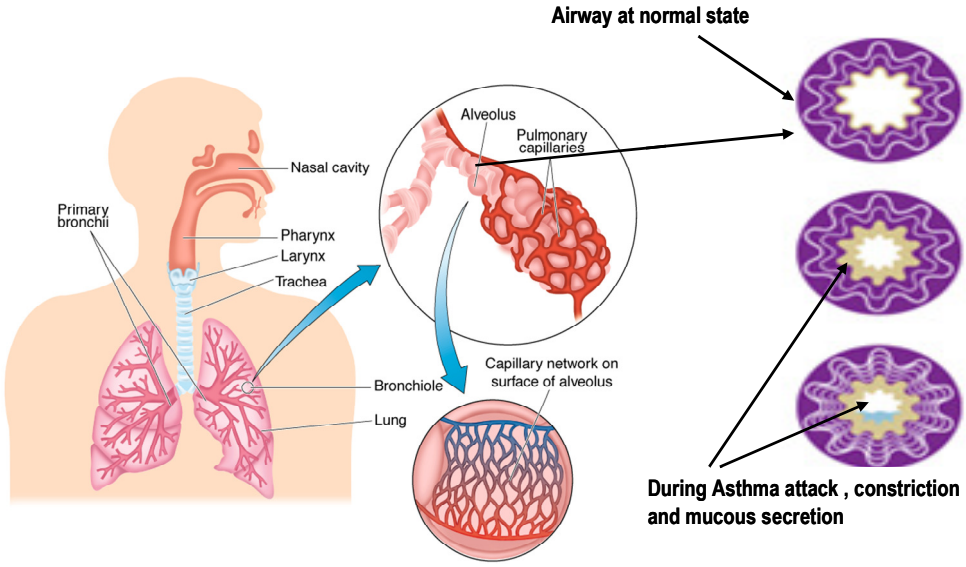


Figure 1.1 The respiratory system and status during asthma attack (adapted from Willmore & Costill, 2004)

1.1. Epidemiology of asthma

Asthma is one of the most common and prevalent problems worldwide affecting over 300 million individuals. In different countries asthma affects from 1% to 18% of the population and it is evident that in both developing and developed countries prevalence of asthma increases as communities adopt modern or 'western lifestyles', becoming urbanised (Bousquet et al., 2005). It has been projected that with urbanisation continuing to increase worldwide, there is likely to be a marked increase in the number of people with asthma living within the next two decades (GINA, 2011). It is estimated that potentially an additional 100 million people worldwide will be affected with asthma by 2025 (Koshy et al, 2010; Ronchetti et al, 2001). There are large differences in asthma prevalence between countries and the rate of asthma mortality does not correlate well with this prevalence, as those countries with the highest prevalence often have the greatest developed infrastructure for asthma management (figure 1.2) (Beasley, 2004; Masoli et al., 2004; Mallola et al., 2012; Pearce et al., 2007). Furthermore, the information on asthma mortality is unreliable in many countries due to lack of resources and measurement error; however, it is estimated that annual worldwide deaths from asthma are approximately 250,000 (Antó, 2012; Masoli et al., 2004)

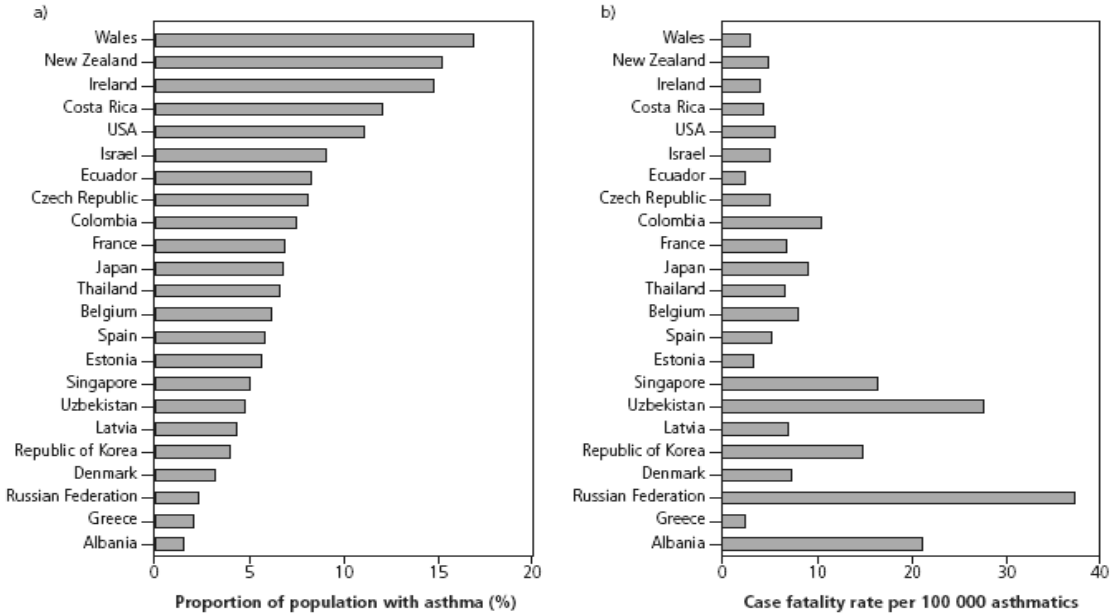


Figure 1.2 Worldwide prevalence and mortality related to asthma (used with permission from Bousquet et al., 2005)

The countries showing highest mortality rates are the ones where controller therapies (e.g. inhaled long-acting β2-agonist (LABA) or leukotriene receptor antagonist (LTRA)) are not yet available. There is a higher mortality rate associated with asthma in developing nations and in poor compared to affluent communities within developed nations, suggesting a role for lifestyle differences such as diet, access to healthcare and exposure to allergens in mortality risk. For example, in the USA mortality rates have increased in those areas where access to healthcare is limited (Al-Hajjaj, 2008; Braman, 2006; Holgate & Douglass, 2010). It is estimated that 15 million disability–adjusted life years (DALYs) are lost annually due to asthma and this represents 1% of all DALYs lost (Bousquet et al., 2005; Murray,1994; Ourania et al.2010). Current estimates suggest that over five million people in the UK suffer from asthma and despite the availability of effective treatments; the condition is still under-diagnosed and under-treated (Asthma UK, 2011). The economic cost of asthma is considerable both in terms of direct medical costs (which include hospital admissions and cost of medication) and indirect costs (e.g. time lost from work, disability–adjusted life years and premature death).

In Europe (excluding UK) the cost of asthma is estimated at 17.7 billion Euros a year in addition to lost productivity estimated at 9.8 billion Euros (Bahadori et al., 2009) while in the USA the economic burden of asthma was estimated to be US$12.7 billion (Campbell et al., 2008). ‘The burden of lung disease report’ by British Thoracic Society (BTS, 2006) indicated that the total cost of all respiratory diseases including asthma for the UK National Health Service was approximately £2.9 billion. Mortality rates from respiratory disease are higher in the UK than the European average. The BTS has identified asthma to be the third major reason for mortality due to respiratory diseases in the UK after tuberculosis and pneumonia (BTS, 2006).The other countries in Europe who have a worse mortality record than the UK are the former Soviet Union countries with relatively under-funded, less sophisticated health services. Poor asthma control is a major challenge of asthma management and can lead to poor economic and clinical outcomes; consequently a number of strategies are being developed by health authorities in different countries to better manage the disease in order to reduce associated costs (Bahadori et al., 2009; Bousquet et al., 2005; BTS, 2012; Weiss & Sullivan, 2001).

1.2. Risk Factors

There are many risk factors for asthma (table 1.1). Factors which influence the risk of asthma are divided into the following categories: (1) those that cause the development of asthma (primarily genetic factors), (2) those which trigger asthma symptoms (mainly environmental factors), and (3) those that do both (Chung & Adcock, 2000). For example, atopy is the genetic predisposition for the development of an immunoglobulin E (IgE)-mediated response to common aeroallergens, and this is a strong identifiable predisposing factor for developing asthma (NHLBI, 2007).

There is an interaction of both environmental and genetic factors that influence susceptibility to asthma and the development of asthma symptoms (Holgate, 1999; Mukherjee & Zhang, 2011). Recent genetic studies have identified a number of genes, which influence the inception, progression and severity of the disease (Beghe et al., 2003; Kormann et al., 2008; Salam et al., 2007; Wan et al., 2011). Genome-wide association studies (GWAS) in mild asthmatic adults, have identified a widely replicated region on chromosome 17q12-21 containing genes ORMDL3, CCL11 and GSDML, that influences asthma susceptibility (Gudbjartsson et al., 2009; Moffatt et al., 2007; Ober et al., 2008). These associated loci have also been replicated in a recent large-scale collaborative study published by the GABRIEL consortium (adults, n= 10,365 cases and n=16,110 controls), investigating mild asthma and single nucleotide polymorphisms (Moffatt et al., 2010). Additionally Wan et al., (2011) completed the largest GWAS of moderate and severe asthma sufferers (adults, n = 1026 individuals of European ancestry) and confirmed the association between the ORMDL3/GSDMB loci on chromosome 17q12-21 and severe asthma (Wan et al., 2011). Given these findings, researchers have established there are genetic differences governing the disease severity. However, more comprehensive studies are required to understand the relationship between gene expression and symptoms of asthma severity for asthma subtypes such as exercise induced asthma and allergic asthma.

Table 1.1 Risk factors for asthma (adapted from NHLBI, 2007)

Risk factor	Examples
Host factors	Family History & Genetics E.g. Genes predisposing to atopy Obesity, sex
Environmental Factors	***Allergens*** Indoor allergens – domestic mites, furred animals Outdoor – Pollens, fungi, moulds ***Viral infections*** ***Tobacco smoke*** Passive smoking Active smoking Smoking during pregnancy ***Indoor/outdoor air pollution*** ***Modern Lifestyle*** Diet Housing environment
Irritants at workplace	Occupational sensitizers

There are a number of environmental factors which also play a significant role in the pathogenesis of asthma which include nutrition, pollution and seasonal changes (GINA Executive and Science Committees., 2007; Holgate et al., 2007). Additionally factors such as the maturation of immune responses and the timing of respiratory infection in the early years of life are emerging as important factors affecting susceptibility to asthma (Wood & Gibson, 2009). A number of large epidemiological studies have suggested that the dietary pattern associated with western cultures (western diet) is negatively associated with respiratory health (Allan & Devereux, 2011; Devereux & Seaton, 2005, Kim et al., 2009; Miyake et al., 2012) . Western diets high in salt, fat, and low in antioxidants, have been shown to increase the risk of developing asthma in children and adults (Hijazi et al., 2000; Mickleborough, 2008; Murphy et al., 2002; Wickens et al., 2005). In the last few decades, there has been significant rise in the therapeutic potential of fish oil for various inflammatory conditions including cardiovascular rheumatoid arthritis, inflammatory bowel disease and asthma. Evidence from observational studies suggests that an increased intake of omega 6 (n-6) polyunsaturated fatty acids (PUFAs) accompanied by a low intake of fish oil [omega 3 (n-3) PUFA] may play a causal role in increased asthma incidence (Black & Sharpe., 1997; Calder., 2006; Hodge et al., 1994; Peroni et al., 2012).

The relationship between dietary PUFAs (n-3 and n-6) and pulmonary function will be the major focus of this thesis and these studies will be reviewed, in detail, in the following sections.

There are racial and ethnic differences in the prevalence of asthma in addition to the socio-economic and environmental factors identified above (Antó, 2012). Researchers have shown that African Americans not only have a higher prevalence of asthma than Caucasians, they also have higher asthma-associated morbidity and mortality. In addition, African Americans living in low-income urban areas have higher prevalence and mortality than African Americans living in high-income urban areas (Silvers & Lang, 2012). Additionally, upper respiratory tract infections and exposure to allergens to which the asthmatic may be sensitive are further risk factors for symptom onset. Some seasonal asthma symptoms can be identified such as pollen induced asthma in summer months. Occupational asthma is triggered by exposure to work-based environmental stimuli that could be a chemical or a protein. There are also some asthmatics that develop exacerbations of their symptoms while taking aspirin or other non-steroidal anti-inflammatory drugs (NHLBI, 2007; Kumari & Rana, 2012).

Sex differences have an influence on the pathophysiology of asthma. It has been shown that the prevalence of asthma is higher in males than in females before adolescence, but that this trend is reversed post-adolescence (Almqvist et al., 2008; Chen et al., 2008; Osman, 2003). The female sex hormones (progesterone and estrogen) can potentially amplify asthma and other allergic diseases (Lim & Kobzik, 2008; Macsali et al., 2009; Vrieze et al., 2003). In general population, early menarche (<12 years) leads to higher risk (2.8 fold) of asthma after puberty compared to late menarche (>14 years) (Salam et al., 2006). Oestradiol (a sub type of oestrogen) and progesterone can inhibit peripheral monocyte production of Interleukin 1(IL-1), which is a strong mediator of inflammation. Additionally studies have shown that oestradiol exhibits anti-inflammatory properties in human leukocytes (Haggerty et al., 2003; Morishita et al., 1999). Consequently, during premenstrual and menstrual phases studies have reported changes in pulmonary function, increases in asthma exacerbations and also hospitalisations (Farha et al., 2009). During the menstrual cycle, there is variation in skin test response to allergens (Kalogeromitros et al., 1995), exhaled breath nitric oxide levels (Mandhane et al., 2009), pulmonary function and adenosine-airway hyperresponsiveness (Choi et al., 2011; Stanford et al., 2006; Tan

et al.,1997) according to the levels of sex hormones. In several studies, the use of oral contraceptives has been found to have a beneficial effect in reducing the variability of pulmonary function over the menstrual cycle (Matsuo et al., 1996; Stanford et al., 2006). Dravata et al., (2010) also recently reported that women had high AHR in the pre-menstrual phase (mid-luteal phase) and cyclical variations in AHR were observed to be smaller among women taking oral contraceptives. The role of sex hormones and use of oral contraceptives (pill) in the severity of asthma symptoms is established. Thus, these factors must be considered in future asthma studies. During this thesis, female participants were studied (chapter 5) and considerations were made in assessing the relationship between oral contraceptive pill usage and lung function.

1.3 Diagnosis and asthma classification

Diagnosis of asthma is predominantly determined by measuring symptoms (episodic breathlessness, wheezing, cough and chest tightness), peak expiratory flow and other parameters of spirometry (Miller et al., 2005a,b). Episodic symptoms after allergen exposures, seasonal variability and a positive family history and atopy are useful diagnostic guidelines (Levy et al., 2006). The presentation of asthma can vary from person to person and asthma may be intermittent with mild to severe episodes requiring treatment (Chung & Adcock, 2000; Reddel et al., 2009). Asthmatics may experience intermittent symptoms for a period of few minutes and in some cases this may be life threatening. Asthma sub groups have been characterised to address the complexities of the disease and for better understanding of the symptoms. A phenotype or subgroup identifies the clinically relevant properties of the disease, but does not show the direct relationship to disease aetiology and pathophysiology (Agache et al., 2012; Wenzel et al., 2012). Table 1.2 shows a classification of asthma into main sub groups including early onset allergic, eosinophilic, aspirin triggered, exercise induced, obesity related and asthma related to airflow obstruction. Exercise induced bronchoconstriction (EIB) is a sub group of asthma, which affects up to 90% of people with asthma and approximately 10% of the healthy population (ATS/ACCP, 2003; Anderson & Kippelen, 2005, 2012; Rundell et al., 2002). This thesis is focussed on investigating the diagnosis/characterisation of mild-moderate asthmatics and once the asthmatics are characterised the effect of omega 3 supplementation in EIB prone individuals will be investigated. This section will review

current diagnostic techniques and the classification of different asthma subtypes with particular focus on EIB.

Table 1.2 Asthma sub groups (adapted from Agache et al., 2012; Wark et al., 2005; Wenzel et al., 2012)

Asthma subgroup	Clinical and physiological features
Early onset allergic	Allergic symptoms and other diseases *Markers- Specific IgE, Th2 cytokines*
Late onset eosinophilic	Sinusitis, less allergic *Markers - IL-5, corticosteroid –refractory eisonophilia*
Aspirin-exacerbated respiratory disease (AERD)	Severe asthma attacks, rhinorrhea and nasal congestion.
Infection induced	Enhanced airway eosinophilia
Asthma associated with apparent irreversible airflow Limitation	FEV_1/FVC ratio below the lower limit of normal for age and FEV_1 < 90% predicted in a patient taking corticosteroids, after acute administration of a rapid onset bronchodilator.
Exercise-induced	**Mild symptoms, intermittent with exercise.** **Reduction in post exercise FEV_1** *Markers: Th2 cytokines, mast cell activation, cysteinyl leukotrienes* Note: 10% of healthy population also suffers from exercise induced symptoms
Obesity related asthma	Mainly Women are affected; very symptomatic, AHR
Eosinophilic asthma	Poor asthma control, increased bronchodilator response, lower lung function and exacerbations. More common in aspirin-sensitive asthma. Some association with expired NO levels and requires sputum analysis for diagnosis.

1.3.1 Diagnostic methods

Pulmonary function test (PFT)

The main diagnostic technique for asthma is an assessment of pulmonary function to identify airflow limitation; this method has been used to demonstrate the reversibility of lung function abnormalities. On its own, the lung function test does not lead the clinicians directly to an aetiological diagnosis and requires other measurements for confidence. The measurement of pulmonary function is combined with an assessment of symptoms such as dyspnoea and wheezing, together they provide reliable information about the about different aspects of asthma control (Kerstjens et al., 1994; Miler et al., 2005a,b). Spirometry is the primary method for pulmonary function test (PFT). The most important aspects of spirometry include forced vital capacity (FVC), which is the maximum volume of

air an individual can expel from the lungs, during expiration made as forcefully and completely starting from full inspiration. The forced expiratory volume (FEV_1) in one second, which is the maximum volume of expired air volume in the first second of a FVC manoeuvre. Peak expiratory flow (PEF) is the maximum expiratory flow achieved from a maximum forced expiration, starting from the point of maximal lung inflation, and is recorded using a PEF meter. Table 1.3 shows major measures and their definitions derived from a FVC manoeuvre.

Table 1.3 Major pulmonary function outcomes, terminology and definitions (adapted from ATS/ERS guidelines (Miller et al., 2005a,b)

Terminology	Definition
FVC (Forced Vital Capacity)	The maximal volume of air exhaled with maximally forced effort from a maximal inspiration, i.e. vital capacity (VC) performed with a maximally forced expiratory effort, expressed in litres at body temperature and ambient pressure.
FEV_1 (forced expiratory volume in one second)	FEV_1 is the maximal volume of air exhaled in the first second of a forced expiration from a position of full inspiration, expressed in litres.
FEV_1/FVC	The ratio of FEV_1 to FVC - it indicates what percentage of the total FVC was expelled from the lungs during the first second of forced exhalation - expressed as FEV_1/FVC ratio.
PEF (Peak Expiratory Flow)	PEF is the maximum expiratory flow achieved from a maximum forced expiration, starting without hesitation from the point of maximal lung inflation, and is recorded using a PEF meter. It is measured in either L/sec or L/min - this is a useful measure to see if the treatment is improving obstructive diseases like asthma.
FEF25%-75% - Forced expiratory flow	This measurement describes the amount of air expelled from the lungs during the middle half of the forced vital capacity test.

Predicted values of FEV_1, FVC and PEF based on age, sex and height have been derived from population studies (Hankinson et al., 1999) and these values are regularly revised by ATS/ERS, BTS and other regulatory authorities. Due to ethnic differences in spirometric values appropriate predictive equations for FEV_1 and FVC have been established for each population. The normal values are higher in young people (age<20 years) and lower in older people (>70 years). Since lung disease could potentially result in a decline in FEV_1, a useful indicator for air flow limitation is the ratio of FEV_1/FVC. The normal FEV_1/FVC ratio is greater than 75-80%, and possibly greater than 90% in children (Miller et al., 2005a; GINA, 2011). Any values less than these values suggest airflow limitation. These predicted values are useful for judging whether a given value is abnormal or not. Recommendations for standardisation have been published and it has been outlined that spirometry is reproducible, but is effort dependent (Miller et al., 2005a,b; Pelligrino et al., 2005).

Consequently, instructions indicating the correct procedure for a normal inhalation and exhalation manoeuvre are provided to all patients prior to the spirometry test . Conventionally, a fixed criteria of ≤80% (for FEV_1/FVC ratio has been used for distinguishing abnormal pulmonary function from normal, however for more precise and accurate estimates, the lower limit of normal (LLN) should be considered as a better definition. The LLN is a limit of normality obtained from a matched control population and allows for a better characterisation of airflow obstruction (Cerveri et al., 2009, Miller et al., 2011; Pellegrino et al., 2005). Using this cut off a given test can be abnormal if the measured value is below the LLN for FEV_1/FVC. The LLN is defined as 1.65 standard deviations below the FEV_1/FVC ratio (Pellegrino et al., 2005). In PFT the normal reference ranges were derived from healthy non-smoking subjects and are dependent on the age, sex, height and ethnicity of the subjects (Hankinson et al., 1999; Miller et al., 2009). However, in a clinical setting, the use of this LLN diagnosis has been found to be difficult. It involves additional statistical calculations that are not yet incorporated into the predicated equations for routine PFTs; prohibiting a quick interpretation of the normality of an individual's results. This issue is currently being considered by the ATS/ERS task forces who are focussing on designing and updating predictive equations that can be used to obtain LLN to ensure accurate diagnosis is made in a clinical environment without any bias (Miller et al., 2009). The limitations of the fixed criteria will be discussed in chapter 3 which was designed to characterise asthmatics based on a fixed criteria (80% ratio) and LLN from a control population. For the assessment and monitoring of asthma it is important to understand the relationship between asthma severity and control. Asthma severity is based upon the criteria described by various regulatory authorities as shown in table 1.4 (ATS/ERS, 2005; NHLBI, 2007). The classification of severity is determined by symptoms, lung function, reliance on inhalers, and interference with normal activity (NHLBI, 2007; GINA, 2011). Asthmatics with mild-moderate asthma have been suggested to have an abnormal pulmonary function with a predicted FEV_1/FVC ratio of (>70% and <100%) (Miller et al.,2005a).

Table 1.4 Severity of asthma characterisation based on symptoms, lung function tests, inhaler usage and interference with normal activity; normal FEV1/FVC: 8–19 years = 85%, 20 –39 years = 80%, 40 –59 years = 75%, 60 –80 years = 70% (ATS/ERS, 2005; Miller et al., 2005a; NHLBI, 2007)

Components of severity	Intermittent	Mild Persistent	Moderate Persistent	Severe Persistent
Symptoms	≤ 2days/week	>2days/week	Daily	Throughout the day
Lung Function	**FEV_1 >80% predicted and normal between exacerbation** **FEV_1/FVC normal**	**FEV_1 ≥70% predicted** **FEV_1/FVC normal**	**FEV_1 60-70% predicted** **FEV_1/FVC reduced by 5% from normal**	**FEV_1 less than 60% predicted** **FEV_1/FVC reduced > 5% from normal**
Rescue Inhaler Use	≤ 2days/week	>2 days per week , but not daily	Daily	Several times/day
Interference With Normal Activity	None	Minor limitation	Some limitation	Extremely limited

During assessment of asthma severity, moderate asthma symptoms may include one or more of the following: deterioration in symptoms, deterioration in lung function and increased rescue bronchodilator use. These symptoms could last for 2 days or more, but not be severe enough to warrant systemic corticosteroid use and/or hospitalisation (Reddel et al., 2009). Furthermore it has been suggested that emergency room visits for asthma (e.g., for routine sick care), not requiring systemic corticosteroids, may be classified as moderate exacerbations (worsening of symptoms) (Oddera et al., 1996; McGrath et al., 2012). Mild asthma symptoms are not justifiable with present methods of analysis; this is because symptoms or changes in flow rates during these episodes lie just outside the normal range of variation for an individual patient. Thus, it is likely that a number of individuals with mild-moderate asthma can be incorrectly classified as normal which could have serious implications on the quality of life of these individuals. Mild-moderate asthmatics may have airway conditions which may result in a temporary loss of asthma control rather than early stages of severe asthma symptoms. The extent of change in these measures is dependent on the age, gender and population studied (Miller et al., 2005b; Reddel et al., 2009). Mild-moderate asthmatics are considered ideal subjects when conducting research in nonclinical settings where there is no access to severe asthma. For this reason, asthmatics with mild-moderate symptoms will make up the study population in this thesis.

1.4 Dyspnoea (symptom of breathlessness)

Dyspnoea scores are collected during asthma assessment in most clinics along with PFTs and is used to estimate the condition experienced by individuals who complain of unpleasant or uncomfortable respiratory sensations (ATS, 1999; Parshall et al., 2012). There is interplay between physiological and behavioural factors in producing respiratory discomfort along with spectrum of phrases used by patients to describe their sensations. Brain imaging studies have demonstrated that there is an association of activated cortico-limbic areas with perception. Additionally, there may be a role of exogenous and endogenous opioids which could possibly modulate perception of dyspnoea (Parshall et al., 2012). Shortness of breath is often triggered by physiological responses such as exercise and sometimes individuals just perceive symptoms of breathlessness. In the normal population, dyspnoea is intensified as oxygen uptake increases with muscular activity and under conditions of heavy exertion (such as exercise) is considered normal. The significance of dyspnoea is inversely related to intensity of exercise provoking the symptoms; consequently, dyspnoea at rest is considered more severe than dyspnoea during intense exercise (ATS, 1999; Scano & Stendardi, 2006; Yorke et al., 2011). A visual analogue scale and the category ratio (CR)-10 scale developed by Borg and colleagues are major instruments used to quantify symptoms during asthma diagnosis and assessment. The Borg scale incorporates non-linear spacing of verbal descriptors of severity corresponding to specific numbers (Borg, 1982). Table 1.5 shows the CR-10 scale.

Table 1.5 Borg scale used for rating perceived breathlessness (Borg, 1982)

Scale	Severity
0	No Breathlessness(At All)
0.5	Very Very Slight (Just Noticeable)
1	Very Slight
2	Slight Breathlessness
3	Moderate
4	Some What Severe
5	Severe Breathlessness
6	
7	Very Severe Breathlessness
8	
9	Very Very Severe (Almost Maximum)
10	Maximum

1.5 Asthma pathophysiology

Asthma is a heterogeneous disease with respect to immunopathology, clinical phenotype, response to therapy and natural history. The severity of asthma symptoms are governed by environmental factors or are caused by insufficient anti-inflammatory treatment (Barnes, 2001; Brightling et al., 2011; Grainge et al., 2011; Holgate & Douglass., 2008; Tattersfield et al., 2002). Thus, the clinical spectrum of asthma is highly variable with airway inflammation being a consistent feature. The pattern of inflammation in asthma is associated with AHR (clinically measured by histamine or methacholine challenge) which leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or early morning. These episodes are generally associated with airflow obstruction within the lungs that is often reversible either spontaneously or with treatment. In most asthmatics, inflammation is largely restricted to the conducting airways but with an increase in disease severity, the inflammatory infiltrate spreads to the small airways and in some cases adjacent alveoli (Kraft et al., 1998). T-lymphocytes play a significant role in organising the inflammatory response in asthma. The release of specific patterns of inflammatory mediators (prostaglandins, cytokines), results in the recruitment and survival of eosinophils and in the maintenance of mast cells in the airways (Barnes., 2006).

Lymphocytes can differentiate into two major, phenotypically distinct, memory Th cell populations, namely T helper 1 cells and T helper 2 cells (Th1 and Th2, respectively) which have distinct inflammatory mediator profiles. Th1 cells characteristically produce cytokines including interleukin-2 (IL-2) and interferon-γ (IFN-γ), induce macrophage activation and are very effective in controlling infection caused by intracellular pathogens (Holgate & Douglass, 2010; Bloemen et al., 2007). Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13 as primary cytokines and the Th2 cells also help B lymphocytes in producing antibodies (figure 1.3).

Cytokines are proteins which act as chemical messengers and have been recognised to regulate the development, growth or activity of target cells by binding to specific receptors on their surface (Calder, 2001). Chemokines are a sub-group of cytokines involved in chemotaxis, they are critical for cell migration during routine immune surveillance, inflammation, and development activities (Allen et al., 2007). Cytokines are produced as response to a trigger and various inflammatory cytokines mediate the systemic effects.

However, the overproduction of inflammatory cytokines has been found to play an important role in some of the symptoms of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases and asthma (Rottem et al., 2002). Many inflammatory cells (macrophages, mast cells, eosinophils and peripheral blood mononuclear cells (PBMC)) have the ability to synthesise and release these proteins. Furthermore, structural cells such as epithelial cells and ASM cells can also produce a range of cytokines, subsequently playing a role in the inflammatory response (Barnes, 2002; Holgate & Douglass, 2010; Szefler et al., 2012).

Another feature of asthma is the response to triggers such as exercise and allergic sensitisation; the airways recognise common triggers and in turn generate a Th2 type cytokine response to them. Asthma is also found to involve local epithelial, mesenchymal, vascular and neurologic events, which direct the Th2 lymphocytes to the lung. Repeated bouts of increased inflammation in asthma may lead to damage to the airway epithelium and subsequent abnormal repair leads to structural changes in the airway walls of asthmatic subjects (collectively referred to as airway remodelling) (Hallstrand et al., 2005; Shifren et al., 2012). Figure 1.3 illustrates the role of different cells and mediators involved in the asthmatic inflammatory response to a trigger. There is recruitment and activation of leukocytes in response to the trigger. Following activation, the cells work actively to neutralise the antigens, subsequently they repair any damage; finally, the cells are removed with resolution of the inflammatory process (Rasmussen et al., 2009; Widgerow, 2012).

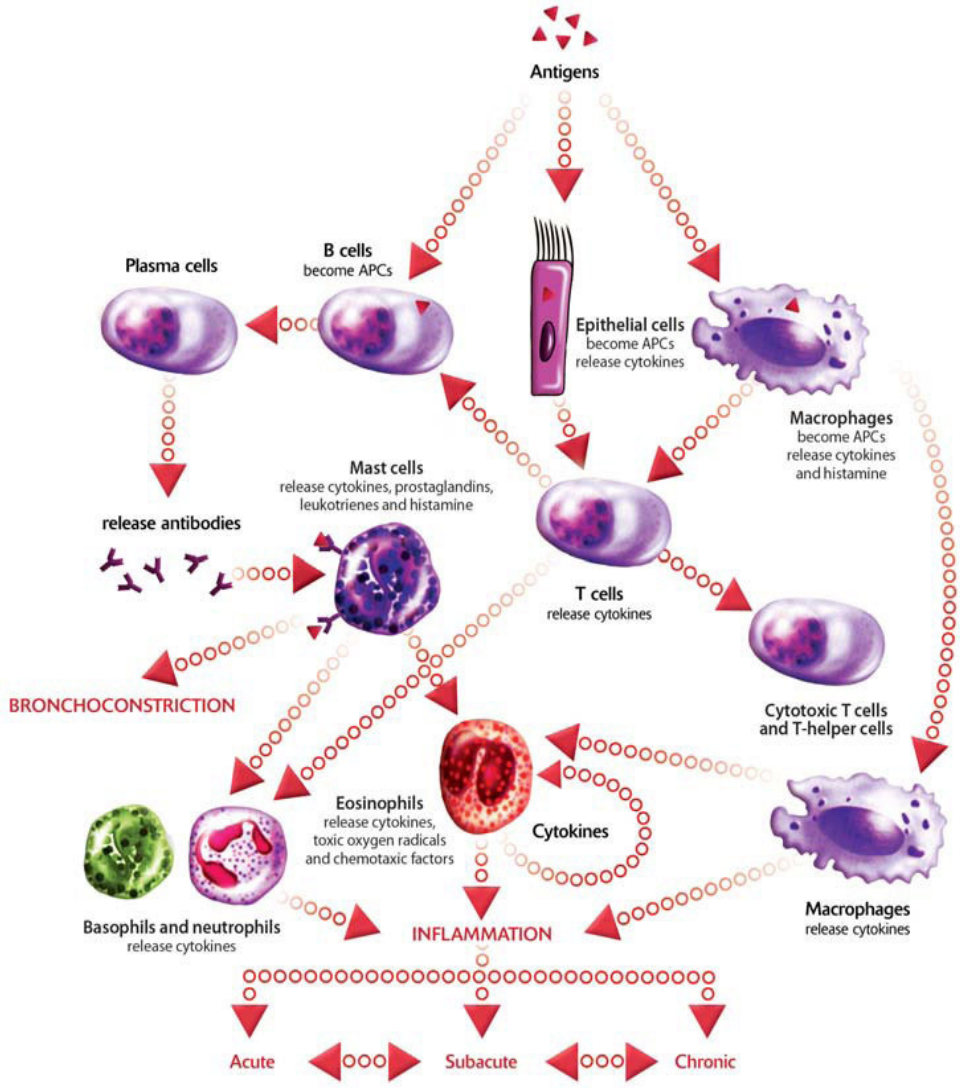


Figure 1.3 Cells and inflammatory mediators involved in inflammation (Asthma pathophysiology, Asthma knowledge centre, 2011 with permission)

Th2 derived cytokines such as IL-5 and IL-4 are responsible for eosinophil differentiation and survival. Asthmatics will often present with have elevated levels of these cytokines in sputum and blood samples (Robinson, 2010). In addition, Asthmatics will have higher levels of IL-13 in airway eosinophils and systemic T cells, furthermore IL-13 shares its activity with IL-4 in switching B cells to IgE production in asthmatics (Humbert et al., 1997; Wills-Karp, 2004). Following therapeutic approaches, blocking the IL-13 receptor a chain (with a fusion protein that can also block IL-4 receptor) has been shown to reduce AHR in animal models of allergic asthma (Kraft, 2011; Wills-Karp, 1998). IL-6 is produced by Th2

cells and other cell types such as epithelial cells and is a marker of airway (local) as well as systemic inflammation and elevated levels are found in bronchoalveolar lavage (BAL) and blood of asthmatics (Canöz et al., 2008; Tanni et al., 2010).

Another Th2 cytokine, IL-9, may play a vital role in sensitizing responses to the cytokines IL-4 and IL-5 and is currently a drug target for asthma (Robinson, 2010). Other cytokines such as IL-1ß, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) are released from different types of cells (e.g. macrophages and epithelial cells), are considered important in increasing the inflammatory response, and prolonging eosinophil survival in airways. TNF-α can cause an increase in histamine in allergic asthma, causing vasoconstriction by secondary production of endothelin-1 (a peptide mediator) (Barnes, 2011; Chalmers et al., 1997, Goldie & Henry, 1999). Both IL-1ß and TNF-α have the ability to activate pro-inflammatory transcription factors including nuclear factor-kB (NF-kB) and activator protein-1 which in turn activate a number of inflammatory genes in the asthmatic airway (Janssen-Heininger et al., 2009; Liu et al., 2010). While cytokines coordinate the chronic inflammation, chemokines play an important role in the selective recruitment of inflammatory cells from the circulation. For example, eotaxin is relatively selective for eosinophils, while thymus- and activation-regulated chemokine and macrophage derived chemokines recruit Th2 cells (Barnes, 2008; Lloyd & Hessel, 2010).

Other cytokines including interferon (IFN)-γ, IL-10, IL-12 and IL-18 have a regulatory role and can attenuate the inflammatory process. IL-10 is described as a cytokine synthesis inhibitory factor which regulates the expression of a number of inflammatory cytokines (IL-1ß, TNF-α and GM-CSF), and inflammatory enzymes (prostaglandin desaturase and COX) (Baine et al., Barnes, 2001; 2011; Biedermann et al., 2004; Holgate et al, 2010; Larché, 2007; Ngoc et al., 2005). IL-10 secretion and gene transcription are defective in macrophages and monocytes isolated from asthmatics and this could potentially facilitate the inflammatory process in asthma (Barnes, 2001; Borish et al., 1996). IL-10 can inhibit eosinophilic inflammation by the suppression of IL-5 and GM-CSF and can influence eosinophil apoptosis and reduce endogenous nitric oxide (NO) production by down-regulation of IL-1 (Bloemen et al., 2007; Holgate et al., 2010; John et al., 1998; Oswald et al., 1992). Since asthmatics have a distinct inflammatory profile, these cytokines have been used to distinguish asthmatics from normal healthy individuals. In this thesis, the

levels of IL-10, IL-6 and of TNF- α were measured in asthmatics to evaluate the relationship of the cytokines with airway inflammation.

An increase in the activity of Th2 cells in asthma may be due to the reduction in regulatory T cells which normally inhibit Th2 cells and an increase in the natural killer (NK) T cells (Akbari et al., 2006; Larche et al., 2003). Peripheral blood mononuclear cells (PBMC) consist of mononuclear cells, which include monocytes, lymphocytes (T-cells, B-cells) and NK cells. Due to their extensive role in inflammatory response, PBMC have emerged in recent years as an attractive cell type for investigating several inflammatory disease states (Baine et al., 2011; Bluth et al., 2008; Manfred et al., 2009). Macrophages and polymorphonuclear neutrophils phagocytose invading pathogens and cellular debris. Neutrophils are increased in the airways and sputum of persons with severe asthma, during acute exacerbations, and in the presence of smoking (NHLBI, 2007). Inside the neutrophils, there is a fusion of the newly formed phagosomes with lysosomes to form phagolysosomes, these structures contain degrading enzymes and produce reactive oxygen species (ROS) to kill trapped organisms and cellular debris (Pettersen & Kenneth*.,* 2002; Serhan et al., 2008; Widgerow, 2012).

Generation of inflammatory mediators, recruitment and activation of inflammatory cells and infection can cause epithelial cells to produce more inflammatory mediators or injure the epithelium itself (Polito & Proud, 1998). A range of mediators have been implicated in asthma, each having different effects on the airways. The epithelial cells, B cells and macrophages serve as antigen presenting cells (APCs) to activate T cells at different stages during the immune response (figure 1.3). Monocytes are able to differentiate into macrophages and dendritic cells in the presence of Granulocyte macrophage-colony stimulating factor (GM-CSF), the latter requiring IL-4 (Holgate & Douglass, 2010; Robinson, 2004; Webb et al., 2007). Dendritic cells have been found to sample allergens from the airway surface and migrate to regional lymph nodes where they interact with regulatory T cells and facilitate the production of Th2 cells (van Rijt et al., 2011). The precise role of macrophages in mediating tissue damage and airway pathology is largely unknown. There is some evidence from animal and human studies suggesting that these cells are a good source of inflammatory mediators such as leukotrienes (LTs), ROS and a variety of lysosomal enzymes (Bang et al., 2011, Peters-Golden, 2004, Holgate, 2008). LTs are a family of inflammatory lipid mediators synthesized from arachidonic acid by a

variety of cells, including mast cells, eosinophils, basophils, and macrophages (Peters-Golden, 2004).

The LTs, LTC_4, LTD_4 and LTE_4 are potent vasoconstrictors of human airways; these mediators affect microvascular and bronchial dilation, increase AHR, and have been implicated in the pathogenesis of asthma (Broughton et al., 1997; De Caterina & Basta, 2001; Wong, 2005). Following the development of potent specific leukotriene antagonists, it is now possible to evaluate the role of these mediators in asthma. LTD_4 agonists have been shown to protect (by up to 50%) against exercise- and allergen-induced bronchoconstriction and this confirms that LTs play a role in bronchoconstrictor responses (Hansbro et al., 2011; Walsh, 2011). Prostaglandins (PGs) are another group of lipid mediators that have been shown to regulate muscle contraction, immune response and inflammation (Broughton et al., 1997; De Caterina & Zampolli., 2004; Lunn &Theobald, 2006). PGs exert strong effects on airway function and there is increased expression of the inducible form of cyclooxygenase (COX-2) in asthmatic airways; however, the inhibition of their synthesis with COX inhibitors, such as aspirin or ibuprofen, may demonstrate some effect in reducing symptoms in some but not all asthmatics (Barnes, 2011). PGD_2 is a bronchoconstrictor produced predominantly by mast cells. Deletion of the PGD_2 receptors in mice significantly inhibits inflammatory responses to allergen and inhibits AHR, suggesting that this mediator may be important in asthma (Bloemen et al., 2007; Swedin et al., 2009). Furthermore, PGD_2 may provide a link between mast cell activation and allergic inflammation via activation of chemoattractant receptor of Th2 cells (Matsuoka et al., 2000; Ricciotti & FitzGerald, 2011). Overall, while inflammatory mediators such as LTs and histamine are suggested to be involved in acute and sub-acute inflammatory responses and in exacerbations it is suggested that cytokines are important in maintaining chronic inflammation in allergic diseases.

Nitric Oxide (NO) is an endogenous regulatory molecule involved in the pathogenesis of asthma. The synthesis of NO in the airways is mediated by a family of enzymes that are collectively called nitric oxide synthases (NOS) (Deykin & Kharitonov, 2002; Palmer et al., 1987). The NOS can exist as constitute isoforms (cNOSs) including endothelial NOS (eNOS) and neural NOS or as an inducible isoform (iNOS) (Puckett & George, 2008; Ricciardolo, 2003). The inducible isoform (iNOS) is found in the epithelium of the bronchial wall, which is the key source for elevated levels of Fraction of exhaled nitric oxide FeNO

seen in asthmatics. Alveolar concentration of FeNO is usually low except in diseases such as alveolititis (Lehtimaki et al., 2001). During an exhalation process, the air from alveolar compartments move to the bronchial compartment; thus, the NO from the bronchial wall diffuses inside the airway lumen leading to an increase in NO levels in the expired air. Increased concentration of NO is observed when exhalation is slow, as this allows a longer time for the NO to diffuse in the airways (Deykin et al., 2002; Kharitonov et al., 1996; Zitt, 2005). Patients with asthma usually exhibit 2-3 fold higher levels of NO in expired air compared to healthy adults (Saleh et al., 1998). Healthy adults can exhibit values between 5-35 ppb (parts per billion) at the standard flow rate of 50 ml/s (Saleh et al., 1998; Zitt, 2005). There are a number of factors such as age, gender, lifestyle (including cigarette smoking), environment and diet, which affect FeNO levels. For example, there is an increase in FeNO levels with age; in addition males appear to have higher levels of FeNO levels than women; in addition during exercise there is exhalation of high volumes of NO (Franklin et al.,1999; Hoffmeyer et al., 2009). The elevated levels of NO in expired air in asthmatics are indicative of eisonophilic inflammation; however, a direct pathogenic role of this gas in asthma has not yet been established (Jatakanon et al., 1998; Lim et al., 2000; Sandrini et al., 2010). Monitoring of NO levels in expired air is an attractive marker for assessing the efficacy of anti-inflammatory treatments (Dweik et al., 2011). In this thesis the effect of dietary supplementation with n-3 PUFA on FeNO levels were assessed to evaluate the anti-inflammatory effects of n-3 PUFA.

1.6 Exercise induced bronchoconstriction (EIB)

Exercise-induced bronchoconstriction (EIB) is a sub group of asthma in which there is transient narrowing of airways after vigorous exercise (McFadden & Gilbert. 1994, Anderson & Holzer, 2000). Occasionally this transient narrowing of airways can occur during exercise (Beck et al., 1994; Rundell et al., 2003; Suman et al., 1999). For patients with EIB a brief period of exercise or increase in ventilation triggers airflow obstruction which lasts for 30-90 minutes in the absence of treatment (Anderson & Kippelen, 2010; Hallstrand, 2012). Clinical focus in asthma therapy has been on the severe disease state, however a large number of asthmatics have mild to moderate symptoms. Recent public health guidelines from the Department of Health (UK) acknowledge the relationship between a lack of physical activity and chronic disease including asthma and consequently, physical activity is encouraged across the population (Department of Health, DH, 2011). EIB is most common in those with clinically diagnosed asthma, and up to 90%

of people with asthma experience EIB at some point during their lives. The majority of those affected by EIB consider exercising to be a major precipitant of their symptoms, and subsequently their involvement in physical activity is affected. EIB has also been reported in non-asthmatics including school children, armed force recruits, and athletes and approximately 10% of the healthy population show symptoms of EIB at some point during their lives (ATS/ACCP, 2003; Anderson & Kippelen, 2005, 2012). Based on the wide prevalence of EIB in asthmatic and healthy population, EIB is considered a limiting factor for physical activity for a large number of individuals. This thesis will focus on classification of mild-moderate asthmatics and EIB using different PFT classification methods and will further evaluate the relationship of n-3 PUFA with EIB to elicit the possibility of using n-3 as a therapy for EIB.

Diagnosis of EIB – the exercise challenge test

The observed symptoms of EIB include coughing, wheezing, chest tightness, shortness of breath or excess mucus production following exercise. However, self-reported symptoms are neither reliable nor specific for EIB. Approximately 50% of elite athletes who report symptoms related to EIB with exercise do not have EIB. While 50% of those who report no symptoms for EIB will test positive on the exercise challenge test for EIB (Parsons et al., 2007; Rundell et al., 2001). Thus, it is essential to support the diagnosis of EIB by performing a relevant exercise challenge test (Rundell & Sue-Chu, 2010).

An exercise challenge test involves exercising at increasing intensities until heart rate response of 85-90% of estimated maximal heart rate is achieved. This will usually occur in the last 4-6 minutes of the test. Following an exercise challenge test, spirometry is performed at 0, 5, 10, 15, 30 and 45 minutes post exercise or until complete recovery; whichever is earlier (Rundell & Sue-Chu, 2010). Following an exercise challenge in susceptible individuals, there is a period of bronchodilation with improved air-flow as shown in figure 1.4. In addition, there is a decline in airflow after exercise, which may be measured by FEV_1 or peak flow. The exercise challenge test primarily involves recording a post exercise reduction in forced expiratory volume in 1 second (FEV_1) of 10% to 15% of the pre-exercise value. The value for FEV_1 may start falling during exercise however; the lowest value will usually be measured 5-12 minutes after the end of the exercise challenge test. The reduction in FEV_1, if severe, is linked to a decrease in oxygen saturation with

hyperinflation of the lungs (Anderson & Kippelen, 2010). In adults, a ≥10% decline in FEV_1 at any time-point within 30 minutes of ceasing exercise is considered a diagnostic of EIB. The decline in FEV_1 is usually maintained over two time points and any un-sustained decline may be due to respiratory muscle fatigue and does not indicate EIB (Crapo et al., 2000; Sterk et al., 1993; ATS/ACCP., 2003).

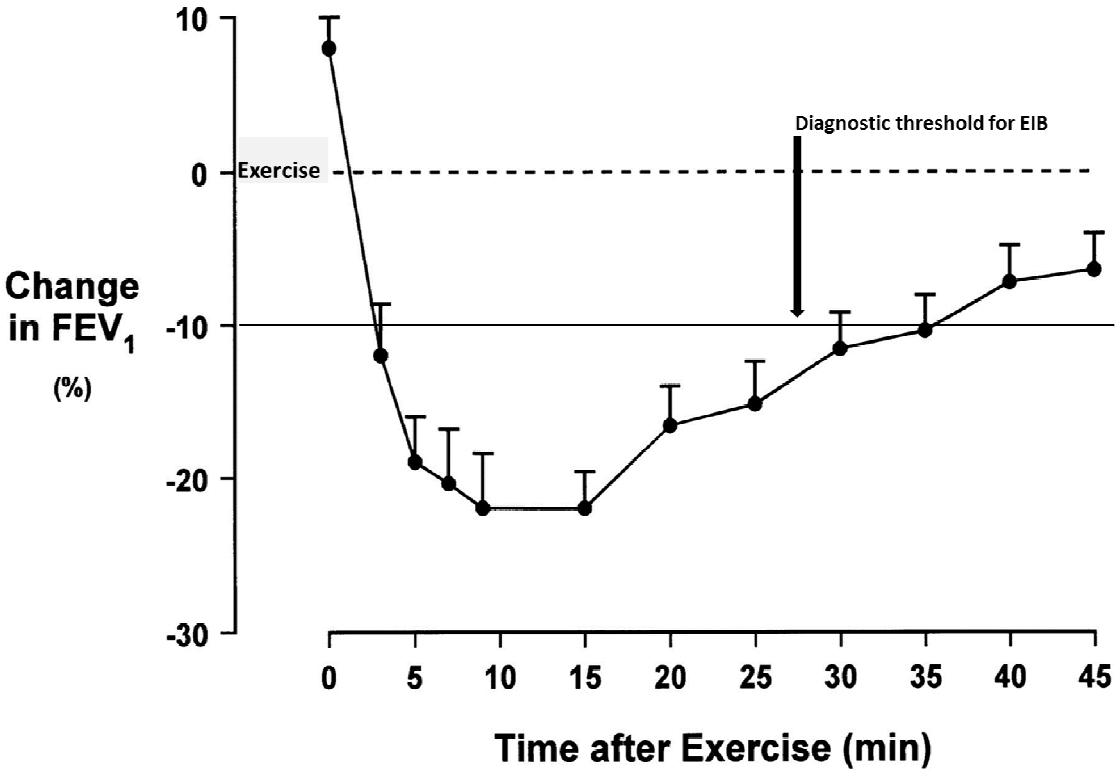


Figure 1.4 Changes in FEV_1 following an exercise challenge test (adapted from O'Byrne, 2000)

Different exercise modes have been investigated to evaluate the most appropriate model for assessment of EIB. It has been suggested that when performed at the same metabolic rate, running is the most effective exercise model for testing EIB, followed in decreasing order by cycling, swimming and walking. With regards to duration and intensity, the maximum effect of a treadmill challenge on lung function often occurs within 6-8 minutes of running at an incline of approximately 10-15% at a constant speed but this is dependent on the level of fitness of the participant (Anderson et al., 1975; Silverman & Anderson, 1972). In this thesis an exercise challenge test (using a motorised treadmill) was conducted for an assessment of EIB in EIB prone participants, further information will be

provided in the chapter 2 (general methods). An exercise challenge test is used mainly for the diagnosis of EIB, assessment of EIB severity and efficacy of preventative drug therapy (Crapo et al., 2000). This test is considered an indirect challenge because it is associated with the release of mediators, which act on the airway smooth muscles to cause contraction. Thus, the exercise test is unlike the direct challenges of histamine and methacholine which act directly on the airway smooth muscle (receptors) to cause bronchoconstriction independent of the presence of inflammatory cells (Rundell & Sue-Chu, 2010).

Proposed EIB mechanisms

There are two possible mechanisms through which EIB occurs: an osmotic path and a thermal path (figure 1.5). Water content and quality of inspired air are additional factors affecting the exercise challenge tests. The water content of inspired air (humidity) affects the respiratory loss of water during exercise. In susceptible individuals, air with low water content is more likely than air with high water content to provoke bronchoconstriction (Rundell & Sue-Chu, 2010; Strauss et al., 1978).

Two hypotheses have been suggested as to how exercise results in the narrowing of the airways i) the osmotic hypothesis and ii) the thermal hypothesis. In the osmotic pathway, EIB is caused due to loss of water by evaporation from the airway surface while inhaling large volumes of air in a short period of time. Following water loss there is airway cooling and dehydration of the airway surface. On the contrary, when exercise is performed with inhalation of hot and humid air, there is a significant reduction or complete inhibition of EIB (Anderson & Kippelen, 2010; Carlsen et al., 2008). Overall, the increase in the osmolarity of the airway surface results in release of pro-inflammatory mediators from mast cells and possibly sensory nerves (Anderson,1984; Anderson & Daviskas, 1992). Subsequently, these mediators can act on bronchial smooth muscle to cause contraction and narrowing of airways. In the airway epithelium of asthmatics, there are large numbers of mast cells, furthermore there are other cells such as epithelial cells, glandular cells and sensory nerves which are affected by airway dehydration and osmotic changes (Anderson & Kippelen, 2012; Anderson & Daviskas, 2000; Chen &Horton, 1977; Strauss et al.,1978).

The alternative thermal hypothesis suggests that EIB is a vascular event resulting from airway cooling during exercise followed by a reactive hyperaemia that happens after the airways re-warm upon competition of exercise (Deal et al., 1979).

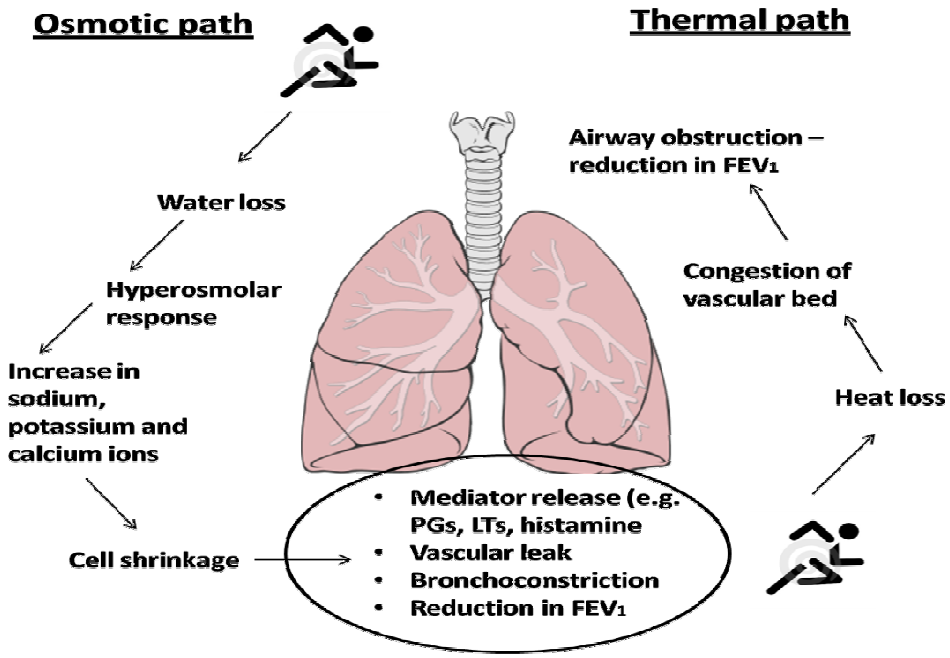


Figure 1.5 Schematic diagram displaying the two different pathways describing the pathophysiologic events leading to EIB in a classic case of a patient with asthma (modified from Castro & Kraft, 2008)

During exercise, heat and water move out of the airways to equilibrate the inspired air to the temperature and humidity of the lower airways. Exercise serves as a trigger to the airway epithelial cells; at the epithelial cell surface there is a transfer of water from the osmotically sensitive epithelial cells via the tight junctions, as well as a thermal gradient. In the presence of osmotic stimuli, inflammatory cells including mast cells and leukocytes are activated leading to sustained inflammatory mediator release in association with smooth muscle contraction (Gulliksson et al., 2006; Hallstrand, 2012). Thus, these stimuli arising from exercise or hyperpnoea are sensed by the airway epithelium, which leads to secretion of inflammatory mediators from leukocytes residing adjacent to the epithelium (Bolger et al., 2011; Romberg et al., 2011).

Cysteinyl leukotrienes (CysLTs) are a family of inflammatory lipid mediators synthesised from arachidonic acid by a variety of cells, including mast cells, eosinophils, basophils, and macrophages (Peters-Golden et al., 2006). Based on non-invasive methods such as exhaled breath condensate (EBC) and urinary markers it has been shown that cellular airway inflammation and generation of CystLTs are associated with severity of EIB (Gulliksson et al., 2006). The levels of CysLTs in EBC and urine have been found to be elevated in asthmatics with EIB and significantly increase after an exercise challenge test. Subsequently, a change in CystLTs in EBC following an exercise challenge test was associated with the severity of EIB (Bikov et al., 2010; Mickleborough et al., 2006). Induced sputum studies show an increase in CystLTs and other bronchoconstrictive eicosanoids such as PGD_2 in the airways following the exercise challenge test thus supporting the non-invasive findings (Barreto et al., 2009; Hallstrand, 2012).

Mast cells are regarded as effector cells of the immune system, but lately they have been shown to have a role in initiating and sustaining complex pathophysiological processes underlying asthma, EIB and other allergic diseases (Bradding et al., 2006; Carter and Bradding, 2011). Mast cells are activated by allergens through high affinity IgE receptors and osmotic stimuli following exercise and increased numbers of mast cells in airway smooth muscles may lead to AHR (Anderson & Kippelen, 2010; Hallstrand, 2012). Mast cells along with eosinophils are the predominant cells in the inflammation of allergic asthma and EIB. When present in increased numbers, these cells release basic proteins such as eosinophil peroxidase and also have the ability to synthesisee eicosanoids such as prostacyclins (PGI_2), CystLTs and PGD_2 and thromboxane A_2 (TXA_2). As described in the asthma pathophysiology section of this chapter, there is a release of a range of tissue damaging superoxide and a range of cytokines and chemokines which increase inflammation and cause bronchoconstriction (Holgate et al., 2009; Kay et al., 2004; Kariyawasam & Robinson., 2006). Furthermore, activated mast cells irrespective of their phenotype, release preformed granule-associated mediators such as histamine, tryptase and other proteases, heparins causing bronchoconstriction (Bradding et al., 2006; Galli et al., 2005). The degranulation of these cells has been suggested to occur during the exercise challenge and is accompanied by a release of histamine and tryptase into the airways after exercise challenge (Hallstrand et al., 2005).

Recent studies have improved our understanding about the pathophysiology of asthma and EIB and suggest that exercise is a stimulus to the airway epithelium, adjacent leukocytes and systemic leukocytes; resulting in sustained production of CystLTs and PGD_2 release in association with smooth muscle contraction leading to EIB symptoms. It has been suggested that mast cells, eisonophils and leukocytes are the principal sources of inflammatory cytokines. Over 100 different mediators derived from inflammatory cells have been found to play a role in the pathogenesis of asthma, and EIB which have been discussed in detail in the asthma pathophysiology section (pages 15-21)..

Implications of airway inflammation in asthma therapy

Resolution of inflammation

Asthma is a condition of the airways in which persistent chronic inflammation leads to changes in airway structure and function (Bloemen et al., 2007; Wouters et al., 2009). As previously discussed, in most individuals, this chronic inflammation is expressed as symptoms such as coughing, mucous production, chest tightness, wheezing and shortness of breath (GINA, 2011). Natural mechanisms and mediators of resolution have been studied in recent years highlighting the role of resolution of inflammation in the pathophysiology of chronic inflammatory diseases including asthma. Resolution is now suggested to be an active process that terminates acute inflammation (Serhan, 2000, 2011b). Towards the end of an inflammatory process, there is neutralisation and elimination of pathogens, followed by removal of cellular components to prevent excessive tissue damage. Neutrophils don't discriminate between microbes and host targets while hyper-expression of endothelial and leukocyte adhesion molecules, along with circulation of soluble adhesion molecules and production of inflammatory mediators, cause host tissue damage (Calder, 2006; Nathan & Ding, 2010). The immune system of the body is required to have a strict control of the inflammatory process and an inadequate inflammatory response could render the body more susceptible to infection. Insufficient resolution is caused by persistent inflammation and there is some evidence to suggest that this could be due to problems with suppressor genes signalling mechanisms. (Levy et al., 2012; Wells et al., 2005). Complete resolution is an ideal outcome of acute inflammation, subsequently the resolution of inflammation is very important and the effectiveness of the immune system is key to this process.

The immune system needs to rapidly 'switch off' the inflammatory response to prevent a chronic situation from occurring. Macrophages and their activated products such as TNF-α, IL-6 and IL-1 are important components of the inflammatory process. These products are responsible for neutralisation of pathogens and related agents and for limiting tissue damage (Anders & Scherer, 2005; Holgate, 2008; Martin & Stockley, 2006; Widgerow, 2012). Depending on their circumstances, cells and compounds may be pro-inflammatory or anti-inflammatory in nature. The mechanism of resolution is continuous with a decrease in the cell number there is a reduction in the levels of pro-inflammatory cytokines and eicosanoids 'switch' from being inflammatory in nature (LTs, PGs etc) to anti-inflammatory or specialized pro-resolving mediators such as lipoxins (LXs), resolvins (RVs) , protectins (PDs) and maresins (Serhan, 2011a, figure 1.6). These mediators have the potential to control the duration and magnitude of inflammation (Serhan, 2008; Serhan & Petasis, 2011a). As a result of inflammatory processes alveolar oedema (abnormal build-up of fluid in the air sacs of the lungs) is possible, leading to hypoxaemia (deficiency of oxygen). Thus, lung-specific resolution mechanisms include clearance of oedema, repair of the airway epithelia, and restoration of pulmonary surfactants (Aoki et al., 2010; Levy et al., 2012).

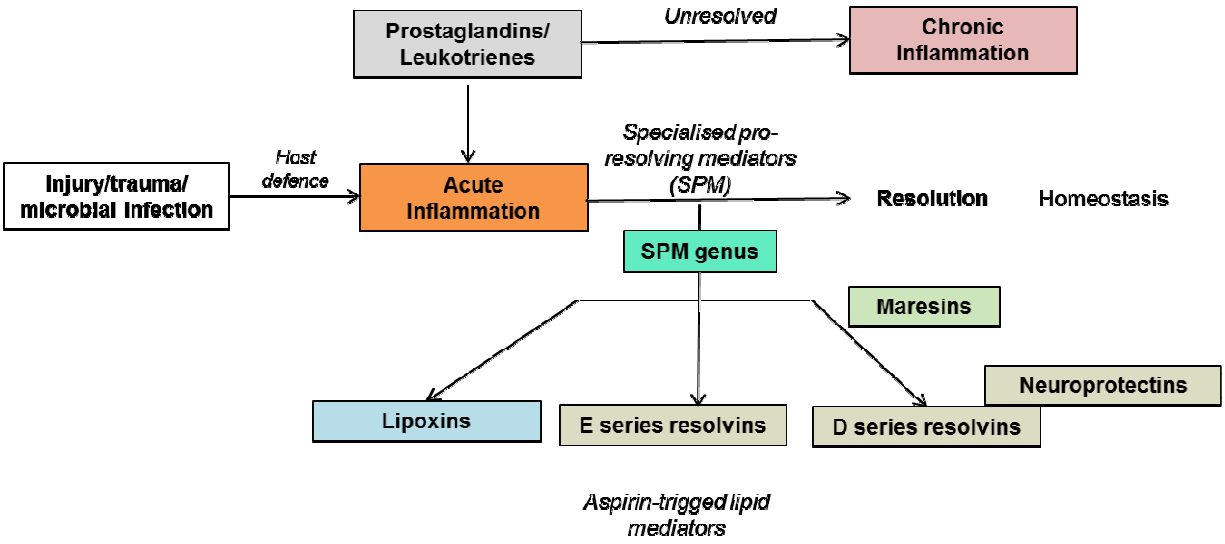


Figure 1.6 Chemical mediators of inflammation and resolution mediators (adapted from Serhan, 2011b)

Issues related to asthma therapy

Asthma is emerging as a chronic health problem worldwide and despite effective treatments there remains high mortality and morbidity which have implications for public health services. Based on the increased understanding of the inflammatory processes in asthma, various therapeutic approaches have been targeted at interrupting these processes (Barnes, 2002; MacRedmond & Dorscheid, 2011). Current asthma treatments target inflammation in one of two ways (acute rescue remedies vs. long-term preventatives) as shown in figure 1.7. The existing pharmacological therapies together with long and short acting β agonists and corticosteroids have proved effective in asthma management in the majority of patients; but they still have issues associated with their use. Tachyphylaxis has complicated the use of β-adrenoreceptor agonists while the known systemic and local side effects of inhaled corticosteroids include osteoporosis and glaucoma (Barnes, 2010; GINA, 2011). Leukotriene modifiers and IgE monoclonal therapy for reduction in IgE has been found to be effective in asthma treatment (Holgate, 2008).

There is a current impetus on identifying specific therapies that target a single inflammatory mediator and are less likely to have major health side effects (Barnes, 2010). These specific therapies have been suggested to be effective for various sub groups of asthma including those with mild-moderates symptoms and EIB. Pharmacological therapies with widespread effects including kinase inhibitors might be more effective but have major health side effects. For the therapy of EIB, inhaled corticosteroids have been identified as the most effective anti-inflammatory treatment available, aiming at reducing AHR and reducing the severity of symptoms. However, inhaled corticosteroids demonstrate both systemic and local health side effects, which affect the sports/physical activity of individuals. Adrenal suppression at high doses, growth retardation in children and adolescents and reduction in bone density have been reported with some inhaled corticosteroids (Carlsen et al., 2008; Egan et al., 1999; Priftis et al., 2006). Although these health side effects are not very common, this possibility needs to be considered especially during treatment of long term use of corticosteroids, which can increase the risk of osteoporosis later in life.

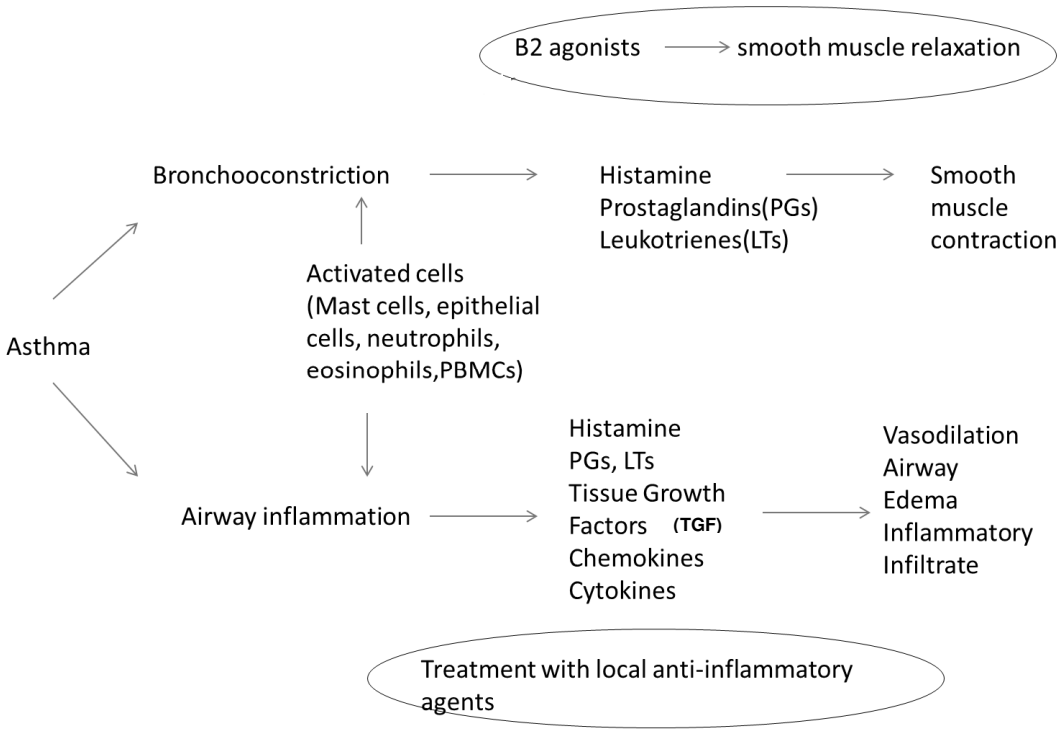


Figure 1.7 Targets for anti – inflammatory asthma therapy

The other anti-inflammatory treatments for EIB include leukotriene antagonists, disodium cromoglycate, nedocromile sodium, β2-agonists, and ipratropium bromide have well established, long term heath side effects providing an impetus for non-pharmacological therapies among researchers and clinical experts (Millward et al., 2010, Mickleborough et al., 2006; Spector & Tan., 2012). In the UK, the NHS and Asthma UK have suggested the use of complimentary therapies alongside conventional medication in asthma (Asthma UK, 2011; NHS, 2012). Consequently, there are both therapeutic and consumer derived interests in identifying potential complimentary therapies for asthma. The recognition of the role of complementary therapy in asthma is limited because these approaches have been insufficiently researched and their effectiveness is largely unproven (GINA, 2011). A range of non-pharmacological treatments including physical conditioning, incorporating a warm-up before and a cool-down period after exercise, performing nasal breathing, avoiding cold weather or environmental allergens, using a face mask or other aid to warm and humidify inhaled air, and modifying dietary intake with omega -3, salt and antioxidants have been identified (Mickleborough et al., 2011). However, to date the efficiencies of all these therapies have not been well established and further investigation is required to validate these therapies with conventional standards (Asthma UK, 2011).

Exploring the potential of non-pharmacologic treatments is important due to the comparatively low risk associated with their use. Since physical activity is a limiting factor for EIB prone individuals, a change in lifestyle and diet could improve the quality of life of these individuals and help them meet the physical activity requirements proposed by the Department of Health (DH). The following sections of the literature review will critically discuss the relationship between fish oil (n-3), PUFAs and their derived mediators and respiratory health in asthmatics, particularly those with EIB.

1.7 Role of omega-3 fatty acids in airway inflammation

1.7.1 Structure and metabolism of omega-3 fatty acids

Fatty acids, both free and as part of complex lipids play an important role in metabolism, storage and transport of energy, gene regulation (Rustan & Drevon, 2001) and as necessary components of all membranes (figure 1.8). Structurally, they are hydrocarbon chains with a carboxyl group at one end and methyl group at the other end. Fatty acids from dietary sources are stored in the body mainly in the form of triacylglycerols; a glycerol molecule backbone to which three, often different, fatty acids are attached (Lunn &Theobald, 2006). Fats have often been perceived as bad components of all nutrients linked to various disease conditions such as cardiovascular disease, obesity, chronic kidney disease (Bagby., 2004), diabetes (López et al., 2010) and other chronic inflammatory diseases (Kennedy et al*.,* 2009; Woodside et al., 2008). The characteristics of a fatty acid are dependent on the length of carbon chain and the presence or absence of double bonds between carbon atoms. Saturated fat has no double bonds and has maximum number of hydrogen atoms bonded to the carbons making them ‘saturated’ with hydrogen; while unsaturated fatty acids have one or more double bonds between adjacent carbon atoms making them vulnerable to peroxidation. Monounsaturated fatty acids (MUFAs) (palmitoleic, oleic, eladic, cis/trans vaccenic and euric acid) have one double bond present in the fatty acid chain while polyunsaturated fatty acids (PUFAs) have more than one double bond present in the fatty acid (Gunstone, 1996; Sanders & Emery, 2003).

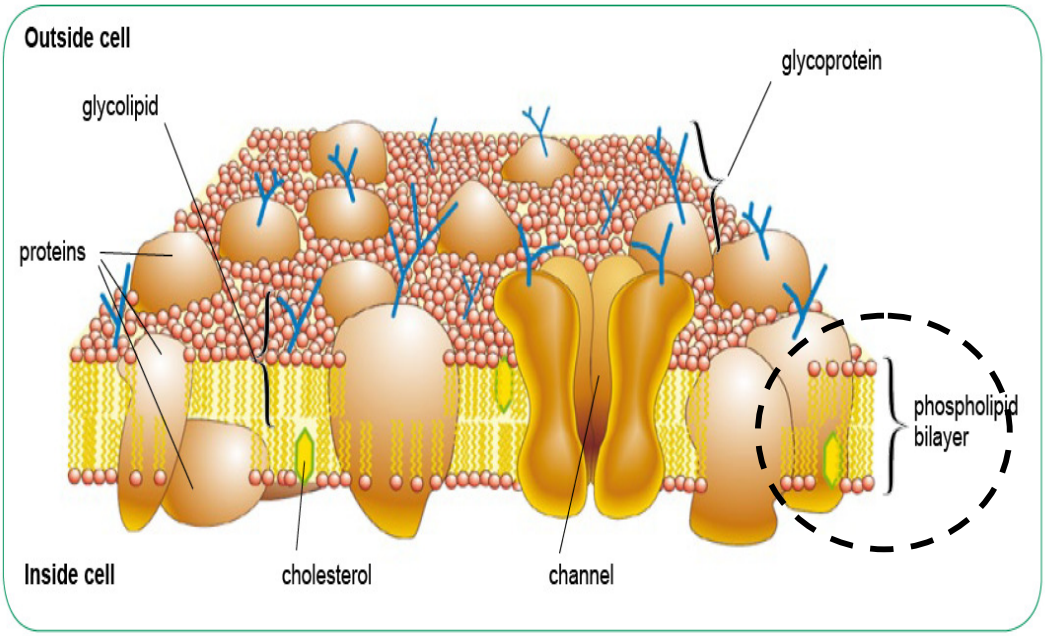


Figure 1.8 Fatty acids are integral components of the cell membrane (phospholipid bilayer) (modified from Jones, 2000)

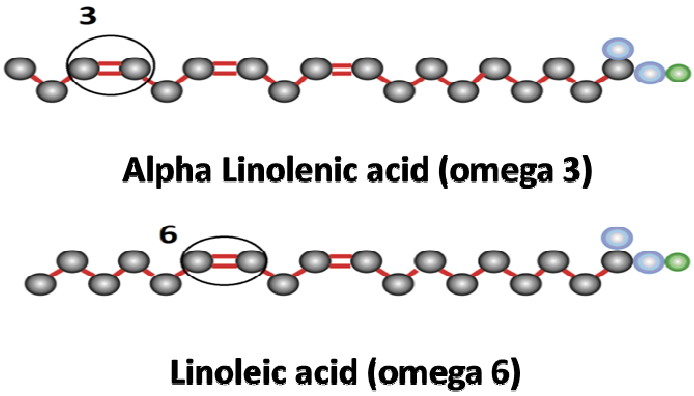


Figure 1.9 Structure of ALA and LA

PUFAs are also known by their shorthand nomenclature, which represents the number of carbon atoms in their chain. Figure 1.9 shows the structure of α-linolenic acid (ALA,18:3 n-3) and linoleic acid (LA, 18:2n-6) demonstrating the position of the carbon bonds responsible for their nomenclature. Omega 3 (n-3) fatty acids are called so because their first carbon double bond is present at carbon number 3 counting the methyl carbon as carbon number 1 while omega 6 (n-6) are called so as their first carbon double bond is present at carbon number 6 (Lunn &Theobald, 2006; Calder & Yaqoob, 2009).

Essential fatty acids and their metabolism

While saturated fatty acids and most MUFA can be synthesised in mammals from non-fat precursors (such as glucose and some amino acids) not all fatty acids can be synthesised *de novo* in mammals, as they cannot insert double bonds before carbon 9 in oleic acid (18:1n-9). Specifically mammals cannot convert oleic acid into linoleic acid (LA, 18:2n-6) or LA into α-linolenic acid (ALA,18:3 n-3) catalysed via delta 12 desaturase and delta 15 desaturase enzymes, respectively. Since ALA and LA cannot be synthesised *de novo*, their intake from food sources is important and they are classified as essential fatty acids. Burr & Burr (1930) conducted experiments on rats and carefully observed the health of the rats over several months. They demonstrated that a deficiency of ALA and LAs led to various deficiency diseases in animals, including severe skin and kidney problems, and some animals often died within weeks. Eventually it was discovered that adding back purified LA and ALA restored the sick animals to health, while the other fatty acids were unable to create this effect. This led to the recognition of LA and ALA as 'essential fatty acids (EFAs)' (Burr & Burr, 1930; Burr et al.,1932). Thus, EFAs (LA and ALA) are those fatty acids, which must be obtained in the diet by humans and other animals to maintain health, these EFA however cannot not be synthesized by the body (Goodhart et al., 1980, Neitzel, 2010).

Human EFA deficiency is not very common in western countries, and most research on EFA deficiency has been conducted in animal models. In countries where protein malnutrition occurs, EFA deficiency may occur owing to either very low dietary EFA intake or lack of enzymes for synthesis. Originally, it was hypothesised that ALA could substitute for LA in the case of deficiency, however this proved not to be true as the product of the metabolism of these two PUFAs differs in function (Holman, 1998; Ratnayake & Galli, 2009; Shireman, 2003). Some of the causes of EFA deficiency in humans include chronic malnutrition (lack of fat and protein, decreased enzyme synthesis), various fat mal-absorption conditions (secondary to pancreatic insufficiency, bowel cancer or serious gastro intestinal disease), and sjogren - larsson syndrome (a genetic enzyme defect in desaturation and elongation). Consequences of EFA deficiency include decreased growth in infants and children, increased susceptibility to infection, and poor wound healing (Cunnane, 2003; Jeppesen et al.,1996; Shireman., 2003; Smit et al., 2004).

Mammalian cells don't have the ability to synthesise LA and ALA, however once these EFAs are obtained from diet they can be metabolised into physiologically active compounds via the introduction of extra double bonds through the process of desaturation and elongation (Ratnayake & Galli, 2009; Wall et al., 2010). Furthermore, LA appears to be an EFA not only because of an immediate cellular function, but because it is the precursor of AA (20:4, n-6) that itself has numerous essential functions. Similarly, the importance of dietary ALA is that it is the precursor of EPA DHA which are found in the phospholipids of cell membranes (e.g. nervous tissue) (Spector, 1999). EPA/DHA have a range of biological functions with EPA demonstrating anti-inflammatory effects while DHA is recognised to be important in premature and newborn infants. Subsequently AA, EPA/DHA have been suggested to be termed as 'conditionally essential' (Burke et al., 1999; Fedacko et al., 2007; Kidd et al, 2007; McCowen & Bistrian, 2005)

Since PUFAs are found in the cell membranes as esterified phosphoglycerides (phospholipids) and the degree of unsaturation introduces 'kinks' into the hydrocarbon chain, the potential of the phospholipids to pack together may be affected. The chains with multiple double bonds pack together better than chains containing fewer double bonds. Hence, membranes comprising greater proportions of long chain PUFAs are more stable (Lunn & Theobald, 2006). Approximately 96% of the dietary ALA is absorbed in the gut and after absorption they can be incorporated into the phospholipids of cell membranes where they affect membrane activities (Burdge, 2006, Burdge & Calder, 2005) or are converted to long chain n-3 fatty acids (Burdge et al., 2002, DeLany et al., 2000). LA is metabolised via desaturation and elongation reactions in the liver, via gamma linoleic acid, to produce arachidonic acid (AA, 20:4n6). In a similar manner, ALA is metabolised in humans by further desaturation and elongation in the liver with desaturation occurring at carbon atoms lower than 9. ALA is converted to steridonic acid (SDA, 18:4, n3) by delta 6 desaturase and then steridonic acid can be elongated to eicosatetraenoic acid (20:4 n3) as shown in figure 1.10. Eicosatetraenoic acid is further desaturated by delta 5 desaturase to yield eicosapentaenoic acid (EPA, 20:5 n3) (figure 1.10). EPA can be further converted to docosapentaenoic acid (DPA, 22:5 n3) and docosahexaenoic acid (DHA, 22:6 n3) as shown in figure 1.10.(Calder & Yaqoob, 2009, Poudyal et al., 2011) (figure 1.10). Since the metabolism of the n-3 and n-6 families requires the same set of enzymes there is competition between the two families. An excess of the n-6 or the n-3 family of fatty acids could affect the metabolism of the other family which may lead to reduction in the

incorporation of these lipids in the tissues which may alter its biological effects (Burdge & Calder, 2005; Hussein et al., 2004).

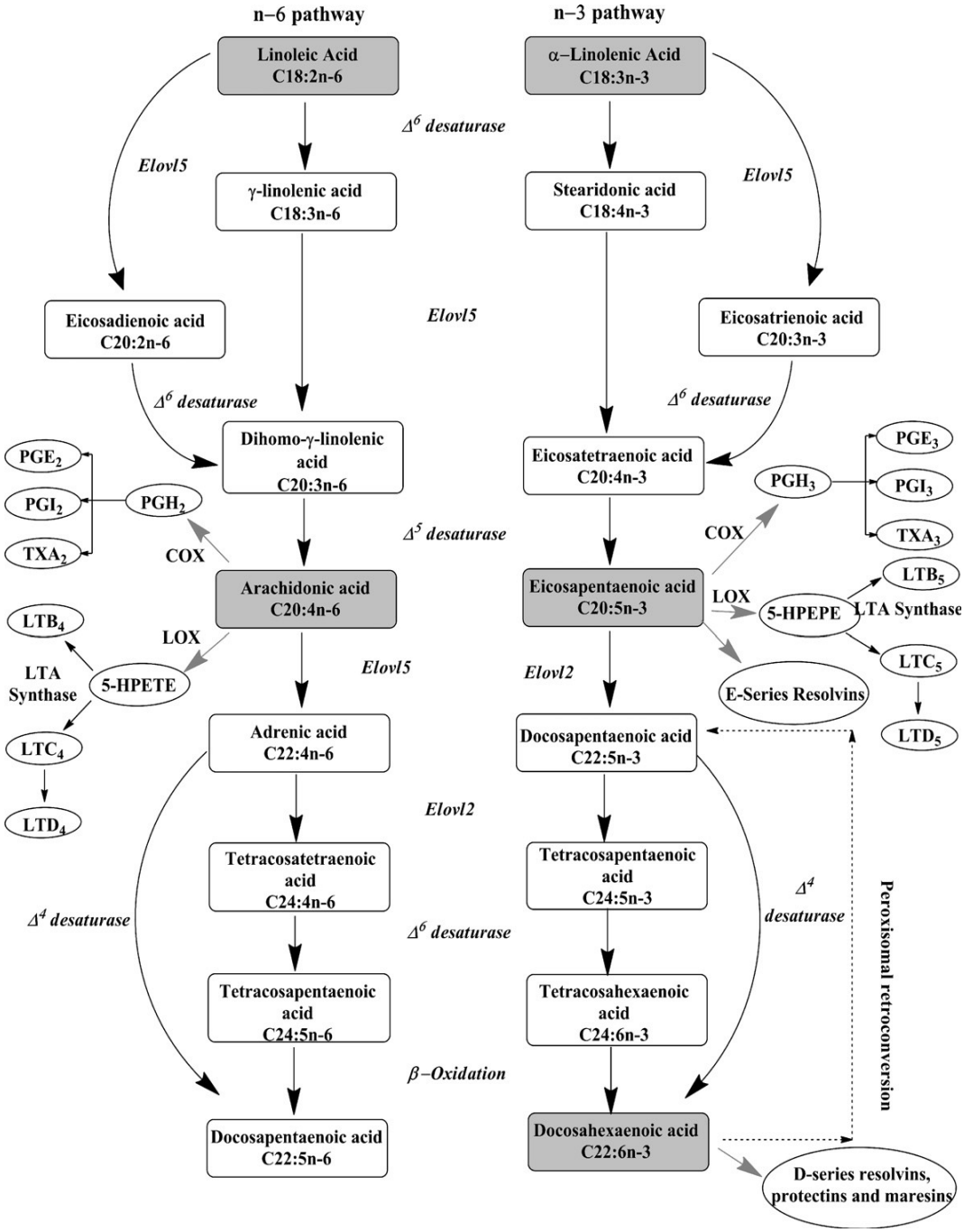


Figure 1.10 Proposed pathway for the metabolism of n-6 and n-3 PUFA (with permission from Poudyal et al., 2011)

1.7.2 Biological Effects of n-3 PUFA and the mechanisms involved

PUFAs are important constituents of the phospholipids of all cell membranes and the characteristic fatty acid composition of different cells and tissues is dependent on the availability of different fatty acids as well as the metabolic properties of the cells and tissues (Calder, 2010b; Calder & Yaqoob, 2009).

The phospholipid component of immune function cells (extracted from the blood) of individuals consuming a typical 'western diet' is composed of approximately 15-20% of fatty acids as AA; 1% EPA and 2-3% DHA per total fatty acid content (Endres et al., 1989; Healy et al., 2000; Rees et al., 2006; Yaqoob et al., 2000). However, there are differences between the phospholipid classes within single cell types with regard to fatty acid content. For example, in neutrophils, alk-1-enylacyl -phosphatidylethanolamine (PE), alkylacyl-phosphatidylcholine (PC) and phosphatidylinositol (PI) phospholipid classes contain >13% AA, while diacyl-PC and diacyl-PE contain <10% AA (Sperling et al., 1993). While immune function cells have a high proportion of AA (15-20%), red blood cells (RBCs) and plasma have relatively lower AA composition (<10%), approximately 1% EPA and <2.5% DHA (Hodson et al., 2008; Poppit et al., 2005; Rees et al., 2006).

Typically, PBMC *total lipid composition* constitutes of palmitic acid (~20%), stearic acid (<20%), oleic acid (~15-20%), LA (~10%) and ALA (<1%) per 100% of fatty acids. In addition, high levels of AA (15-20%) and low levels of EPA (<1%) and DHA (<3%) are found in PBMC total lipid composition (Damsgaard et al., 2008; Yaqoob et al., 2000). PBMC *phospholipid* composition is reported to be constituted typically of palmitic acid (15-20%), stearic acid (~20%), oleic acid (~15-20%), LA (~8%), AA (15-20%), EPA (<1%) and DHA (~3%) (Kew et al., 2004; Miles et al., 2004; Thies et al., 2001). Different phospholipid classes within PBMC and different subsets of PBMC have also been shown to have differences in fatty acid composition (Hon et al., 2009; Miles et al., 2004). Therefore, depending on the type of cells analysed, differences in fatty acid content are likely to be observed. Since tissue and blood fatty acid profiles have been shown to be modified by dietary intake, they have been used as compliance markers for dietary supplementation studies (Garcia-Larsen et al., 2011; Hodson et al., 2008; Jeppesen et al., 2012; Meneses et al., 2009). However it is important to understand if phospholipid or total lipid content of cells are an adequate marker of dietary intake. In adults, there are a number of epidemiological and supplementation studies evaluating the effects of dietary n-3 PUFA

intake on asthma. In a population based study by McKeever and co-workers (2008) (n=13,820, ages 42.2 ±11.2 years), individual fatty acid intakes (estimated by food frequency questionnaires; FFQs) were analysed and related to symptoms of asthma. The results show that high intake of n-3 fatty acids does not protect against asthma, however higher consumption of several n-6 fatty acids including LA and AA were found to be associated with a significant reduction in FEV_1, particularly in smokers (McKeever et al., 2008). In another population-based study of Respiratory Health in Northern Europe, 16,187 subjects aged 23–54 years completed a postal FFQ and it was reported that a minimum level of weekly fish intake (>1 serving per week) in adulthood was associated with protection against asthma. Participants who never had fish were found to have an increased risk for asthma (Laerum et al., 2007). It should be highlighted that the inconsistencies from the population-based studies are possibly due to the methods used. Most studies that have found associations between fatty acid intake and asthma have used indirect measurements of fatty acid intake including FFQs or other dietary recall methods (Wood et al., 2004).

The presence of n-3 fatty acids in the phospholipids of plasma, RBCs and even whole blood has been used as a marker for compliance in various supplementation studies. In addition incorporation of fatty acids in inflammatory cells/membranes have been studied in inflammatory disease states (Harris, 2010; Miles et al., 2011; Mickleborough et al., 2006; Rizzo et al., 2010; Schubert et al., 2009; Stenius-Aarniala et al., 1989; Versleijen et al., 2012). Thus, it can be argued that studies using a direct marker of fat intake may provide a reliable method for evaluating the relationship between asthma and dietary n-3 PUFA. Some but not all studies show evidence to suggest there is a possible agreement between dietary FFQs and plasma fatty acid levels; in a study by Wood and co-workers, (2004), it was found that the n-3 PUFAs and n-6/n-3 ratio in plasma phospholipids were not consistently associated with asthma or atopy. The only positive association with current asthma was found with dihomoγ-linolenic acid in plasma phospholipids. In this study, there was a good agreement between the dietary FFQ estimated fatty acid intake and the plasma fatty acid levels (Wood et al., 2004). Recently the Global Allergy and Asthma European Network of Excellence (GA^2LEN) have shown that a reasonable association exists between estimates of dietary n-3 PUFA and total plasma phospholipid composition using the GA^2LEN FFQ within the European countries that took part in the study (Garcia-Larsen et al., 2011). During supplementation studies, while dietary estimates

provide information about the dietary behaviour of individuals, the total cell or phospholipid content of fatty acids can be used as a reliable marker of incorporation. In this thesis (chapter 4), the effect the n-3 PUFA supplementation on the pulmonary function of EIB prone individuals will be investigated and to support the study; incorporation of EPA/DHA will be evaluated in the total PBMC lipid composition.

Dietary supplementation with fish oil can alter fatty acid profiles and there is an increase in overall EPA and DHA in different cell types including PBMC following supplementation (Damsgaard et al., 2008; Yaqoob et al., 2000). The time-course studies demonstrate that incorporation of EPA and DHA into human inflammatory cells starts within days after supplementation; and reaches near maximum within four weeks and the incorporation occurs in a dose response manner (Kew et al., 2004; Rees., et al. 2006; Thies et al., 2001; Yaqoob et al., 2000). Within the cell membranes, EPA and DHA can compete with and replace n-6 PUFAs (primarily AA) (Blonk et al., 1990; Harris et al., 1991; Calder, 2012; Katan et al., 1997; Sanders et al., 1983; Healy et al., 2000; Mickleborough et al., 2006; Witte et al., 2010). The modification of phospholipid content can influence the membrane-linked enzyme systems and cell signalling pathways that affect the physiological responses of cells (Mickleborough & Rundell, 2005; Surette, 2008).

One of the mechanisms for the action of n-3 PUFA is the altered pattern of lipid mediator synthesis; subsequently the fatty acid composition of cell membranes has an important role for the regulation of the response and functions of these cells (Tvrzicka et al., 2011). The metabolites from EPA and AA form the basis for regulatory signals (eicosanoids). Eicosanoid synthesis involves PUFA mobilisation from the cell membrane by various phospholipase enzymes, most notably phospholipase A_2 (PlA_2). Following the mobilisation; the free AA or EPA/DHA acts as a substrate for eicosanoid production via COX (cyclooxygenase) and LOX (lipoxygenase) pathway. PGs, prostacylins and TXs are formed by the action of COX while LTs and hydroxy fatty acids are formed by the action of LOX enzymes.

Eicosanoid production from the two families of fatty acids (n-3 and n-6) follow different pathways; however they share the same set of enzymes and the content of n-3 to n-6 in the diet will affect which pathway will be active (Wall et al., 2010). The nature of the eicosanoids produced from the two families of fatty acids varies in biological activity. For

the pathophysiology of any inflammatory condition, the timing of eicosanoid generation, the concentration of eicosanoids produced and the subsequent sensitivity of the target cells and tissues to the eicosanoids produced is important (Calder, 2006, 2012). AA is one of the most tightly regulated fatty acids in cell membrane phospholipids as it affects the way cells behave, and its actions have far ranging effects (Calder., 2012; Seeds & Bass, 1999). Diets high in LA or AA could potentially result in over-activity of AA derived eicosanoids which could lead to an over active immune system which has been hypothesised to form the basis of chronic inflammatory disease states such as asthma, CVD, diabetes, and cancer (Calder, 2006; Levick et al., 2007; Mickleborough, 2008; Miller, 2006; Reiss & Edelman, 2006).

There is a competition between n-6 and n-3 fatty acids for the production of eicosanoids via the COX and LOX enzymes (figure 1.11). Since inflammatory cells contain a high proportion of AA and a low proportion of EPA, AA is the major substrate for eicosanoid synthesis (Lee et al., 1985; Calder., 2012; Endres et al., 1989; Rees et al., 2006; Sperling et al., 1993). AA metabolised via COX produces 2-series PGs and the 2-series TXs. Monocytes and macrophages produce large amounts of prostaglandin E_2 (PGE_2) and prostaglandin F_{2a} (PGF_{2a}) while neutrophils and mast cells produce prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) respectively (Pavord, et al., 1993,1995; Sastre & Pozo., 2012; Wall et al., 2010). Increased levels of these PGs have been found in urine and sputum samples of individuals with EIB compared to healthy individuals (Anderson & Kippelen, 2010; Haverkampet al., 2012; Kippelen et al., 2010). Following the LOX pathway (5-LOX), AA is metabolised to hydroxy and hydroperoxy derivatives, such as 5-HETE and 5-hydroperoxyeicosatetraenoic acid (5-HPETE), and the CystLTs - LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4). Neutrophils, monocytes and macrophages produce LTB_4, while LTC_4, LTD_4, and LTE_4 are produced by mast cells, basophils, and eosinophils. LTB_4 is a strong leukocyte chemotactic agent and an activator of neutrophils. LTB_4 increases vascular permeability, enhances blood flow, acts as chemotactic agent for leukocytes and induces release of reactive oxygen species ROS and lysosymal enzyme. In addition, LTB_4 promotes secretion of inflammatory cytokines such as TNF-α, IL-1ß and IL-6 by macrophages (Tilley et al., 2001; Wall et al., 2010). Similarly other CystLTs (LTC_4, D_4 and E_4) increase vascular permeability, increase mucous secretion, increase hypersensitivity and cause bronchoconstriction (Anderson & Kippelen, 2010; Duong et al., 2008; Moloney et al., 2003; Gulliksson et al., 2006).

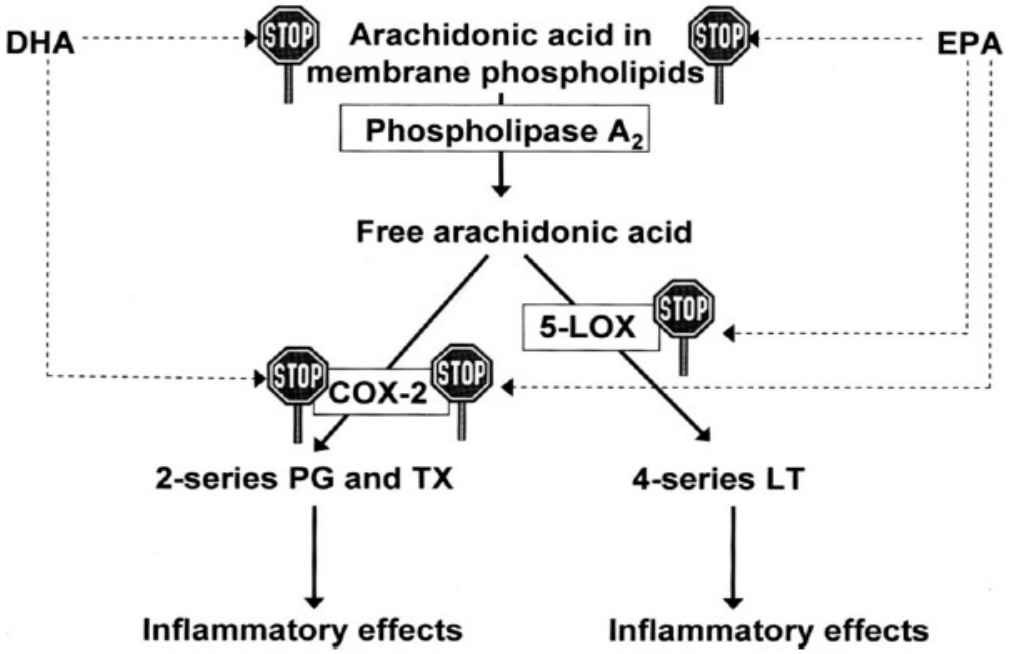


Figure 1.11 Suggested mechanism of the inhibitory effect of EPA on AA, COX and LOX pathways (from Calder with permission, 2006)

When present in excessive concentrations, the AA derived eicosanoids can cause damage to host tissues, lead to formation of thrombi, and facilitate inflammatory disorders (Bagga et al., 2003; Calder, 2006; Levick et al., 2007 Miller, 2006;). Despite the pro-inflammatory effects of AA derived eicosanoids, it is now recognised that not all the metabolites from AA act in the same manner. Inhaled PGE_2 has been found to promote bronchodilation in normal subjects (Walters & Davies, 1982) but may cause constriction in patients with asthma because of activation of reflex cholinergic bronchoconstriction (Pavord & Tattersfield, 1995; Tilley et al., 2001). However, recent studies have shown a contradictory outcome; for example inhalation of PGE_2 is shown to improve abnormal airway physiology and inflammation that characterise asthma (Bloemen et al., 2007; Ying et al., 2004). Additionally, it has been suggested that PGE_2 acts as an inhibitor of the production of two important pro-inflammatory cytokines (TNF-α and IL-1β) by monocytes and macrophages (Calder & Yaqoob., 2009; Dooper et al., 2002; Miles et al., 2002). Gauvreau and co-workers (1999) have shown that there is a significant decline in pulmonary function (immediate and late phase FEV_1 and methacholine responsiveness) 24 hours after allergen challenge. In atopic asthmatic subjects, inhaled PGE_2 before an allergen challenge test results in decreased eosinophils and PGD_2 in the BAL, which suggests that PGE_2 restrains allergic inflammation (Hartert et al., 2000). Furthermore, PGE_2 is also found

to inhibit 5-LOX, and to henceforth reduce the production of 4 series LTs (Levy et al., 2002), and to induce 15-LOX production of lipoxins (Levy et al., 2002; Vachier et al., 2002) which have anti-inflammatory effects (Calder, 2010b; Gewirtz et al., 2001; Vachier et al., 2002).

PGI_2 has been found in some studies to reduce allergic inflammation and it is hypothesised that PGI_2 can possibly suppress Th2 activity and eosinophil recruitment (Kremmyda et al., 2011; Moore & Peebles, 2006; Park & Christman, 2006; Wang et al., 2011; Yeh et al., 2011). There is increasing evidence from *in vitro* studies to suggest that PGI_2 analogues have therapeutic value for asthma due to their anti-inflammatory effects. Kuo and co-workers (2010 a, b), for example, have recently reported that PGI_2 analogues can enhance the expression of a regulatory cytokine (IL-10) and suppress the activity of pro-inflammatory TNF-α via epigenetic regulation. Since, however, a few studies have conversely shown pro-inflammatory effects of PGI_2, including enhanced expression of a Th2-related chemokine *in vitro* and increased neutrophil recruitment (Kuo et al., 2010 a, b) the mechanisms for the anti-inflammatory effects of PGI_2 observed in some studies require further verification to establish their exact role in Th2 regulation. It appears, therefore, that some AA derived metabolites may have both pro- and anti-inflammatory effects affecting inflammatory processes. Some AA derived eicosanoids such as lipoxins (e.g. Lipoxin A_4) have only reduced inflammatory effects which could be important in resolution of inflammation associated with asthma. It has been demonstrated that lower levels of lipoxin A_4 are associated with increased asthma severity, EIB and related respiratory disorders (Bozinovsk et al., 2011; Levy, 2010; Hallstrand, 2012; Tahan et al., 2008).

EPA is a competing substrate for cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes and this competition with AA leads to decreased expression of COX-2 and 5-LOX. This in of itself has been suggested as a potential mechanism for the proposed anti-inflammatory benefits of n-3 PUFA, in that this competition leads to an altered eicosanoid production. Ordinarily, AA is metabolised by COX into biologically active 2-series prostanoids, however when EPA is utilised as a COX substrate the resultant prostanoids are of the alternative 3-series. There is some evidence to suggest that PGE_2 has both pro and anti-inflammatory functions depending upon the nature of the inflammatory insult (Fujitani et al., 2002; Rajakariar et al., 2007) and while there is less know about the inflammatory activity of the equivalent 3-series prostanoids some studies have presented

data to show that PGE_3 is a less potent inducer of IL-6 production by macrophages than PGE_2 (Bagga et al., 2003).

In an *in vitro* study by Wada et al., (2007) specificities of prostanoid enzymes and receptors towards EPA-derived (3-series PGs) and AA-derived (2-series PGs) were compared. There was a significant decrease in the formation of 2-series PGs via PGHS-2 (PG endoperoxide H synthase - 2) and this was demonstrated to occur only to the extent that AA levels in phospholipids were decreased by EPA replacing AA (Wada et al., 2007). Approximately two- to three-fold higher activities were observed for AA derived mediators compared to EPA derived ones with the different receptors studied. For example, lower potencies were observed for PGE_3 compared with PGE_2 towards the EP1, EP2, EP3 and EP4 receptors (Wada et al., 2007). In a separate *in vitro* study it has been demonstrated that EPA derived PGD_3 antagonises the effect of PGD_2-mediated migration of neutrophils across endothelial cells (Tull et al., 2009). These studies directly comparing the effect of EPA and AA derived lipid mediators provide some evidence to confirm the competition between n-6 and n-3 fatty acids for the production of eicosanoids and go some way towards identifying the possible mechanisms involved.

It has also been shown that 5 series LTs can be produced in the macrophages of fish oil fed mice (Chapkin et al., 1992) and in the neutrophils of humans supplemented with fish oil for several weeks (Lee et al., 1985; Sperling et al., 1993). LTB_5 derived from EPA has been shown to be 10-100 fold less potent as a neutrophil chemotactic agent than AA derived LTB_4, thus LTB_5 is a much weaker inducer of inflammation (Goldman et al., 1983; Lee et al., 1985; Wall et al., 2010). In a n-3 supplementation study using EIB it was found that there was a significant reduction in LTB_4 and significant increase in LTB_5 level in activated polymorphonulcear cells by the end of 3 weeks of supplementation and this reduced inflammatory effect was accompanied by an improvement in post exercise EIB symptoms (Mickleborough et al., 2006).

Effect of n-3 PUFA supplementation on Th1 and Th2 responses and cytokines and lipid rafts

The modified cell membrane phospholipid fatty acid content with n-3 PUFA supplementation facilitates the formation of 'lipid rafts' which have been studied in T cells. The relationship between lipid rafts and the immunomodulatory effects of n-3 PUFA have been of considerable interest. These rafts are formed by the movement of receptors, accessory proteins, and enzymes within the plane of the cell membrane to co-localise into signalling platforms (Katagiri et al., 2001; Yaqoob, 2009). These rafts in turn influence the activity of membrane proteins including receptors, transporters, ion channels and cell signalling enzymes, these result in the transfer of intracellular signals into the cytosol (Miles et al., 2002). Based on the evidence from cell culture and animal feeding models it has been shown that n-3 PUFA supplementation modifies raft formation in T cells, which in turn impairs the signalling mechanism of these cells (Stulnig et al., 1998, 2001). Thus, the exposure of T cells to modified n-3 PUFA (after supplementation) can alter the chemical structure of rafts which can consequently affect their function (Fan et al., 2003, 2004; Zeyda et al,. 2002). Additionally, the supplementation of n-3 PUFA has been reported to affect cell signalling pathways either by altering the expression and activity of membrane receptors or by modifying the expression of genes by the activation of transcription factors such as NFκB and PPAR-α (Michaud & Renier, 2001; Rudkowska et al., 2009; Tai et al., 2005; Calder, 2012).

Since the eicosanoids have an effect on Th1 and Th2 responses it is hypothesised that fish oils containing n-3 PUFA could potentially influence T cell functions through eicosanoid production. Human supplementation studies with n-3 (EPA and DHA) have shown differential effects on lymphocyte functions. Meydani et al., (1991) showed that with three months of supplementation with 2.4 grams n-3 (EPA+DHA) per day in healthy volunteers, there was a decrease in lymphocyte proliferation and decreased production of IL-2 in women aged 51-68 years but not in younger women. In a different study, Meydani et al., (1993) showed that supplementation of a low fat diet with n-3 PUFA decreased T-cell proliferation in subjects older than 40 years. Furthermore, Thies et al., (2001) have shown that with a low dose (1 g n-3/day (720 mg EPA plus 280 mg DHA) for 12 weeks there was a decrease in lymphocyte proliferation, but IL-2 and IFN-γ production were unaffected in healthy subjects aged 55-75 years. Although there are a number of studies

showing that dietary n-3 PUFA supplementation in humans significantly reduces lymphocyte proliferation in response to mitogens; there are studies, which showed no effect of dietary n-3 supplementation on cytokine production in healthy individuals and those with chronic disease states (Cooper et al., 1993; Blok et al., 1997; Stapleton et al., 2011).

There is some evidence to suggest that EPA is effective in suppressing pro-inflammatory mediator generation and cytokine expression from lipopolysaccharide -stimulated human asthmatic alveolar macrophages (Mickleborough et al,. 2009). The study by Mickleborough et al., shows that pure EPA and EPA-rich media significantly suppressed TNF-α and IL-1β mRNA expression and the production of LTB_4, PGD_2 and TNF-α and IL-1β in lipopolysaccharide-stimulated primary alveolar macrophages obtained from asthmatic patients to a much greater extent than pure DHA and DHA-rich media respectively (Mickleborough et al,. 2009). Several supplementation studies with EPA/DHA in healthy volunteers have shown decreased production of TNF-α, of IL-1β and IL-6 by endotoxin-stimulated monocytes or mononuclear cells (Caughley et al., 1996; Endres et al., 1989; Trebble et al., 2003). However the study by Mickleborough et al., (2009) shows that high or low levels of DHA supplementation alone do not affect T cell proliferation and cytokine production, there is therefore the potential to use EPA alone for inhibiting cytokine synthesis in vitro (Mickleborough et al., 2009).

Overall, the evidence from *in vitro* and *in vivo* studies show that n-3 PUFA supplementation has a potential for inhibiting T cell proliferation and production of cytokines. Inhibition of T cell responses has been observed with higher dosage of n-3 while this effect is not observed at low n-3 levels. These inconsistencies may be related to differences in subject characteristics including age, gender, heath, diet, differences in study design (dose and duration) as well as experimental methods (cell preparation, cell culture, cytokine assays). In conclusion, there is evidence to suggest that fish oils may modulate T cell response and functions independently of eicosanoid production (Alexander et al., 1988; Jolly et al., 1997; Wallace et al., 2001; Kelley et al., 1999; Versleijen et al., 2012). Furthermore, increasing phospholipid EPA/AA ratios in inflammatory cells with dietary n-3 supplementation is likely to be one of the mechanisms, which can potentially facilitate the production of weaker eicosanoids that may exhibit anti-inflammatory effects.

Thus, there are different mechanisms for the immunomodulatory action of n-3 PUFAs, which require further verification in *in vivo* human studies.

1.8 Studies investigating the effect of fish oil (n-3) on asthma

The adult n-3 intervention trials in the last two decades have provided a contradictory picture of efficacy with respect to FEV_1 or PEF. Table 1.6 shows relevant n-3 intervention trials in asthma and EIB with three or more weeks of supplementation, the information included in the table is as complete as possible. Kirsch et al. (1988) compared a high dose n-3 PUFA supplementation (4 gram EPA/day, n=6) with a low dose (0.1 grams/day, n=6) for 8 weeks on asthmatics in a small study (n=12, age 42 to 73 years) and found there was no difference in FEV_1 or symptom scores between the two groups. There was no difference in the lung function determined by PEF between the two groups before the start of supplementation (Kirsch et al. 1988). Hodge and co-workers (1998) reported no change in lung function values in asthmatic children (8-12 years, n=45) however, there was a reduction in TNFα production (by cultured PBMC) compared to baseline; however, the magnitude of change between groups was not significant (Hodge et al. 1998). Peak expiratory flow (PEF) has been reported in some studies as a marker for lung function. Emelynov et al., (2002) showed a significant increase in morning PEF with 8 week supplementation with a low dose of n-3 (50mg EPA+ DHA per day) in mild-moderate atopic asthmatics (n=46; 18-56 years). In addition, Surette et al., (2008) reported a significant improvement in quality of life and asthma management scores (including symptoms) assessed by questionnaires after 3 weeks of supplementation (0.5 gram EPA + 0.75 gram DHA per day; n = 65 mild-moderate asthmatics). Conversely, in two cross over trials there was no significant change in PEF after 10 week n-3 supplementation with > 2grams EPA+ DHA per day (see table 1.6) (McDonald, 1990; Stenius-Aarniala, 1989). Overall, although some literature and our knowledge of the physiological action of n-3 fatty acids suggest that we should potentially see effects of supplementation on lung function there is no consistent effect of n-3 supplementation on FEV_1 or PEF reported which could be attributed to the heterogeneity between the studies. These studies have used a range of doses, as low as 50 mg to > 3grams of EPA/DHA per day and have studied different sub group of asthmatics such as mild-moderate, severe and atopic populations.

In a study by Schubert and co-workers, (2009) (n=23, atopic asthma, table 1.6) dietary supplementation with either an n–3 PUFA-enriched fat blend (0.69 g/day, comprising 450mg EPA and 180 mg DHA/day; n=12 participants) or placebo (n=13 participants) for 5 weeks was provided. After 3-weeks of supplementation, the participants underwent two allergen challenge tests in the remaining two weeks of supplementation. FeNO was significantly lower in the n–3 PUFA group (p=0.01); though the levels of FeNO increased during allergen exposure in both groups, the mean values were 5-fold lower in the n–3 PUFA group. No differences were observed between the asthmatic and control group with regards to asthma symptoms, FEV_1 or the allergen dose required to induce deterioration of lung function challenge. Furthermore, compliance was monitored by plasma and RBC cell membrane fatty acid composition and it was found that two weeks of supplementation led to a 3-fold higher value of EPA in the n-3 PUFA group compared to placebo and these levels were maintained till the end of supplementation in plasma and RBC cell membrane (Schubert et al., 2009).

In another double blind, placebo-controlled pilot study, a shorter duration of supplementation was used (n=20; dose – 0.9 g EPA and 0.65 g DHA/day) for 2 weeks (Moreira et al., 2007). The study showed no changes in FeNO levels, FEV_1 or asthma quality of life questionnaires. However, this study was not a well-controlled study as the participants were on their regular medication of inhaled corticosteroids, which makes it difficult to evaluate the true effect of n-3 PUFA supplementation on pulmonary function and other outcomes. Furthermore, the low dose and duration of supplementation could be a reason why no effect of n-3 supplementation was observed. In addition, the participants in this study had stable asthma following their corticosteroid usage and their FeNO levels were not significantly elevated (28 ppb) compared to healthy individuals (25 ppb). Due to these reasons the exact relationship of n-3 PUFA supplementation on FeNO was difficult to evaluate. In this thesis, asthmatics with elevated FeNO levels will supplemented with EPA/DHA to assess the effect of n-3 on FeNO levels.

Asthmatics have a reliance on pharmacological medication and despite the significant advancement in asthma medication during the last two decades the treatments are still far from ideal (GINA, 2011; Mickleborough, 2008). The asthma medications provide modest protection when taken daily; however prolonged usage of these can result in reduced effectiveness or tachyphylaxis. Some of the earlier intervention trials have not shown any

significant changes to reliance on medication (Arm et al., 1988; McDonald et al., 1999; Thien et al., 1993, table 1.6). Hodge et al (1998) have shown a significant reduction in medication use in asthmatic children after 9 month supplementation with 1.2 gram EPA+DHA per day; while Mickleborough et al (2006) have reported that bronchodilator use was significantly reduced during the last 2 weeks of n-3 supplementation (3.2 grams EPA+2.2 grams DHA per day) in EIB-prone adults. Furthermore, it has been reported that there are improvements in self-reported asthma status and bronchodilator use in subjects consuming a fish oil emulsion (1gram EPA + 1.5 grm GLA per day) for 4 weeks compared to a placebo(Surette et al., 2008). The authors have also reported results from another trial showing an improvement in the asthma quality of life and asthma control based on non-validated questionnaires (primarily based on bronchodilator usage) (Surette et al., 2008).

Chronic inflammation is associated with AHR, which is responsible for recurrent episodes of wheezing, breathlessness, chest tightness, and coughing among asthmatics (Brannan, 2010). The majority of intervention studies show inconsistencies when reporting effects of n-3 on AHR (Thien et al., 2011). Studies (Arm et al., 1988; Thien et al., 1993) have reported AHR in terms of the provocation dose of histamine required to produce a 35% fall in specific conductance and showed no effect of n-3 supplementation on AHR. However, Nagakura et al., (2000) reported AHR as the provocative concentration of acetylcholine causing a 20% fall in FEV_1 for each subject and saw a reduction in acetylcholine responsiveness in the fish oil group but not in the control group. Schubert et al., (2009) reported a reduction in AHR after an allergen challenge with n-3 supplementation however, this change failed to reach significance (Schubert et al., 2009).

Based on the above discussed trials it can be concluded that there is a possibly a beneficial role of omega -3 on asthma and EIB. It is widely shown that n-3 PUFA exert a range of anti-inflammatory effects and do not show any health side effects. The advantages of using n-3 PUFA supplementation in asthma has been widely reviewed and their effectives as a complementary therapy for is acknowledged (Schachter et al*.,* 2004; Thien et al*.,* 2011). Furthermore, there are some studies which show that there may be subgroups of asthmatics (EIB and allergic asthma) benefit greatly and others who do not benefit from long-chain n-3 PUFA (Broughton et al., 1997; Mickleborough et al., 2003, 2006; Schubert et al., 2009). These inconsistencies in the studies are possibly due to the different dosages and duration studied. In addition, the studies have investigated different

sub groups of asthma with each having its own characteristic inflammatory pattern. Further studies pertaining to different sub groups are required where specific physiological and biochemical characteristics of these groups are monitored with n-3 supplementation. In addition, a number of studies on inflammation have suggested a threshold for an anti-inflammatory effect exhibited by n-3 to be exerted in the range of 1.3 -2.7 grams EPA per day (Calder, 2006, 2012; Dignass et al,. 2004; Kelley et al., 1998; Grimble et al., 2003). Thus, appropriate dose should be considered when designing the studies. In this thesis, the effect of EPA/DHA supplementation was assessed in EIB prone individuals and a justification of dosage and duration of study based on evidence from other trial is discussed in chapter 4.

Table 1.6 Lists relevant intervention trials of omega-3 supplementation and asthma.				
Studies reporting benefits of n-3 supplementation				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
Arm et al., 1989	RCT, double blind, placebo controlled, parallel design. Comparison of n-3 capsules with placebo supplementation 2-week run-in period followed by 10-week treatment period	**Omega 3**: 3.2g EPA and 2.2g DHA/day **Placebo:** Matched capsules with olive oil/day	N=22 atopic, non-smoking asthmatic volunteers entered and 17 completed the trial Age = 18-42 years. Asthma severity determined from asthma symptoms and PEF measurements. No participants were using oral steroids or theophylline or gave history of aspirin sensitivity	No changes were observed for lung function outcomes, medication usage , dyspnoea **10 fold increase in neutrophil phospholipid EPA content with supplementation.** **50% inhibition of total LTB (LTB_4 and LTB_5) generation by stimulated neutrophils** **Suppression of neutrophil chemotaxis**
Stenius-Aarniala et al. 1989	RCT crossover design. Three arm comparison of fish oil versus evening primrose oil (omega-6 group) versus olive oil using liquid oil supplementation. Two week run-in period followed by *30 week intervention (10 weeks per treatment arm) period.* No wash out period	**Omega 3:** 20 mL/day (3.6g EPA and 2.2 gram DHA) **Evening Primrose oil**: 20 mL/day (72% cis linoleic, 9% gamma linoleic) **Olive oil:** 20 mL/day (mainly oleic)	n=40 asthmatics selected, 36 entered study and 29 completed study. Age = 19-61 years. Asthma severity determined from asthma symptoms and PEF measurements	No differences in PEF, symptoms or medication usage. **Plasma PGE_2 levels increased during the fish oil treatment but there were no changes in other TxB_2, PGF_2 αand 6 keto-PGF_1 α in plasma or urine.** **The plasma fatty acid results for cholesterol esters showed significant increase in EPA and DHA levels.**

Studies reporting benefits of n-3 supplementation				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
Schubert et al., 2009	RCT, double blind, parallel study. 5 week supplementation, after 3 weeks, participants were challenged daily with low dose house dust mite allergen (2 weeks).	**Omega 3:** 450mg EPA and 180 mg DHA/day **Placebo:** Unsaturated and monounsaturated fatty acids	N = 23 house dust mite-allergic asthmatics (13 females and 10 males) Age = 22-29 years; Asthma severity determined from asthma symptom, skin prick, lung function and methacholine challenge	**With 3-week supplementation with n-3, FeNO was lower in n-3 group compared to placebo**. **FeNO was lower in n-3 group compared to placebo during allergen challenge. .** **Significant reduction in eosinophilic cation protein and *in vitro* CystLT release** **Significant increase in EPA levels in RBC and plasma membrane** No improvement in PFT, AHR, and number of serum neutrophils.
Emelyanov et al., 2002	RCT, double-blind, parallel study, placebo-controlled 2 week un-in period, *8-week supplementation*	**Omega 3:** New Zealand green-lipped mussel extract (50 mg EPA and DHA per day) **Placebo** : 150 mg olive oil per day	n=46 mild-moderate atopic asthmatics Age = 18-56 years Asthma diagnosis based on ATS guidelines (clinical history, reversibility of (FEV_1 of 15% and diurnal variability of PEF of>20%, skin prick test to common inhalant allergens	**Mean daytime wheeze was significantly reduced in n-3 group compared to placebo.** **Mean morning PEF was significantly higher during the treatment with n-3 with placebo** No change in mean FEV_1 and evening PEF during the treatment with n-3 .

Studies reporting benefits of n-3 supplementation				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
Surette et al., 2008	*Two trials were reported:* ***Trial 1***: Randomized, prospective, double-blind, placebo-controlled, parallel group trial Supplementation for 4 weeks Subjects were questioned about their asthma management using a non-validated questionnaire after 2 and 4 weeks.	Omega 3 : Low-dose medical food emulsion-containing 0.75 g GLA + 0.5 g EPA, or 1.13 g GLA + 0.75g EPA Placebo: olive oil.	Trial 1: n = 35 atopic subjects with mild-to-moderate asthma.	**Dose of 1.13 g GLA + 0.75g EPA was effective in blocking LT biosynthesis.** **Fasting plasma GLA and EPA levels plateaued within 7 days' daily consumption at all levels of intake, whereas the time to maximum plasma concentration was shorter for GLA than for EPA.** **Significant increase in plasma EPA content with 4 week supplementation**
	Trial 2: Supplementation duration – 4 weeks	Low-dose medical food emulsion (same as trial 1)), daily. Quality of life and asthma management were measured using validated questionnaires,	Trial 2: n = 65 mild-moderate asthma subjects	**Self-reported asthma status and mediation use improved in participants consuming low- and high-dose emulsion between week 2 and week 4.** **Medication use decreased with high dose.** **Quality of life and asthma management scores improved significantly.**

Studies reporting benefits of n-3 supplementation				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
Mickleborough et al., 2006	RCT, double-blind, crossover, placebo controlled trial. 3 week supplementation period in each arm 2 week washout phase	Omega 3: 3.2 g EPA and 2.0 g of DHA) Placebo: Olive oil (volume not specified)	N=16, subjects Age = 23 ±1.6 years Participants with clinically treated mild- moderate asthma with a FEV_1>70% predicted	**n-3 improved pulmonary function to below the diagnostic EIB threshold** **Reduction in medication usage** **Reduction in induced sputum differential cell count percentage and concentrations of LTC_4-LTE_4, PGD_2, IL-1β, and TNF-α before and following exercise on the n-3 diet** **Significant reduction in LTB_4 and a significant increase in LTB_5 generation from activated plymorphonuclear cells on the n-3 diet**
Mickleborough et al., 2003	RCT, double-blind, crossover study. Subjects entered the study on their normal diet, and then received either fish oil capsules containing	Omega 3: 3.2 g EPA and 2.0 g of DHA) Placebo: Olive oil (volume not specified) .	N=10 athletes and n=10 controls. Age (asthmatics = 23.2 ± 1.9 years ; controls = 22.4 ± 1.7 years). Participants with clinically diagnosed EIB.	No effect on pre exercise pulmonary function in either groups **Improvement in post exercise pulmonary function In n-3 group** **Reduction in LTE_4, 9α, 11β-PG F_2, LTB_4,TNF–α, and IL-1β, on the n-3 PUFA diet compared with baseline and placebo diets and after exercise challenge**

Studies reporting benefits of n-3 supplementation (in children)				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
Hodge et al., 1998	RCT, double blind, parallel design Comparison of diet high in omega-6 fatty acids (and diet high in omega-3 fatty acids 2 week run in period followed by six month intervention period Dietary control: n-3 diet participants advised to eat fish at least once per month, omega-6 group to avoid fish.	Omega-3 : 1.2g n-3/day (MaxEPA capsules Omega 6 : placebo capsules: safflower/palm/olive oil .	n=45 asthmatic children Age = 8 to 12 years. Asthma defined as reported episodic wheeze in past 12 months and AHR, PFTs, day and night symptoms and medication and medication usage.	No change in lung function values. **Drop in TNFα production (cell culture for PBMC) compared with baseline (however the magnitude of change between groups was not significant.)** **Incorporation of n-3 in the plasma phospholipid at 3 months, group at 3 and 6 months compared to the omega-6 group.**

Studies reporting no benefits of n-3 supplementation				
Author	**Study design**	**Intervention**	**Participants**	**Outcomes**
McDonald et al., 1990	RCT, crossover design, double blind, placebo controlled Comparison of omega-3 supplementation with placebo supplementation. *10 week intervention period,* 6 week washout and then 10 week cross-over intervention	**Omega 3:** 2.7g EPA & 1.8g DHA/day **Placebo**:15g olive oil/day. Subjects asked to keep their dietary fish intake unchanged throughout study	n=15 non-smoking asthmatics. Age= 28 to 72 years completed the study. n=7 subjects were ex-smokers Asthma severity determined from asthma symptoms and PEF measurements.	**T**here was no significant change in PEF, medication usage or asthma symptoms after n-3 supplementation
(Kirsch, et al. 1988)	RCT, double blind, parallel design. Compared high dose of EPA vs. low dose of EPA supplementation. 6 week run in period, 8-week treatment period. 2 week wash out	High dose of EPA : 4grams EPA/day Low dose of EPA: 0.1grams EPA/day	N=12 patients, aged 42-73 years. Asthma severity determined from symptom index and physical evaluation.	No difference observed in clinical status (symptoms, hospital admissions) or pulmonary function between groups at the start of the study/ at the end of 8 weeks of n-3 supplementation
Thien, et al., 1993	RCT, double blind, placebo controlled, parallel design, Comparison of n-3 supplementation with placebo supplementation. Six month supplementation.	Omega 3: 3.2 gram EPA + 2.2 gram DHA per day Placebo: Olive oil , (volume not specified)	N=37, non-smoking, pollen sensitive adults, Age = 19-42 years. Asthma severity determined by symptom, medication usage, PEF measurements and AHR.	No changes in PFT, medication usage or other parameters including symptoms, airway conductance and AHR

1.8.1 Dietary consumption of n-3 PUFAs and recommendations

Dietary sources and conversion from ALA to long chain n-3 PUFAs

The major dietary sources of LA include plant oils (sunflower and corn oils), cereals, animal fat and wholegrain bread while ALA is found primarily in green leafy vegetables, flaxseed, walnut, and rapeseed oils (Lunn &Theobald, 2006; Sanders & Emery, 2003; Wall et al., 2010). Based on short term isotope labelled studies and long term ALA supplementation trials, it has been demonstrated that there is poor conversion of dietary ALA to EPA, DHA and DPA with limited conversion to all the way to DHA (Brenna et al., 2009; Burdge & Calder, 2005; Goyens et al., 2006). Several studies have shown that supplementation with ALA in adults leads to an increase in EPA and DPA but little or no effect on DHA content in plasma fractions and in circulating blood (Brenna et al., 2009). It is estimated that the amount of ALA converted to EPA ranges from 0.2% to 8% (Burdge & Calder, 2005: Lunn & Theobald, 2006) with young women appearing to have a higher conversion efficacy of up to 21% (Burdge & Wootton, 2002). This greater conversion rate in females is potentially due to the up regulation of delta 6 desaturase by female sex hormones (Calder, 2010a). Supplementation trials with purified EPA ethyl esters consistently show an increase in both EPA and DPA but no change in DHA in the blood (Brenna et al., 2009; Grimsgaard et al., 1997).

Although humans have some ability to elongate dietary ALA to the long chain n-3 polyunsaturated fatty acids (EPA and DHA), the rate of synthesis may not be sufficient to meet the requirements of the body (Lunn &Theobald, 2006). Compared to other food products, seafood presents a rich source of long chain PUFA including EPA, DPA and DHA; however, different types of fish contain different content and ratios of EPA and DHA. For example, oily fish such as mackerel or salmon are rich sources of EPA and DHA and can provide 1.5-3.0 grams of these fatty acids per serving compared to a meal of lean fish (such as cod) which can yield only 0.2-0.3 grams of long chain PUFA per serving (Calder & Yaqoob, 2009). Some effects on cardiovascular risk factors and inflammatory markers have been observed with ALA supplementation studies; however, these effects are weaker in relation to the effects achieved by increasing EPA and DHA (Calder & Yaqoob, 2009; McEvoy et al., 2012; Wang et al., 2006). For example in healthy volunteers, ALA supplementation (~13.4 grams/day) can reduce the production of inflammatory cytokines such as TNF-α and IL-1β by approximately 30% while EPA and DHA (~2.7 grams/day) can

reduce the production of these two markers by >70% (Caughey et al., 1996; Zhao et al., 2004). However due to the poor conversion of ALA to EPA and DHA, it is not possible to achieve adequate EPA and DHA in the body. This emphasises the importance of dietary EPA and DHA and thus justifies the classification of these fatty acids as 'conditionally essential'.

Dietary pattern in the UK for n-3 and n-6 PUFA

The National Diet and Nutrition Survey (NDNS) of adults conducted in 2008/2009 reported on the dietary behaviours and nutritional status of the British population including children over the age of 1.5 years and adults (Pot et al., 2012). The surveys suggest that intake of n-6 PUFA as a percentage of food energy has changed slightly from the previous survey (2001-2002) with a lower percentage reported for all age groups. The absolute intake as a percentage of food energy ranged from 3.9% for toddlers to 5.3% for adults. The direction of change for n-3 PUFA was upwards for all groups compared with previous surveys, although the differences in absolute terms were very small. Thus, the dietary ratio of n-3/n-6 in current UK population is approximately 1/5. Additionally, the survey demonstrated that the most common dietary supplementation consumed in all age groups was fish oils (including cod liver oil) along with multivitamins and /or minerals. Cod liver oil and other fish oils were consumed by 9% of toddlers and 7% of those aged 4 -10 years,3% of those aged 11-18 years, 11% of adult men and 13% of adult women (Pot et al., 2012).

The present dietary recommendation in the UK for EPA and DHA are in the range of approximately 0.45 grams/day (3.15 grams/week). And this can be achieved by the consumption of at least two servings of fish per week (one white and the other oily fish) (Lunn & Theobald, 2006; Wall et al., 2010). What the current and/or previous NDNS surveys do not provide, however, is intake data for individual n-3/n-6 fatty acids. This limits our knowledge about the consumption of long chain n-3 and n-6 PUFA in UK population, a limitation which been highlighted previously by the British Nutrition Foundation (Lunn & Theobald, 2006). Based on the data available from the NDNS survey, adults in the UK consume either approximately 100 grams of lean fish or approximately 50 grams of oily fish per week. This consumption pattern is similar to intakes in Eastern European, North American and Australian countries. Lean fish intake is higher is Southern European countries while oily fish intake is higher in Japan (Calder &Yaqoob, 2009; Givens & Gibbs, 2006; SACN, 2004). This recommendation is primarily based on epidemiological evidence

and supplementation studies demonstrating reduced cardiovascular mortality, morbidity and risk with increased dietary consumption of fish or fish oil supplementation (SACN, 2004; Lunn &Theobald, 2006). Those individuals who may not be willing to change their dietary habits and eat more oily fish could potentially achieve the recommended intake of EPA and DHA by consumption of fish oil capsules/liquid. A typical 1-gram fish oil capsule per day can provide 0.3 grams of EPA+DHA/day which would allow many individuals to meet the basic recommendations (de Decker et al., 1998). Furthermore, encapsulated preparations of fish oil with a higher amount of EPA and/or DHA are also available and it is possible to achieve intake 5 times higher (or more) than the recommended levels of EPA and DHA by taking a single fatty acid supplement per day (Calder, 2010a).

There are other sources of EPA and DHA for those who do not eat fish or are vegetarians. Enrichment of food products that are not naturally rich with n-3 (EPA and DHA) is an upcoming method to provide dietary n-3 supplementation, for example, fish oils can be added to food products such as yoghurts, milk, bread etc. In addition, farm animals can be fed with n-3 PUFA resulting in enrichment of eggs, milk, etc. with n-3 (Gibbs et al., 2010; Givens et al., 2005; Calder & Yaqoob, 2009). These enriched food products have the advantage that individuals do not need to alter their dietary pattern to increase their n-3 levels. However, the levels of enrichment may be limited in animal feeding approaches due to the metabolic processes in animals (Givens & Gibbs, 2008).

Due to increased awareness of n-3 PUFAs from fish and fish oils being an important dietary component, it is questioned whether fish-oil producers will be able to meet the future demands (Surette, 2008). Consequently, there is a potential for alternative n-3 sources which have been developed or are in progress. For example, oils naturally enriched in DHA have been extracted from cultured microorganisms like the algae *Crypthecodinium cohnii* are used in infant formulations (Fedorova-Dahms et al., 2011; Nitsan et al., 1999). Similarly, novel plants like *Echium plantagineum* have a potential for cultivation as their seed oil naturally contains stearidonic acid (SDA) which is an intermediate in the metabolism of n-3 PUFA. Though SDA is found in very small quantities in the diet, it has been shown to be metabolised more effectively than ALA, resulting in enrichment of cells and tissues with EPA and DHA (Surette, 2008). Moreover, transgenic varieties of common plants including canola, soybean and safflower are being developed that can produce seed oils enriched with SDA, EPA, and DHA (Surette, 2008; Taneja &

Singh, 2012). Thus, with the levels of current enrichment it is possible to achieve additional intake of a few hundred milligrams of n-3 per day when combinations of different enriched food products are consumed. Overall the intake of long chain n-3 PUFA is UK is still considerably below the recommended and there is a potential for dietary change (DH, 1991; Lunn & Theobalth, 2006)

1.8.2 The role of new lipid mediators (resolvins, protectins and maresins) in resolution of inflammation

In the last decade, several lipid mediators have been identified that have a potential role in the resolution of inflammation. Towards the end of an inflammatory process, there is neutralisation and elimination of pathogens, followed by removal of cellular components to prevent excessive tissue damage (Calder, 2006; Nathan & Ding, 2010). The mechanism of resolution is continuous with a decrease in the number of inflammatory cells, there is a reduction in the levels of pro- inflammatory cytokines and eicosanoids 'switch' from being inflammatory in nature (LTs, PGs etc.) to anti-inflammatory or specialized pro resolving mediators such as LXs, Rvs, PDs and maresins (Serhan, 2011, Figure 1.12). These mediators have the potential to control the duration and magnitude of inflammation (Serhan, 2008; Serhan & Petasis, 2011a).

These Rvs, PDs and maresin mediators are produced in humans from n- 3 PUFAs (EPA and DHA). The accessibility, affordability and lack of health side effects related to fish oil supplementation have generated interest in these potent mediators for research studies in humans with or without inflammatory diseases (Calderon et al., 2012). Serhan and collaborators identified, characterised and explained families of pro resolving lipid metabolites from EPA and DHA using lipidomics approach (Serhan et al., 2010, 2011; Serhan & Savill, 2005). There are two classes of resolvins, the E series derived from EPA and D series derived from DHA (figure 1.12). Although the focus of this thesis is on the mediators derived from n-3 PUFA, this figure highlights that AA derived lipid mediator called lipoxin can also exhibit inflammation-resolving effects. It has been suggested that once the inflammatory process reaches initial resolution phases, there is a 'switch' from the inflammatory nature of AA derived metabolites (LTs, PGs etc) to anti-inflammatory or specialized pro resolving lipoxins which stop leukocyte recruitment and help promote generation of lipid mediators such as Rvs and PDs (Levy et al., 2012; Serhan & Savill, 2005).

Resolution of inflammation in airway diseases involves the removal of inflammatory cells from injured tissues/wounds, which is driven by apoptosis of leukocytes and elimination from the tissues (Rossi et al., 2006; Uller et al., 2006). The LXs demonstrate their anti-inflammatory effects by reducing the formation of ROS by leukocytes, decreasing

transendothelial migration of white cells, and promoting non-phlogistic phagocytosis (Kohli & Levy, 2009). It is now evident that monocytes act in a non-phlogistic manner under the influence of anti-inflammatory metabolites such as LXs and are recruited as mature macrophages to phagocytose apoptotic cells and infectious agents (Maddox & Serhan, 1996; Serhan et al, 2011a,b). LXs also stop neutrophil infiltration and hence stop local inflammatory signals (Takano et al., 1997, 1998; Serhan, 2011). In asthmatics, there is decreased generation of LXs and this is particularly explained by the deregulated expression of LX biosynthetic genes which vary by disease severity and anatomic compartment (Planaguma et al., 2008). The only study assessing the relationship between LXs and EIB has been conducted in children (6-17 years, n=12) and it was reported that children with EIB have lower levels of lipoxin A_4 (LXA_4) than healthy controls (Tahan et al., 2008). Overall, it has been suggested that LXs are generated in airways during airway inflammation and any reduction in the generation of LXs could lead to persistent inflammation and contribute to the pathogenesis of asthma (Christie et al., 1992; Celik et al., 2007; Levy et al., 2012; Tahan et al., 2008).

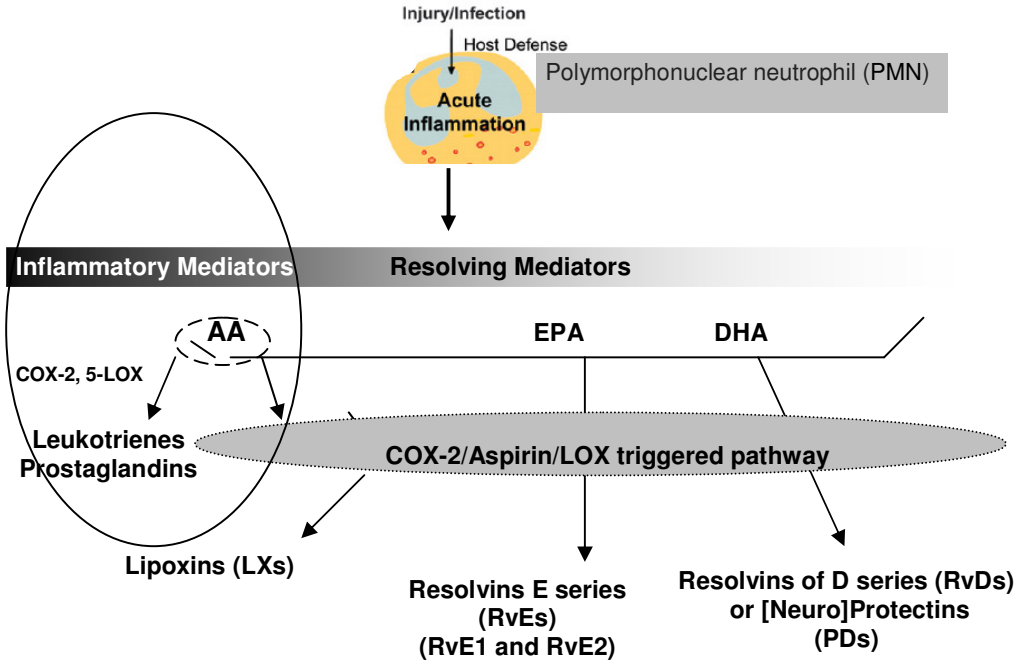


Figure 1.12 Lipid mediators derived from AA, EPA and DHA.

The DHA derived D series resolvins are involved in resolution by preventing TNF-α from making pro inflammatory cytokines which would be responsible for cascading neutrophil infiltration (Arita et al., 2005, Serhan, 2011a; Uddin & Levy, 2011; Van Dyke & Serhan, 2003). A group of D series resolvins are aspirin triggered after acylation of the COX-2 enzyme by aspirin and its interaction with DHA. It has been hypothesised that Rvs and PDs are a part of molecular mechanisms that highlight the role of aspirin in enhancing the conversion of EPA and DHA to Rvs of E and D series (Aoki et al., 2010; Ariel & Serhan, 2007;Kohli & Levy, 2009). The E series resolvins are found in two major forms – resolvin E1 (Rv E1) and resolvin E2 (Rv E2). The RvE1 has been found to exhibit its activity by responding to neutrophils. RvE1 impedes the migration of polymorphonuclear cells (PMN) to the site of inflammation, stops the PMN response to inflammatory cytokines and promotes the clearance of inflammatory cells via phagocytosis by macrophages (Levy, 2010). Furthermore, RvE1 has been found to block the synthesis of pro inflammatory cytokines and induce apoptosis and phagocytosis by up-regulation of chemokine receptor type 5 (Ariel, 2006). Fredman and co-workers have recently demonstrated regulatory functions of RvE1 by inhibition of adenosine diphosphate activation of human platelets. This highlights a new cellular mechanism of action by EPA, which leads to resolution of vascular inflammation. This may be vital for management of inflammatory disease states (Fredman et al., 2010).

RvE1 has been shown to be involved in the suppression of the production of cytokines such as IL-1, IL-2, IL-6 and TNF-α (Arita et al*.,* 2005; Hong et al*.,* 2003; Marcheselli et al*.,* 2003), as well as facilitating wound healing (Gronert*,* 2005). Recent animal models have shown that RvE1 regulates IL-23 and LXA_4 to promote resolution of allergic airway inflammation in a mouse model of asthma (Haworth et al., 2008; Hisada et al*.,* 2009). RvE1 can act along with LXs as resolution phase mediators to regulate IL-17 while only RVs have the ability to regulate IL-23 and IFN-γ levels (Haworth et al., 2008). In other animal models, it has been shown that RvE1 is highly potent when supplied intraperitoneally before and during sensitization and aeroallergen challenge phases (Aoki et al., 2008). This concept has been further investigated to confirm that administration with RvE1 in allergic asthma (murine models) can prevent the development of AHR, mucous metaplasia, eosinophil accumulation, and Th2 cytokine generation e.g. IL-13 (Aoki et al., 2010). Haworth and co-workers have reported in murine models that NK cells expresses CMKLR1 (a receptor for RvE1), and depletion of NK cells lead to a reduction in RvE1

mediated resolution of allergic inflammation. Subsequently these findings signify novel functions of NK cells in facilitating resolution of adaptive immune responses and emphasise that NK cells are possible targets for specialized resolution phase lipid mediators for clearance of activated T cells from injured or inflamed lungs (Haworth et al., 2011). While the functions of RvE1 have been investigated thoroughly, there is less information available about the specific activity of RvE2. This mediator has been reported to be produced by neutrophils and acts in similar manner as RvE1 (Calderon et al., 2012; Serhan & Petasis, 2012).Rvs have been reported to reduce leukocyte endothelial interactions and leukocyte adhesion in turn promoting the resolution of inflammation (Van Dyke & Serhan, 2003; Widgerow, 2012). The two forms of E series resolvins have been hypothesised to have separate receptors as there is an additive effect when the two types of resolvins are administered together (Levy, 2012).

PDs, maresins and D series resolvins are DHA derived lipid mediators and they function as anti-inflammatory molecules by blocking the activation and migration of neutrophils to sites of inflammation and reduce the production of pro-inflammatory cytokines (Serhan et al., 2011; Stables & Gilroy, 2011). In healthy individuals, airways and other mucosal surfaces have been found to be enriched with DHA while those individuals with asthma/cystic fibrosis have low levels of DHA (Freedman et al., 2004). There is little evidence related to the effects of D-series resolvins, maresins, and other DHA-derived mediators in asthma and only protectin D1 (PD1) has been investigated. To date no receptors for PDs have been found, although like RvE2 there is a combined effect with RvE1 suggesting distinct receptors for the two mediators (Fredman et al., 2010; Levy et al. 2012). PD1 has been reported to facilitate the expression of CCR5 ligands on neutrophils and inhibit NF-κβ induction, which prevents the migration of neutrophils (Hong et al., 2003; Serhan et al., 2006). Based on murine models of allergic asthma *in vivo*, low PD1 levels are found during asthma exacerbations and PD1 has been shown to display broncoprotective effects (Levy et al., 2007). In a direct comparison with Lx stable analogue and RvE1 in acute self limited murine peritonitis, PD1 has displayed the lowest infiltration for leukocytes and the shortest resolution time interval (time taken from maximal leukocyte infiltration to 50% of maximum) and these functions are suggestive of effective biological activity of PD1 in resolution of inflammation (Bannenberg et al., 2005).
The biological characteristics of these new anti-inflammatory and pro-resolving mediators and the pathways, which drive the formation and actions of these molecules, have

provided a new concept for treating inflammatory diseases. The majority of studies conducted have been in animal models including mice, rats and rabbits, with limited studies on humans. Most recently, in a clinical trial designed by Resolvyx, the phase 2 results show that the agonist resolvin produced dose-dependent improvement in both the signs and symptoms of dry eye in humans, and didn't show any health side effects (Brooks, 2009; Resolvyx, 2012; ClinicalTrials.gov identifier : NCT00799552). This first clinical study of the effect of resolvins in humans will help improve the understanding of the agents that can stimulate the resolution mechanisms and resolve acute inflammation along with chronic inflammation to reduce human diseases where uncontrolled inflammation forms the basis of their pathophysiology (Serhan, 2011a). Early phase trials are currently on going using natural and synthetic resolvins in various disease conditions such as asthma, inflammatory bowel disease, and other related inflammatory diseases; however, no information about the appropriate dosage of these compounds have been publicised. Based on a renal re-perfusion study with 23-28 g mice, intravenous resolvin dosage ranged from 0.01-0.1 mg/kg (Duffield et al., 2006),while in another study investigating the effect of RvE1 in the asthma mouse model, a reduction in airway mucous, AHR and leukocyte bronchoalevolar lavage was achieved with intravenous dosages of 50-200 ng/mouse (Haworth et al., 2008). Furthermore, Xu and colleagues have recently shown that a dose of only 10 ng/mouse of RvE1 and RvD1 is sufficient to reduce inflammation and pain via regulation of the central and peripheral nervous system (Xu et al., 2010).

To summarise, these lipid mediators have been identified to have potent action (in the nanomolar and picomolar range) in a variety of cell types *in vitro*, as well as in many *in vivo* models of inflammatory diseases (Serhan, 2008). These include conditions such as periodontitis colitis and intestinal inflammation (Arita et al., 2005; Gewirtz et al., 2002), asthma and airway inflammation (Levy et al., 2012), cystic fibrosis, acute lung injury (El Kebir et al., 2009) and Alzheimer's disease (Lukiw et al., 2005). Human supplementation studies are required to evaluate the action of these novel lipid mediators in other diseases including EIB. Furthermore, dose response studies are required to elucidate the most appropriate dose for the anti-inflammatory effects. Since Rvs, PDs and maresins are biological molecules derived from n-3 PUFA which are integral components of cell membrane phospholipids, these lipid mediators can play a possible role in cell signalling, which modulate more than one (and often several) tissues via pleotrophic effects. Furthermore, these characteristics distinguish the lipid mediators from industrially

produced drugs and what have been conventionally regarded as primary biological therapeutic agents, which have shown to exhibit more limited and specific effects (Serhan, 2011b). However, the major challenges that have limited the application of these new mediators are the standardisation of appropriate methods for measurement of these in a laboratory setting. Other areas of interest in recent years have been a comparison of the health benefits of EPA and DHA supplementation versus treatment with Rvs or PDs. The differences, similarity and acceptance of health benefits of dietary n-3 PUFAs and/or their mediators will be dependent on factors such as costs, safety and public health implications that are affected as a result of adopting a particular treatment approach (Calderon et al., 2012). Thus, well designed trials are required to understand the efficacy of these novel mediators in inflammation.

1.9 Summary, aims and hypothesis

Asthma is a chronic inflammatory disease that has serious implications for quality of life and public health. The standard classification guidelines for asthma are based on fixed criteria for FEV_1/FVC ratio (80% ratio) derived from predicted equations from a reference population. There are issues related to the reliability of predicted values and 80% ratio; for this reason, the classification guidelines have been questioned. A LLN derived from healthy control population is proposed for asthma classification to minimise these issues.

Clinical focus in asthma therapy has been on the severe disease state, however a large number of asthmatics have mild to moderate symptoms. Recent public health guidelines from the Department of Health (UK) acknowledge the relationship between lack of physical activity and chronic diseases including asthma and consequently, physical activity is encouraged across the population. EIB is a sub type of asthma that affects approximately 90% of asthmatics and 10% of the healthy population; thus, EIB is a limiting factor for physical activity for a large number of individuals.

Though the currently available pharmacological therapies for asthma and EIB are effective, long-term usage of these therapies are associated with issues of tachyphylaxis and health side effects. Complementary therapies are becoming gradually more popular among asthmatics for asthma management. Increasing evidence from observational and intervention studies have suggested the possible anti-inflammatory effects of fish oil on various chronic inflammatory diseases including asthma and there is, therefore, an impetus towards using fish oil as a complimentary therapy. There is no major health side effects associated with the intake of fish oil, consequently fish oil is an attractive non-pharmacological intervention which could benefit asthma. The anti-inflammatory effect of fish oil has been suggested to be linked with a change in cell membrane composition. The altered membrane composition with fish oil supplementation (primarily EPA and DHA) can modify lipid mediator generation by producing less inflammatory eicosanoids.

A newly identified group of lipid mediators produced from the oxidation of n-3 (EPA and DHA) include resolvins and protectins, which have been suggested to be key players in the resolution of inflammation. Reduced inflammation attenuates the severity of asthma

including symptoms (dyspnoea) and exerts a bronchodilatory effect. There is a paucity of data relating to fish oil supplementation and EIB with studies that have been conducted focussing primarily on athletes. To address some of the issues highlighted above and to respond to identified gaps in current literature, this thesis consists of a series of studies designed to move forward the field of asthma research. This thesis addresses issues related to disease severity, classification of EIB, the impact of EPA and DHA supplementation on post exercise pulmonary function in EIB prone males and finally the impact of resolvins on EIB prone females. The aims and hypotheses for this thesis are listed below. The details of how each of these three research hypotheses were addressed are summarised here and expanded upon in chapter 3, 4 and 5 where study findings are presented and discussed.

The first aim of this thesis was to characterise mild-moderate asthmatics based on 80% ratio and then separately characterise the same individuals using the lower limit of normality (LLN) from a control population.
Once the asthmatic population was identified, it was hypothesised that there would be a statistical difference between the dyspnoea score and circulating levels of inflammatory cytokines (IL-6 and TNF-α) of asthmatics and controls.

The second aim of this thesis was to assess the effect of EPA/DHA supplementation on post exercise FEV_1 in mild – moderate EIB prone males.
It was hypothesised that there would be a statistically significant improvement in post-exercise FEV_1 after three-weeks of EPA/DHA supplementation. To support this study EIB prone, mild – moderate asthmatic males were recruited using the previously derived LLN. To evaluate the effects of EPA/DHA supplementation on pulmonary function in EIB-prone males, dyspnoea score, and FeNO levels were simultaneously measured. Additionally, the effect of EPA/DHA supplementation was assessed on the lipid composition in PBMC. Circulating levels of IL-6, IL-10 and TNF-α were measured to evaluate the changes in cytokines with supplementation.

The third aim of this thesis was to investigate the effect of EPA-derived bioactive mediators (E series Resolvins) on post exercise FEV_1 in mild – moderate EIB-prone females.

It was hypothesised that there would be a statistically significant improvement in post-exercise FEV_1 after three-weeks of resolvin supplementation. To support this study, EIB prone mild –moderate asthmatic females were recruited using fixed cut off value of 80% for FEV_1/FVC. To evaluate the effects of resolvin supplementation, dyspnoea score and FeNO levels were simultaneously measured. Circulating levels of IL-6, IL-10 and TNF-α were measured to evaluate the changes in cytokines with supplementation.

Chapter Two
General Methods

2 General Methods

2.1 Study overview

This chapter provides a summary of the general methods used in this thesis and gives details of procedures and rationale for the methods used. The methods described are in the order they were developed, modified and applied during the study. A summary of methods and statistical analysis for each separate study are presented in the relevant chapters. Risk assessments were conducted for phlebotomy and analysis of blood products, and were annually reviewed by the departmental safety officer. For all the chemicals used in the testing, relevant Control of Substances Hazardous to Health forms were completed and reviewed by the laboratory manager. Standard operating procedures were followed for the existing lab protocols, and relevant instruction manuals were followed when using commercial kits. When conducting tests with participants, a safe system of work was maintained taking particular note of requirements for protective clothing and equipment and appropriate ventilation.

2.2 Ethical approval

The study protocols were approved by the Loughborough University Ethical Advisory Committee. Generic protocols were used for venepuncture (G03-P8), and specific protocols were used for exercise testing (GO9-P8) and Omega 3 supplementation (GO9-P7).

2.3 Participant recruitment

Participants were recruited from a population of Loughborough University's students and residents of Leicestershire, UK with the help of student support groups, advertisements and talks (see appendix 2). There was no financial incentive offered and full written consent was obtained from all participants. The participants completed standard health screen questionnaires and other related procedures (appendix 2). Male and female participants aged 18-30 years were recruited as per the requirements of individual studies, and the details are provided in respective chapters.

2.4 Venous blood sampling

A certified phlebotomist following standard health and safety procedures outlined by the Loughborough University collected venous blood. Blood for PBMC analysis was collected in an anti-coagulant treated tube (K-EDTA), and coagulated blood was collected for serum separation for ELISA. A NVQ in Phlebotomy was obtained during the course of this thesis and the course involved two day training followed by a day of supervised practical session; the certificate is attached in appendix 2. The research design for the studies conducted in this thesis was such that the blood samples were collected at the same time of the day at each collection point (±30 minutes), and the sample analysis was conducted within 2 hours of collection.

2.5 Physiological measurements

2.5.1 Pulmonary Function Tests (PFTs)

Spirometry is a physiological test that measures how an individual inhales or exhales volumes of air as a function of time. Volume or flow may be the main signal measurement in spirometry. This is invaluable as a screening test of general respiratory health in the same way that blood pressure provides important information about general cardiovascular health. In clinical practices, spiormetry may be supported by other tests such as symptom score, FeNO measurements and allergy tests for aetiological diagnosis. Spirometry was performed on all participants in a sitting position using a calibrated computerised spirometer (Superspiro; Micro Medical; Rochester, Kent, UK). These spirometers are gold standard devices based on the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines. The entire protocol for PFTs was performed as per the guidelines (Miller et al., 2005a,b). The main quality control and testing parameters are reported below.

The spirometer was evaluated for calibration on a monthly basis by a laboratory technician using a mechanical syringe to test the range of exhalations that are likely to be produced by the test population. Quality control was maintained in the laboratory by recording and maintaining the following: 1) a log of calibration results; 2) the documentation of repairs and other alterations which returned the spirometer to original configuration; 3) computer

hardware and software changes; 4) if the spirometer was relocated (taken on field trips etc). Calibration checks were performed before further tests began. The spirometer specification was maintained as per the ATS/ERS guidelines and was capable of accumulating volume for >15 s (longer times are recommended) and measuring volumes of ≥8 L with an accuracy of at least ±3% of the reading or ±0.05 L, whichever was greater.

FEV_1 and FVC manoeuvres are the two main aspects of PFTs used in the assessment of respiratory disorders. FVC is the maximal force of air exhaled with maximally forced effort from maximal inspiration (vital capacity) and is expressed in litres at body temperature and ambient pressure. FEV_1 is the maximal volume of air exhaled in the first second of a forced expiration and is expressed in litres. There are three distinct phases to the FVC manoeuvre, which are as follows: 1) maximal inspiration; 2) a ‘‘blast’’ of exhalation; and 3) continued complete exhalation to the end of the test. To ensure the accuracy of the test, the participants were instructed to inhale rapidly and completely, the breathing tube (mouthpiece) was inserted into the participant’s mouth and it was ensured that the lips were sealed around the mouthpiece. The FVC manoeuvre started with no hesitation, the participants were prompted to ‘blast’ and not just ‘blow’ the air from their lungs, and then he/she was encouraged to fully exhale. As per the guidelines, enthusiastic coaching of participants was done to ensure that the participant provided the maximum effort. If the participants felt ‘dizzy’ during the manoeuvre, the procedure was stopped and testing was scheduled for a different day. Loss of consciousness could follow this dizziness due to prolonged interruption of venous return to the thorax; subsequently the participants were closely monitored during the manoeuvre. Participants were required to perform three acceptable FVC manoeuvres based on the ATS/ERS recommendation of ≤5% variation in between-manoeuvre recoded values (Miller et al., 2005a,b). By the end of a typical manoeuvre, a flow volume curve is generated which is used in the interpretation of a PFT. The main parameters tested were FEV_1, FVC, PEF and FEF 25-75%. Curves depicting the parameters tested in lung function tests are shown in figure 2.1, and flow volume loops in the lung function of asthmatics compared to normal individuals are shown in figure 2.2.

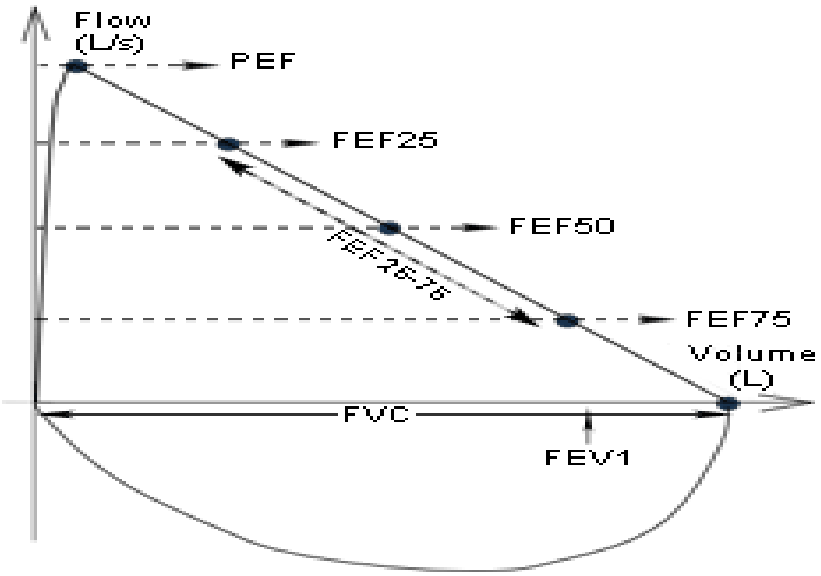
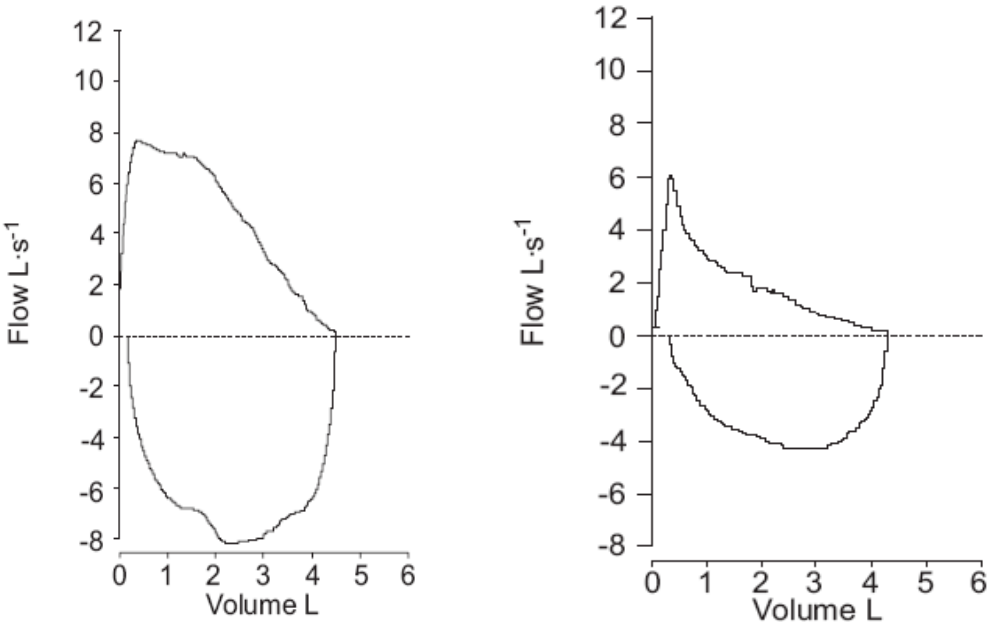


Figure 2.1 A flow volume loop showing the parameters tested during a pulmonary function test. PEF = Peak Expiratory Flow, FEV1 = Forced expiratory volume in 1 second, FVC = forced vital capacity, FEF 25-75% = forced expiratory flow between 25% and 75% of the FVC (FEF 25–75%) has also been known as the maximum mid-expiratory flow (adapted from Miller et al., 2005a)



2.2. (A) 2.2. (B)

Figure 2.2 (A) Flow–volume loop of a normal subject (B) Moderate airflow limitation in a subject with asthma (adapted from Miller et al., 2005a)

2.5.2 Exercise challenge testing

Exercise testing was performed based on the ATS/ACCP (American College of Chest Physicians) statement on cardiopulmonary exercise testing (2003) and Rundell & Sue-Chu, 2010. The purpose of the exercise challenge test was to assess EIB symptoms in adults with stable asthma.

Participant preparation

Participants were requested to avoid alcohol, coffee (for 8hrs) and strenuous physical exertion (for 24 hours) before the exercise challenge, and medication usage was reported before testing. Caffeine is classified as a methylxanthine drug (like theophylline, a long acting bronchodilator) which has been shown to improve pulmonary function for up to four hours (Welsh et al., 2010). Furthermore, moderate alcohol consumption is also found to exhibit anti-inflammatory effects, improved mucociliary clearance, direct bronchodilator, and antioxidant effects (Sisson et al., 2007; Stanton et al, 2010). In addition, respiratory muscle training and physical activity could improve lung function (Emtner et al., 1996; Sutbeyaz et al., 2010). Inclusion criteria for participants for each study are reported in respective chapters.

Pulmonary function tests (PFTs)

PFT procedures were followed as described in the section above. Spirometry was performed at baseline prior to the exercise challenge test. For the exercise challenge test the participant exercised on a motorised treadmill at increasing intensities until they attained a level that elicited a heart rate response of 85-90% of maximal heart rate in the last 4-6 minutes of the session. Target heart rate was elicited from a calculated estimate of maximal heart rate (Gellish et al., 2007). The desired work intensity was achieved by increasing the incline while running at a constant speed within the first two minutes of the challenge. The relative humidity of the room was constant (approximately 50%), as was the room temperature (18-22℃). During the exercise test, heart rate was continuously monitored by a polar heart rate monitor.

Spirometry was performed at 0, 5, 10, 15, 30 and 45 minutes post-exercise or until complete recovery; whichever was earlier. At each time point three repeatable FEV_1 values were measured and the greater of the two was recorded. The participants were not released from the lab until the FEV_1 had returned to 95% of the baseline value. If a participant shows a decrease of >50% in FEV_1, the administration of short acting ß2 agonists (bronchodilator) to reverse the response is recommended, and participants were instructed to bring their bronchodilator during testing to ensure medication was available if required. However, during the study there were no instances where administration of medication post-exercise was necessary.

Post exercise FEV_1 values were compared to pre-exercise baseline values and percentage change was calculated using the following equation. A ≥10% fall in FEV_1 at any time point after exercise is considered diagnostic for EIB (Haby et al., 1994; Sterk et al., 1993), and this criteria was used during the testing.

$$\frac{\text{Pre-exercise } FEV_1 - \text{post-exercise } FEV_1}{\text{Pre-exercise } FEV_1} \quad X\ 100$$

.....equation 1

2.5.3 Fraction of exhaled breath nitric oxide (FeNO) measurement

Airway inflammation is a central characteristic of asthma and related lung diseases. As a consequence, monitoring of airway inflammation using FeNO measurements is becoming popular as an adjunct test to routine spirometry. Invasive techniques like bronchoscopy with lavage and biopsy, or the analysis of induced sputum can be used for direct sampling of airway cell and inflammatory mediators. Exhaled breath nitric oxide measurement is a suitable non-invasive measurement technique ideal for repeated monitoring of patient groups. ATS have highlighted FeNO measurement as progressing from being a research tool to having a clinical application in asthma and suggest that it may be used as an additional test in asthma diagnosis (ATS/ERS, 2005; Dweik et al., 2011). Online measurement of exhaled breath was conducted using the portable NIOX MINO analyser (NIOX MINO, Product code 03-1000). The NIOX MINO is a newer, smaller, hand held

portable device for the measurement of airway inflammation based on the established NIOX monitoring system, which detects and measures levels of exhaled nitric oxide. The NIOX system uses a chemiluminescence gas analyser, which can measure low concentrations of NO while the NIOX MINO uses electrochemical sensor technology. It has been suggested that there is a good agreement between the FeNO values measured with the stationary chemiluminescence gas analysers and hand held NIOX MINO (Kim et al., 2012). The built in controls of the NIOX MINO provide reliable values as per the ATS/ERS guidelines.

Ambient NO levels may affect a single breath analysis of FeNO, so the use of NO free air (containing <5ppb) for inhalation is preferred (Silkoff et al., 1997). The quality controls system of the NIOX MINO provided filtered air to eliminate any influence from ambient NO. The participant sat on a chair in an upright position, wearing a nose clip to minimise any nasal NO inhalation. The participants inserted a mouthpiece and inhaled over 2-3 seconds through the month to total lung capacity (TLC) or near TLC if TLC was difficult, and exhaled through a mouthpiece that was connected to the analyser. Exhalation was immediate as breath holding may affect FeNO levels. Inhaling to TLC is recommended as this is a constant point in the respiratory cycle and most asthmatics accustomed to spirometry are familiar with inhaling this volume.

The other two critical factors to ensure that reproducible and standardised measurements of lower respiratory tract FeNO are achieved are that there must be exclusion of nasal NO and standardisation of flow rate. The exclusion of nasal NO is important in view of high nasal NO levels relative to lower respiratory tract. FENO values vary considerably with exhalation flow rate because of variation of airway NO diffusion with transit time in the airway, thus standardisation of the exhalation flow rate is required. The NIOX MINO flow control system maintained an exhalation rate of 0.005 L/second regardless of the patient's skill. A visual display guided the patient to maintain an appropriate pressure range while exhaling. The results for FeNO are expressed in parts per billion. Three acceptable manoeuvres based on the ATS/ERS recommendations were carried out as per the (ATS, 2005). Quality control procedures were completed as per the manufactures guidelines.

2.5.4 Dyspnoea

Dyspnoea is a subjective experience of breathing discomfort that consists of qualitatively distinct sensations that vary in intensity. The experience derives from interactions among multiple physiological, psychological, social, and environmental factors, and may induce secondary physiological and behavioural responses (ATS/ERS, 1999). In asthma, the assessment of dyspnoea is an integral part of assessment and management of the disease. The severity of dyspnoea and the qualitative aspects of unpleasant breathing experiences differ widely among patients. Physical exercise can potentially act as a stimulus for both, physiological and perceptual responses. The significance of dyspnoea as a clinical marker of asthma is inversely related to the intensity of exercise provoking the symptom; subsequently dyspnoea at rest is considered more severe than dyspnoea during intense exercise. In the 1970s, Borg first described a scale ranging from 6-20 to measure perceived exertion during exercise (Borg, 1970) and this scale has been modified to a 10 point scale with verbal expressions of severity anchored to specific numbers (Borg, 1976; 1982). The reliability and validity for Borg ratings of breathlessness have been widely accepted (LeBlanc et al., 1986; Muza et al., 1990; Silverman et al., 1988; Skiner et al., 1973). For this study, a standard Borg scale for breathlessness was used (chapter 3, 4 and 5) and care was taken to provide consistent, specific instructions when using the scale.

2.6 Biochemical methods

2.6.1 Estimation of levels of omega 3 and omega 6 in PBMC

The method employed here involved isolation of PBMC followed by extraction of the lipid and esterification of the fatty acids. Quantification of the n-3 and n-6 was performed by gas chromatography-flame ionisation detection (GC-FID). Specifically, this method required isolation of PBMC from whole blood followed by fatty acid transesterification to produce volatile fatty acid methyl esters (FAME), which could be identified and quantified by GC-FID. The protocol for esterification and GC-FID was modified from Masood et al., 2005 and was validated to ensure that the method was appropriate to meet the requirements of the study. The original fatty acids of interest were ALA, LA, EPA, DHA and AA only. In the literature, DPA is considered to be an important fatty acid but was not specifically

identifiable using the modified method. Caution is warranted while interpreting the findings as only 70% of the peaks were identified using the modified method.

2.6.2 Specimen collection and handling

Fresh blood was collected and processed within 2 hours of collection as stipulated in research protocol (reported on page 72) in order to extract PBMC. PBMC are a group of circulating mononuclear cells and typically constitute 85-90% lymphocytes and 10-15% monocytes (Calder et al., 2002). Different subsets of monocytes and lymphocytes show significant diurnal variations with higher counts in the evening compared to the morning (Shantsila et al., 2012; Watson et al., 2010). In addition, a delay of greater than 2-4 hours in sample analysis has been shown to increase the number of mononuclear cell subsets significantly (Shantsila et al., 2012). The research design for the studies conducted in the thesis was such that the blood samples were collected at the same time of the day at each collection point (±30 minutes), and the sample analysis was conducted within 2 hours of collection. Furthermore, monocyte numbers for different subsets such as Mon1 ($CD14^{++}$, $CD16^{-}$ and $CCR2^{+}$), increase after exercise followed by a significant reduction after 1 hour (Scharhag et al., 2005; Smith et al., 1998; Shantsila et al., 2012). Blood was collected before exercise from each participant for this study. PBMC were not counted; and the results for n-3 and n-6 fatty acids were expressed as percentages.

2.6.3 Isolation of PBMC

The Ficoll Paque PREMIUM (Catalogue number 28-4039-56 AA, GE Healthcare, UK) method was used for isolation of PBMC in this study. This method involves layering of anticoagulant-treated blood on the Ficoll-Paque PREMIUM solution, followed by centrifugation for a short period of time (30-40 minutes) (Figure 2.3, A). Differential migration during centrifugation results in the formation of layers containing different cell types. The lowermost layer comprises the RBCs that have been aggregated by the Ficoll, and therefore sediment completely in the Ficoll-Paque PREMIUM layer. The layer immediately above the erythrocyte layer contains granulocytes, which at the osmotic pressure of the Ficoll-Paque PREMIUM solution attain a density great enough to migrate through the Ficoll-Paque PREMIUM layer. The mononuclear cells are found at the interface between the plasma and the Ficoll-Paque PREMIUM layer (Figure 2.3, B). The

other slowly sedimenting particles that are found at the interface are washed in a balanced salt solution removing platelets, density gradient medium, and plasma leaving a pellet of mononuclear cells. A balanced salt solution was prepared for washing of PBMC, made from two stock solutions (appendix 1)

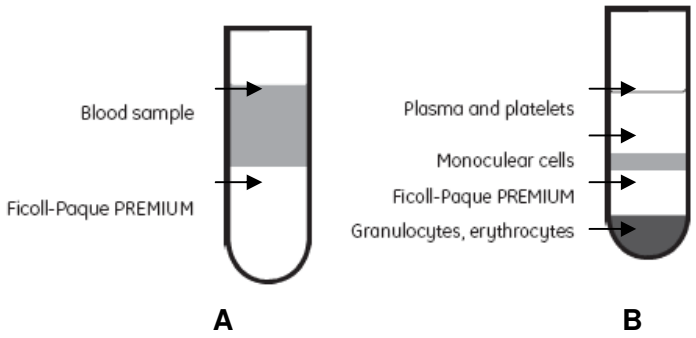


Figure 2.3 (A) Layering the diluted blood sample on Ficoll-Paque PREMIUM, (B) After centrifugation, the phase separation.

2.6.4 Procedure for isolation of mononuclear cells

The Ficoll-Paque PREMIUM was shaken several times to ensure thorough mixing. Ficoll (13.5 mL) was added to a 50ml centrifuge tube. In a separate 50mL centrifuge tube, 9 mL of anticoagulant-treated blood (K-EDTA) was added to an equal volume of balanced salt solution to make a final volume of 18 mL. The blood was mixed gently and thoroughly using a Pasteur pipette. Care was taken while mixing to ensure minimal damage to the blood cells. The diluted blood samples (18 mL) were slowly layered on to the Ficoll-Paque. It is important to assure when layering the sample not to mix Ficoll-Paque and the diluted blood sample. The mixture was centrifuged at 400 x g for 30 min at 18–20℃. Following centrifugation, the upper layer containing plasma and platelets was extracted using a sterile Pasteur pipette, leaving the layer of mononuclear cells undisturbed at the interface. There was a very fine layer of mononuclear cells obtained from 9 mL of blood and the extraction step required careful handling of the test tube. The technique required 3-5 minutes, and a micropipette was used to extract mononuclear cells with precision to avoid any mixing with plasma and the Ficoll layer.

Washing the mononuclear cells to remove platelets

Using a sterile Pasteur pipette the layer of mononuclear cells was transferred to a sterile centrifuge tube. Attention was paid to remove the entire interface except for a small amount of Ficoll-Paque PREMIUM and supernatant. Removing excess Ficoll-Paque PREMIUM causes granulocyte contamination, removing excess supernatant results in unnecessary contamination by platelets and plasma proteins. This step was optimised by extracting 1-3 mL of the mononuclear cell layer containing Ficoll-Paque, and finally 1-1.5 mL of mononuclear was considered optimum for washing.

Wash 1: At least three volumes (27 mL) of balanced salt solution was added to the mononuclear cells in the centrifuge tube. The cells were suspended by gently drawing them in and out of a Pasteur pipette. After centrifugation at 100 x g for 10 min at 18–20°C, the supernatant was removed.

Wash 2: The mononuclear cells were re-suspended in ~27 ml balanced salt solution by gently drawing them in and out of the Pasteur pipette. This second density centrifugation step was important to remove any red blood cell contamination. After centrifugation at 60 – 100 × g for 10 min at 18–20°C, the supernatant was removed. The extracted mononuclear cells settled at the bottom of the centrifuge tube were suspended in the 1:1 phosphate buffer saline (PBS). Two aliquots were made depending on cell recovery, one aliquot with 1.1 dilutions and one with no dilution (cells only). Any excess buffer was evaporated under nitrogen and stored at -20ºC until further analysis.

2.6.5 One step fatty acid esterification

After isolating the PBMC, the next step was to extract the total cell fatty acids from the PBMC and derivatise them to volatile methyl esters that could then be identified by GC based upon their molecular size. Fatty acids in their free, underivatised form may be difficult to analyse because these highly polar compounds tend to form hydrogen bonds, leading to adsorption issues. Reducing their polarity makes them more amenable to analysis. In addition, derivatisation of fatty acids to volatile esters is helpful to distinguish between the very slight structural differences exhibited by unsaturated fatty acids (Gunstone, 1993; Knapp, 1979).

For the extraction of fatty acids from cells and tissues, most methods are based on the principals and techniques used by Folch (1959) or Bligh & Dyer (1959), although there is no one particular method which can be accepted as the most appropriate method for fatty acid extraction. The choice of the method is dependent on the type of fatty acids to be analysed, and whichever method is adopted, validation is required to determine its suitability for the intended use. These methods have been widely applied and used in the preparation of FAMEs for a range of laboratory analyses using different types of cells (Damsgaard et al., 2008; Garcia-Larsen., 2011; Healy et al., 2000; Lee et al., 1985; Mickleborough et al., 2006; Rees et al., 2006). There are standard methods for derivatisation of lipids from cells and tissues in a quantitative manner (Blau & Halket, 1993; Christie, 2003), and the most widely used methods are acid catalysed esterification or transesterification (& Roy, 1986; Morrison & Smith, 1964). Masood et al. (2005) have proposed a modified method for measurement of fatty acids in plasma samples. This method is derived from Lepage & Roy (1986) and had the advantage over the original of being faster, more efficient and cost effective for plasma samples. In addition, the Masood et al., (2005) method does not require the use of any toxic chemicals, which were previously necessary in the Lepage & Roy (1986) protocol. For this thesis, the Masood et al., (2005) method was further adapted for PBMC analysis and subsequently optimised and validated to assess its suitability for the current study.

Masood et al. (2005) method adaptation:

A simplified, one-step transesterification reaction proposed by Masood et al. (2005) required the same stock solutions that were conventionally required by the Lepage and Roy (1986) method. The 1.9 ml of stock solution required for each sample included 1.7 ml of methanol, 100µl of acetyl chloride, and 100 µl of the internal standard solution (containing 10µg of 23:0 methyl ester). Briefly, 50µl of PBMC and 1.9 ml of the stock solution were combined in screw-capped glass tubes. The tubes were capped tightly and heated at 100ºC for 60 min. The tubes were cooled down to room temperature. Hexane (0.75 ml) was added, and the tubes were vortexed for about 30 seconds. The upper organic phase was collected, and re-extraction with hexane was done for the lower phase to enhance lipid extraction. The combined hexane solution was evaporated under nitrogen

to dryness, and the dry residue was then re-dissolved in 60µl of hexane. A maximum of 1µl of this sample was injected into GC

The modified method developed for this thesis had limited level of toxicity due to the use of non-toxic reagents. Condensation lids were used with a constant supply of water to prevent any evaporation of esters. The Masood et al. (2005) method was scaled up to esterify 100µl of PBMC samples. For this, a stock solution of 3.6 ml was made up comprising of 200µl of acetyl chloride that was added to 3.4 ml of methanol. PMBC (100µl) was added to the 3.6 ml of the stock solution in screw-capped glass tubes. The tube was capped and heated at 100 ℃ for 60 min. After the methylation, the tube was cooled on ice. Analytical grade n-Hexane (1.5 ml) was added to the tube and vortxed for 30 seconds. The upper organic phase was collected with a Pasteur pipette. This extraction procedure was repeated to maximise lipid yield. The hexane phases were combined for absolute recovery, and finally nitrogen gas was passed through to reduce the volume (~20-30µl).

Cell counting was not carried out to estimate the concentration of the extracted PBMC. This was considered as not required because only the percentages of each fatty acid relative to all fatty acids in a chromatogram were required for estimation of the ratio of fatty acids of interest. This provides only an estimate of the percentage of each fatty acid present in the PBMC and does not provide the concentration or quantification of the cells. To achieve quantification, the cells are required to be counted using an appropriate technique (e.g. hemocytometer). This could be considered for future studies. Furthermore, no internal standard was used, as relative percentages of different fatty acids were required for the analysis. In the absence of an internal standard, relative quantification was used in GC analysis by the use of an external 37-component FAME mix (Supelco, catalogue number 47885-U). The modified method was fast and efficient requiring less than 4 hours for isolating PBMC samples followed by esterification and analysis by GC-FID. The method was also relatively cheaper than other methods published in the literature for isolation and analysis of PBMC. The cost per sample for processing was less than £4.00 including all reagents and materials required for isolation and analysis of PBMC using GC-FID.

2.6.6 Gas chromatography-flame ionization detection (GC-FID)

After the esterification, the samples (1µl) were loaded into a GC-FID (HP 5890 Series) for analysis. The samples were run on an HP-5 column 30 m, 0.32 mm, 0.10 µm (Agilent, catalogue number 19091J-313) followed by FID analysis. Helium was initially used as a carrier gas, and later on replaced by nitrogen to meet the requirements of the laboratories. A comparison was undertaken to assess the efficiency of the two carrier gases, and it was found that the resolution of fatty acids was similar irrespective of the carrier gas; the results will be shown in the validation section of this chapter. There was 5% variation in resolution with nitrogen compared to helium; however, the overall CV% was less than 10% for all identified peaks. The carrier gas was set at 80 bars and hydrogen was used as the make-up gas. The split ratio was adjusted to 15:1 to prevent overloading of the column. A temperature programme was used with a starting temperature of 160°C and a ramp of 10°C/minute to achieve a final temperature of 270°C in 11 minutes. The run was held at 270°C for the following 4-5 minutes. The total run time was up to 18 minutes; in addition, the purge valve was switched off for 0.3 min in the beginning to ensure most of the FAME sample was put on the column. This method allowed identification of approximately 80% of the total fatty acids in the FAME and this included the n-3 and n-6 PUFAs of interest for analysis in this thesis. A range of different injection techniques and syringes were tested to obtain quantifiable sharp peaks. Finally, a 1 cm needle was used with up to 1µl sample injection. This standardisation was important for accurate measurement of the area under the curve, which would affect resolution and henceforth influence identification of the peaks.

2.6.7 Result interpretation

Each sample, when run on a GC, results in individual peaks corresponding to fatty acids (as FAMEs), as shown in figures 2.4-2.10. Each peak is characteristic and unique for each fatty acid and these peaks appear according to the order in which the FAMEs are detected by the FID of GC. The time required by each FAME to appear on the chromatogram (retention time) is equal to the time each fatty acid is retained inside the column, and is characteristic of each fatty acid. FAMEs with shorter chains and fewer or no double bonds have a shorter retention time than the fatty acids with a longer hydrocarbon chain; consequently, they appear first on the chromatogram. The identification of specific fatty

acids is done by comparison of the retention times of FAMEs within a known standard mix of fatty acids.

A standard calibration GC-FID graph was generated with the 37-component FAME mix (Supelco, catalogue number 47885-U). There was no information from the manufacturer (Supleco) about the resolution of 37-component FAME mix on an HP-column. The manufacturer of the standard mix has however reported their resolution on a fused silica column 'equity -1' (15m x 0.10mm, d_f 0.10mm, catalogue number 28039-U, Sigma). The properties of the equity -1 column are comparable to that of HP-5 as per the manufactures guidelines. Both the columns support temperatures as high as 250-270 ℃ and have a bonded matrix of poly methyl siloxane phase. The two columns have been suggested for use as multi-purpose columns that can be utilised for FAME analysis. The main difference between the two columns was the column length, compared to equity-1, HP-5 is a longer column (30m x 0.32mm, d_f 0.25mm), which suggests that there would be a better efficiency and resolution of samples on HP-5. Additionally, there could be a longer retention time for the components tested on HP-5. Thus, the resolution of 37-component mix was compared to that of HP-5 and subsequently all the FAMEs were identified. To accurately identify the order of elution of the fatty acids on HP-5, standard FAMEs for pure DHA, LA and ALA were tested and subsequently their retention times were used for reference. Neat FAMEs for pure DHA and LA were also spiked on the 37-component standard mix to assess the accuracy of resolution of these FAMEs. The retention times were comparable when neat FAMEs were run and when these FAMEs were spiked on the 37-component FAME mix. There were up to ± 0.05 minute differences in the resolution of FAMEs between the two runs, which were considered acceptable for analysis.

After the identification was complete, adjustments were made to remove solvent peaks for analysis. The area under each peak was calculated and the proportion of each peak area to the sum of the area of all peaks was calculated. This proportion represented the percentage of each fatty acid in the total fatty acids analysed (total PBMC lipid content). The data was analysed by the Clarity Lite software (Clarity v.2.4.1.77, DataApex Ltd, 2005). The area under the curve was measured for the samples and standard, and percentage fatty acid content for each of the fatty acids of interest were recorded from the graph and analysed in MS Excel. Docosapentanoic acid (DPA) was not one of the fatty acids of focus in the original research design; the fact that it was not present in the 37-

component FAME mix should not impact the current analysis. However, it would be possible in a follow up study to specifically focus on DPA and identify it in subsequent samples.

2.6.8 Validation of the modified method

The esterification and GC-FID method reported in this thesis is modified from Masood et al., (2005). A validation was required after the modification to the existing method to confirm the performance specifications when compared to the original method. In addition, the validation aimed to evaluate if the method was suitable for use in routine analysis.. Parameters such as accuracy, precision, reportable range, linearity, interference, recovery and analytical specificity should be assessed. There were limitations as to what could be achieved as part of the validation due to constraints on available materials and costs. Justification has been provided in the following section to support decisions not to undertake a particular validation experiment. A 37-component FAME mix obtained from Sigma Aaldrich (ISO 9001: 2008 registration) was used as a reference standard and a measure of quality control during the experiments. All samples were analysed in duplicate to control for intra-assay variations.

Validation parameters

The following parameters were determined for the original [Masood et al., (2005)] and the modified method.

Precision and allowable error based on biological variation

Precision of a method is the degree of agreement among the individual test results, when the procedure is applied repeatedly to multiple samplings of a homogenous sample. Within assay and between assay precision was determined by analysis of replicates of the 37-component FAME mix within a day and between two different days. Mean standard deviation and the coefficient of variation (CV) were recorded for each test. There may be error due to both the imprecision and inaccuracy of the method, therefore the combination of the two errors, or the total analytical error (TEa), determines the quality of the test result (Westgard, 2004). The TEa may be derived from biological variation or derived from manufacturers' specifications. For analytical techniques a mean CV is routinely set at 20%

to account for TEa (NIH, 2010). Since there was no manufacturers' guidance about TEa for FAMEs derived from PBMC on HP-5 column, an acceptable CV of 20% was set as standard to allow for imprecision, bias, manual error and total error of the tests. This CV was set as the highest error limit for each assay, which reflected the realistic performance expectations of the modified method based on the biological variation.

Accuracy and linearity

Accuracy is the measure of exactness of an analytical method or the closeness of agreement between the measured value and the value that is accepted as a conventional or accepted reference value. The determination of accuracy requires an accepted method compared against the modified method. For this purpose the modified method was compared to Masood et al. (2005) method. A range of standards including the 37-component FAME mix and in house esterified reference oil standards (fish, sunflower and olive oil) were analysed using GC-FID. The test material (PBMC) was analysed using the two GC-FID methods. Additionally, plasma and red blood cells (RBCs) were analysed for comparison. The components of the capsules used in the intervention study (fish oil: Biocare and placebo) were also esterified and analysed using the two methods. Linearity of a method is its ability, within a given range, to obtain results that are directly proportional to the concentration of an analyte in the sample. Linearity describes the degree of the linear relationship between the test results and the concentration. Since the 37-component FAME mix was a complex mixture of different FAMEs it was not practical to efficiently assess the linearity of the experiment. Henceforth linearity was not assessed during this validation.

Interference (specificity)

The interference of a method is defined as the ability of the method to measure the analyte of interest to the exclusion of other components. The aim of this study was to determine the relative percentages of omega 3 and omega 6 PUFAs of interest (AA, EPA and DHA). A temperature program was used for the GC-FID as described in the previous section 2.6.6 which allowed better resolution of the PUFAs of interest. Only those peaks were identified in samples which corresponded to the ones with the same retention time in the 37 component FAME mix as standard. FAMEs derived from PBMC showed some

unidentified peaks as they did not correspond to any of the FAMES in the standard and these peaks were subsequently excluded from analysis.

Validation results

Composition of 37 component FAME mix (standard)

Using the original Masood et al. (2005) method and the modified method on a 37 component FAME mix, up to approximately 70% of peaks were identified in the GC-FID chromatographs respectively. For the modified method there was difficulty in the resolution of the low molecular weight fatty acids due to the nature of the GC-FID programme. The programme was targeted to resolve high molecular weight FAMEs of interest and this could be considered a major technical limitation due to the parameter of GC-FID programme and column used. In future studies, this significant limitation may be resolved with the use of different analytical application in order to resolve more fatty acids. Table 2.1 shows percent of fatty acids in the 37 component FAME mix relative to all fatty acids in the chromatogram based on the manufacturers guidance. A total of 30 FAMEs out of the 37 FAMEs listed by the manufacturer were identified using the modified method and the area covered by these peaks was estimated to be 70% of the total peak area of the GC trace. The within batch precision calculated by observing the mean CV% of all the FAMEs identified on the same day was <5% run on the same day (in five different runs). A between batch precision was calculated by measuring CV% over two different days was less than 20%. Lists of important fatty acids based on their degree of saturation were identified with their mean values and CV% listed in table 2.1. Intra assay CV% for saturated, monounsaturated, and polyunsaturated FAMEs using the modified method were calculated as 10%, 5% and 6% respectively. The mean CV% of total n-3 FAMEs (calculated as a sum of the mean CV% of ALA, EPA and DHA) was 10% and mean CV% for total n-6s (calculated as a sum of the mean CV% of LA and AA) was 7%. The mean CV% of all fatty acids using the Masood et al. (2005) method was less than 10% which is approximately 5% higher than the values reported by Masood et al.,. The inter-assay CV% for all fatty acids identified using the two methods was <20%. The GC-FID graphs showing the fatty acid profiles from the 37 component FAME mix are displayed in figures 2.4, 2.5 and 2.6. The two methods (modified and Masood et al., (2005) method) were directly compared using two sample paired t-tests ($p \leq 0.05$).

Since the manufacturers did not provide any standard reference graph or retention time for the 37-component standard mix on an HP-5 column it was important to check the elution order for the FAMEs. The graph was compared to the output from an equity 1 column (catalogue number 28039-U Sigma) with a similar specification. To assess the accuracy of elution of FAMEs, neat DHA (all-cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester, catalogue number 47570-U, Supelco) was spiked on to the 37 component FAME mix. The 37-component FAME mix contained DHA at 2% by weight and the 1:1 volume/volume spiking enhanced the peak. The neat DHA FAME was analysed by GC-FID separately and the retention time was comparable to the retention time for DHA in the 37-component FAME mix. There was less than 1% CV in the retention times between two runs completed on the same day or different days. Thus, neat DHA FAME served as a standard reference material for determining the accuracy of the modified method.

Table 2.1. Percentage fatty acid composition in a 37 component FAME mix using the two methods, mean % shows the% of each fatty acid in the 37 component FAME mix relative to total fatty acid content. SFA= saturated fatty acids, MUFA= mono unsaturated fatty acids and PUFA=polyunsaturated fatty acids

	Manufacturers guidance (% by weight)	Percentage (%) of fatty acids in a 37 component FAME mix relative to all fatty acids in a chromatogram			
Fatty Acid		**Modified method**		**Masood et al. (2005) method**	
SFA		**Mean%**	**CV%**	**Mean%**	**CV%**
Lauric acid	4	3.8	9.1	3.3	5.1
Myristic acid	4	4.6	17.1	4.1	8.7
Pentadecanoic acid	2	2.7	23.1	2.4	17.4
Palmitic acid	6	6.7	2.1	7.0	9.8
Heptadecanoic acid	2	3.4	0.9	2.5	3.8
Stearic acid	4	3.8	7.4	5.1	0.8
MUFA					
Palmitoleic acid	2	3.1	7.1	2.0	11.4
Oleic acid	4	3.9	5.3	5.4	9.2
PUFA					
Linoleic Acid (LA)	2	2.7	12.8	1.9	14.4
Alpha - linoleic Acid (ALA)	2	1.7	20.2	3.4	0.29
Arachidonic acid (AA)	2	3.4	6.5	2.1	7.3
Eicosapentaenoic Acid (EPA)	2	2.2	9.4	2.4	10.1
Docosahexaenoic Acid (DHA)	2	1.9	3.6	1.81	7.3
Mean ± SD		**3.4 ± 1.30**		**3.3 ± 1.61**	

There were no differences between the means for the two methods when analysed using paired t-test (p>0.05).

Comparison of standard reference oils (olive oil, sunflower oil and fish oil) with manufacturers specifications.

A range of standard reference oils including olive, sunflower and fish oil were esterified and analysed by GC-FID using both methods, the modified and the Masood et al. (2005) method. Olive oil and sunflower oil were obtained from the supermarket, and compared against compositions specified by Sigma Aldrich (specified below) for pure olive oil and sunflower oil respectively. This may not provide an exact comparison owing to the purity of the oils; however, the basic composition of the oil in terms of the main components should be comparable. In addition, the composition mentioned by Sigma Aldrich is for neat oil, and there could have been a particle loss of components during the esterification process.

Hence, considering biological variation, a CV% of up to 20% of the basic composition for olive oil and sunflower oil when compared to Sigma Aldrich values was considered acceptable for this experiment. The esterified standard reference oil FAMEs were diluted in n-hexane (1/10) prior to GC-FID analysis.

Fish oil

Menhaden fish oil (catalogue number: 47116 Sigma Aldrich) was used for this experiment. The fish oil was esterified and analysed by GC-FID using both methods. The GC-FID traces were compared to the manufacturer's specifications. Only 80% of peaks were reported by the manufacturer, and remaining 20% were suggested to be derived from unidentified n-3 fatty acids. The modified method identified a majority of the peaks that were comparable to the manufacturer's specifications and there was greater than 80% recovery for the major fatty acids of interest (EPA/DHA). Oleic acid was not resolved using either of the two methods. The values are reported (in table 2.2) as a comparison to manufacturer's specifications and the GC-FID graph is shown in figure 2.5. Using the modified method approximately 79% of the trace was identified and matched up with the manufacturers' specifications. The remaining 21% of the GC trace was not identified as it did not match up with any of the components listed in the 37-component FAME mix. EPA and DHA were the two main components in the menhaden fish oil corresponding to 16 and 19% of the total respectively. Subsequently, the recovery percentage was between 80-120% and the mean CV% between two runs was calculated as less than 4%. Using the Masood et al. (2005) method, approximately 70% of the peaks in the GC trace were identified with a CV% of less than 5%. EPA and DHA were identified as being 14% and 12% of the total respectively providing 80-120% recovery of these fatty acids in the esterified fish oil standard.

Table 2.2 Percentage fatty acid composition for Menhaden Fish oil, values reported for the two methods are expressed as percentage of each fatty acid relative to all fatty acids in a chromatogram.

	Menhaden Fish oil				
Composition - Percentage by weight	**Manufactures guidance (% by weight)**	**Modified method**		**Masood et al. (2005) method**	
		Mean	**CV%**	**Mean**	**CV%**
Myristic acid	6-9%	8	8	1	1
Palmitic acid	15-20%	17	7	19	4
Palmitoleic acid	9-14%	13	3	12	1
Stearic acid	3-4%	3	1	3	5
Oleic acid	5-12%	1	5		
LA	<3%	0	4	5	1
ALA	<3%	1	3	4	20
Octadecatrtranoicacid	2-4%	--	--		
AA	<3%	--	--		
EPA	**10-15%**	**16**	**6**	**14**	**3**
DHA	**8-15%**	**19**	**5**	**12**	**6**
Mean ± SD		**8.67±7.70**		**8.75±6.36**	

There were no differences between the means for the two methods when analysed using paired t-test (p>0.05).

Olive oil

The olive oil analysed for this experiment was obtained from Napolina Ltd., UK and the manufacturers stated that the pack contained extra virgin olive oil with no indication to purity. Table 2.3 shows recovery of fatty acids in the olive oil standard when compared against manufacturer's specifications (olive oil, catalogue number: 47118, sigma aldrich). There was a greater than 80% recovery for oleic acid using both methods. Using the modified method, oleic acid corresponded to 69% while the remaining 31% of the peak area was covered by stearic acid (3%), linoleic acid (14%) and palmitic acid (14%). The mean CV% between two runs was calculated as less than 5%. Using the modified method there was a recovery of >80% for all fatty acids with oleic acid corresponding to 66% of peak area.

Table 2.3 Percentage fatty acid composition for olive oil, values reported for the two methods are expressed as percentage of each fatty acid relative to all fatty acids in a chromatogram

Olive oil					
Composition - Percentage by weight	**Manufacturers guidance (% by weight)**	**Modified method**		**Masood et al. (2005) method**	
		Mean	**CV%**	**Mean**	**CV%**
Palmitic Acid	<13%	14	6	13	7
Palmitoleic Acid	-	-	-	-	-
Stearic Acid	<3%	3	5	3	2
Oleic Acid	**71%**	**69**	**3**	**66**	**2**
LA	<10%	14	6	18	5
ALA	-	-	-	-	-
Mean ±SD		**25±27.8**		**25± 28.0**	

There were no differences between the means for the two methods when analysed using paired t-test (p>0.05).

Sunflower oil

The sunflower oil analysed for this experiment was obtained from Flora, UK and the manufacturers stated that the pack contained pure sunflower oil with vitamin E. The analysed GC-FID trace was compared to sunflower seed oil (catalogue number: 47123; Sigma Aldrich). There was no pure sunflower oil available from the manufacturer and this was the closest match to sunflower oil. Table 2.4 reports the profile of sunflower oil derived from the GC-FID graph. There was > 80% recovery for linoleic acid, which was the major fatty acid in the sunflower oil using the two methods. Using the modified method, linoleic acid was identified as the primary component (56%) and the remaining 44% of the peak area was contributed by palmitic acid (14%), oleic acid (25%) and stearic acid (5%). When compared to the manufactures composition, there was a 10% difference in the value of the primary component (linoleic acid). Using the Masood et al. (2005) method, linoleic acid was identified as the major peak (63%) followed by oleic acid (26%), palmitic, and stearic acid (4% each). The mean CV% between two runs was calculated as less than 5% for both the methods.

Table 2.4 Percentage fatty acid composition for sunflower oil, values reported for the two methods are expressed as percentage of each fatty acid relative to all fatty acids in a chromatogram

Sunflower oil					
Composition - Percentage by weight	**Manufacturers guidance (% by weight)**	**Modified method**		**Masood et al. (2005) method**	
		Mean	**CV%**	**Mean**	**CV%**
Palmitic Acid	7%	14	0	4	9
Palmitoleic Acid	--	--	--	--	--
Stearic Acid	5%	6	2	4	2
Oleic Acid	19%	25	5	26	5
LA	**68%**	**56**	**5**	**63**	**1**
ALA	1%	--	--	--	--
Mean ± SD		**25.3 ± 21.9**		**24.2 ± 27.8**	

There were no differences between the means for the two methods when analysed using paired t-test (p>0.05).

PBMC total lipids

PBMC total lipids expressed as percentage of a fatty acid relative to all fatty acids in a chromatogram are shown in table 2.5. Approximately 80% of peaks were identified using the modified method while the Masood et al. (2005) method resolved up to 85% of peaks, GC-FID traces are shown in figure 2.3. Some of the low molecular weight fatty acids (butyric, capric, and lauric acid) were not identified using the modified method due to the limitations of the temperature program for GC. These fatty acids were also difficult to resolve when analysed by the Massod et al. (2005) method. Additionally, plasma and red blood cells (RBCs) were analysed using both the methods, and >80% of peaks were identified by both the methods with a CV% of less than 20%. The capsules used in the intervention study (Biocare and placebo) were also esterified and analysed using the two methods; both methods provided >80% recovery of the total fatty acids.

Table 2.5 PBMC total lipids expressed as percentage of a fatty acid relative to all fatty acids in a chromatogram.

Fatty acids	Modified method		Masood et al. (2005) method	
SFA	**Mean**	**CV%**	**Mean**	**CV%**
Myristic acid	9.1	3.7	9.3	0.3
Pentadecanoic acid	2.3	5.3	-	-
Plamitic acid	16.7	2.0	17.8	2.3
Heptadecanoic acid	0.6	0.3	0.3	1.5
Stearic acid	6.5	0.9	10.7	2.3
MUFA				
Palmitoleic acid	3.3	3.9	-	
Oleic acid	16.2	1.1	15.6	0.3
PUFA				
LA	8.5	1.0	10.0	0.6
ALA	0.9	1.2	0.6	3.3
AA	4.6	2.7	7.0	0.6
EPA	1.6	2.3	1.6	4.1
DHA	0.8	0.5	1.0	2.6
Mean ±SD	**5.92 ± 5.7**		**7.3 ± 6.3**	

There were no differences between the means for the two methods when analysed using paired t-test (p>0.05).

Composition of Biocare and Placebo capsules compared against manufacturers specifications

The capsules used in the intervention study were received free of charge from Biocare (catalogue code: 52730) while the placebo capsules were received from Bionovate. The compositions of the capsules as per manufactures guidelines are reported in table 2.6. Applying the modified method, 100 µl of the components from the capsules were esterified and analysed using the GC-FID method. The Bionovex capsules containing resolvinol (E series resolvins) were not analysed since there was no manufacturers GC report for comparison. Biocare capsules when analysed were found to primarily constitute EPA and DHA. The capsule contained 43.65% EPA and 29.10% DHA making up approximately 73% of the total peak area. This accounted for the approximately 2:1 ratio of EPA: DHA, which was suggested by the manufacturer. The remaining 27% constituted of grapeseed oil, which was suggested as a vehicle for the two primary constituents of the capsule. The

mean CV% of all the fatty acids identified was less than 8%. There were no differences between the means for the two methods when analysed using paired t-test ($p>0.05$).

Table 2.6 Composition of the capsules used in the intervention study

Capsules	Manufacturer	Ingredients per capsule
Mega EPA	Biocare Catalogue Code: 52730	1000 mg Fish Oil Concentrate Containing 310mg EPA and 210mg DHA (relative ratio – 2:1) 50mg lecithin and 10mg vitamin E 15i.u. (d-alpha tocopherol) Capsule: gelatine, glycerine and chlorophyll
Bionovex	Bionovate	Derived from Green Lipped Mussels Containing 10mg Resolvinol 0.5% natural vit E (antioxidant) with the oil vehicle being olive oil. Capsule: gelatine
Placebo	Bionovate	Olive oil filled only, with 0.5% vit E. Capsule: gelatine

Nitrogen vs. Helium as carrier gas

Using nitrogen as a carrier gas in chromatography is more cost effective than using helium. An assessment of the efficiency of the GC-FID method using nitrogen as an alternative for helium was conducted. The same GC-FID programme using the modified method was applied and the percentage recovery of fatty acids in the 37-component FAME mix was compared for the two gases. The data suggests that the resolution of peaks are 5% better with helium, however the total CV% for all fatty acids when using nitrogen is under 10%, which was acceptable under the allowable error based on biological variation for this experiment. Thus, nitrogen can be used as an alternative for helium in GC-FID analysis. For the supplementation study, helium was used as the carrier gas with a HP-5 column, however for additional validation results nitrogen was used as the carrier gas. The data comparing efficiency of the two carrier gases is reported in table 2.7.

Table 2.7 Percentage recovery of key fatty acids using nitrogen and helium as carrier gases

	Manufacturer's guidance (% by weight)	Percentage of fatty acids in 37 component FAME mix relative to all fatty acids in a chromatogram			
		Using Helium		***Using Nitrogen***	
SFA		Mean	CV%	Mean	CV%
Lauric acid	4	4.6	2.1	3.8	9.1
Myristic acid	4	4.6	3.7	4.7	17.1
Pentadecanoic acid	2	2.4	0.3	2.8	23.1
Palmitic acid	6	7.3	0.2	6.7	2.1
Heptadecanoic acid	2	2.3	6.3	3.4	0.9
Stearic acid	4	4.8	8.8	3.8	7.4
MUFA					
Plamitoleic acid	2	2.6	2.3	3.2	7.1
oleic acid	4	6.4	2	4	5.4
PUFA					
LA	2	2.5	7.9	2.8	12.9
ALA	2	1.0	5.3	1.8	20.2
AA	2	1.9	0.7	3.5	6.5
EPA	2	2.1	4.7	2.3	9.4
DHA	2	2.0	5.0	2.0	3.6
Mean ± SD		**3.42 ± 1.9**		**3.44 ± 1.3**	

There were no differences between the means for the two methods when analysed using paired t-test ($p>0.05$).

Sample GC-FID traces from the validation

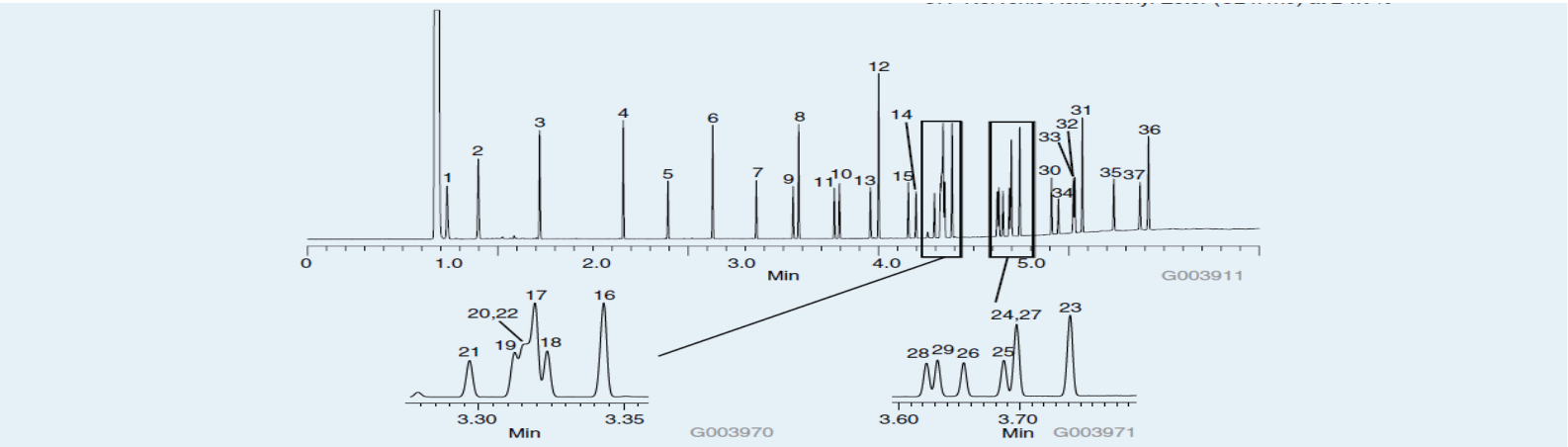


Figure 7. 37-Component FAME Mix on the Equity-1

column: Equity-1, 15 m x 0.10 mm I.D., 0.10 µm (28039-U)
oven: 100 °C, 50 °C/min. to 300 °C (1 min.)
inj.: 250 °C
det.: FID, 300 °C
carrier gas: hydrogen, 50 cm/sec constant
injection: 0.2 µL, 200:1 split
liner: 4 mm I.D., split, cup design
sample: Supelco 37-Component FAME Mix (47885-U), analytes at concentrations indicated in methylene chloride

1. Butyric Acid Methyl Ester (C4:0) at 4 wt %
2. Caproic Acid Methyl Ester (C6:0) at 4 wt %
3. Caprylic Acid Methyl Ester (C8:0) at 4 wt %
4. Capric Acid Methyl Ester (C10:0) at 4 wt %
5. Undecanoic Acid Methyl Ester (C11:0) at 2 wt %
6. Lauric Acid Methyl Ester (C12:0) at 4 wt %
7. Tridecanoic Acid Methyl Ester (C13:0) at 2 wt %
8. Myristic Acid Methyl Ester (C14:0) at 4 wt %
9. Myristoleic Acid Methyl Ester (C14:1) at 2 wt %
10. Pentadecanoic Acid Methyl Ester (C15:0) at 2 wt %
11. cis-10-Pentadecenoic Acid Methyl Ester (C15:1) at 2 wt %
12. Palmitic Acid Methyl Ester (C16:0) at 6 wt %
13. Palmitoleic Acid Methyl Ester (C16:1) at 2 wt %
14. Heptadecanoic Acid Methyl Ester (C17:0) at 2 wt %
15. cis-10-Heptadecenoic Acid Methyl Ester (C17:1) at 2 wt %
16. Stearic Acid Methyl Ester (C18:0) at 4 wt %
17. Oleic Acid Methyl Ester (C18:1n9c) at 4 wt %
18. Elaidic Acid Methyl Ester (C18:1n9t) at 2 wt %
19. Linoleic Acid Methyl Ester (C18:2n6c) at 2 wt %
20. Linolelaidic Acid Methyl Ester (C18:2n6t) at 2 wt %
21. γ-Linolenic Acid Methyl Ester (C18:3n6) at 2 wt %
22. α-Linolenic Acid Methyl Ester (C18:3n3) at 2 wt %
23. Arachidic Acid Methyl Ester (C20:0) at 4 wt %
24. cis-11-Eicosenoic Acid Methyl Ester (C20:1n9) at 2 wt %
25. cis-11,14-Eicosadienoic Acid Methyl Ester (C20:2) at 2 wt %
26. cis-8,11,14-Eicosatrienoic Acid Methyl Ester (C20:3n6) at 2 wt %
27. cis-11,14,17-Eicosatrienoic Acid Methyl Ester (C20:3n3) at 2 wt %
28. Arachidonic Acid Methyl Ester (C20:4n6) at 2 wt %
29. cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester (C20:5n3) at 2 wt %
30. Heneicosanoic Acid Methyl Ester (C21:0) at 2 wt %
31. Behenic Acid Methyl Ester (C22:0) at 4 wt %
32. Erucic Acid Methyl Ester (C22:1n9) at 2 wt %
33. cis-13,16-Docosadienoic Acid Methyl Ester (C22:2) at 2 wt %
34. cis-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester (C22:6n3) at 2 wt %
35. Tricosanoic Acid Methyl Ester (C23:0) at 2 wt %
36. Lignoceric Acid Methyl Ester (C24:0) at 4 wt %
37. Nervonic Acid Methyl Ester (C24:1n9) at 2 wt %

Figure 2.4 2A GC-FID graph showing a profile of 37 component FAME mix on Equity 1 column provided by the manufacturers.

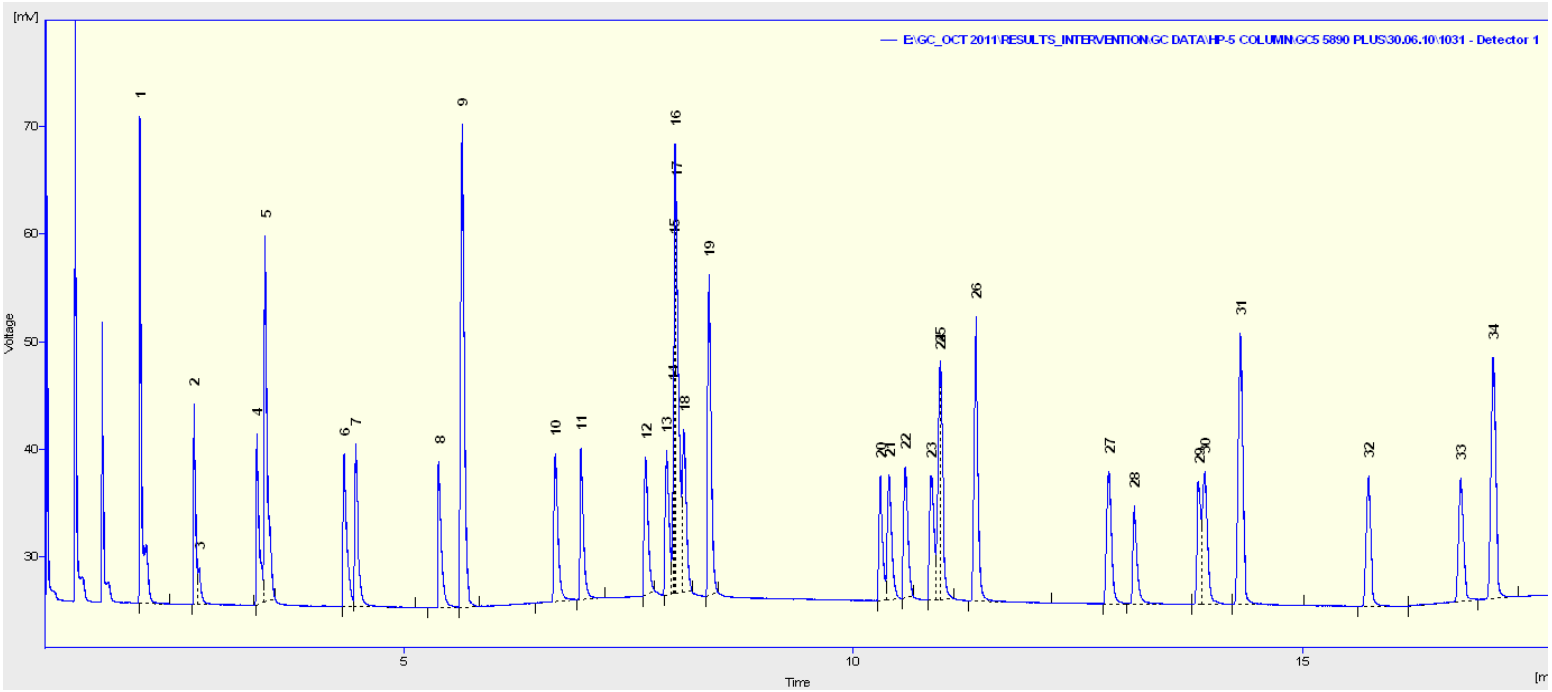
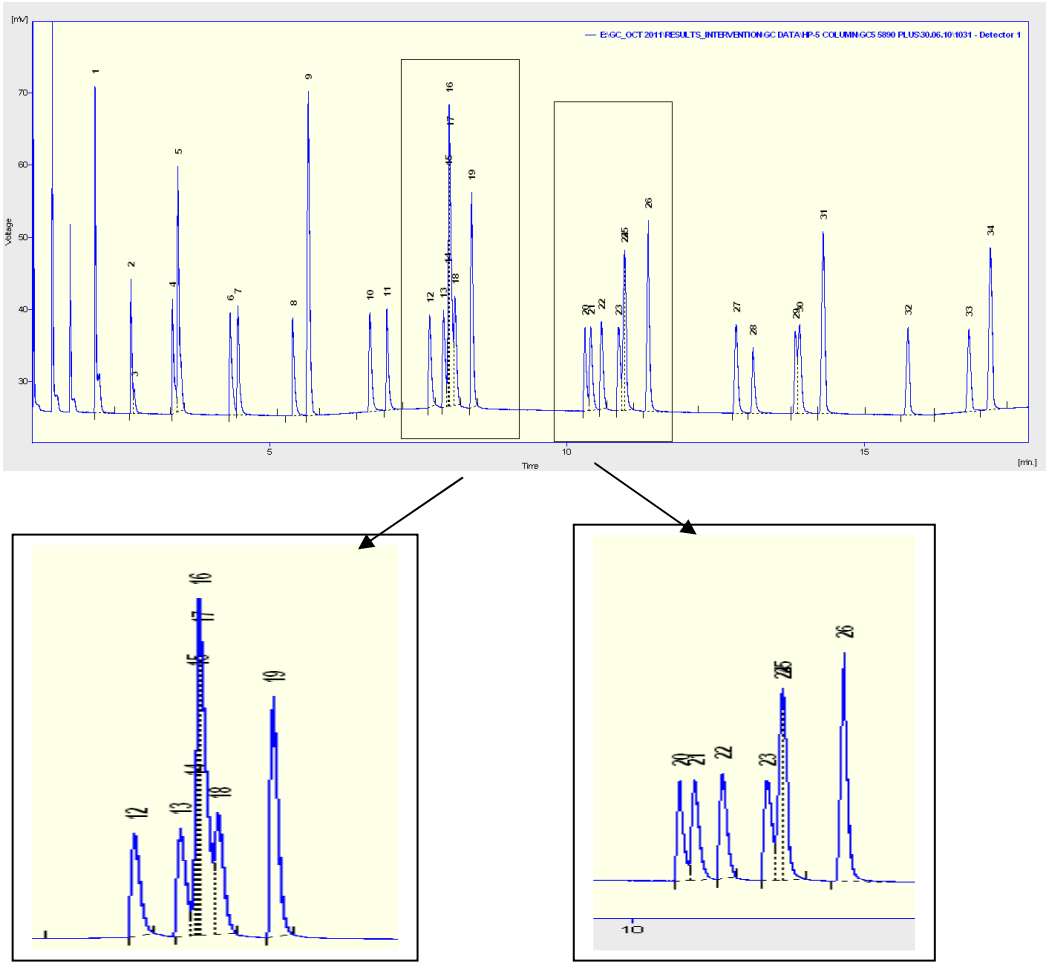


Figure 2.5 A GC-FID graph showing a profile of 37component FAME mix on HP-5 column using the modified method. Key to the profile is shown on next page.



Key

1. Lauric Acid
2. Tridecanoic Acid
5. Myristic Acid
4. Myristoleic Acid
5. Pentadecanoic Acid
6. cis-10-Pentadecenoic
9. Palmitic Acid
8. Palmitoleic Acid
11. Heptadecanoic Acid
10. cis-10-Heptadecenoic Acid
19. Stearic Acid
17. Oleic Acid
14 α-Linolenic Acid
13. Linoleic Acid
15. Linolelaidic Acid
16. γ-Linolenic Acid
18. Elaidic Acid
26. Arachidic Acid
24 Eicosenoic Acid
23. Eicosadienoic Acid
22. Eicosatrienoic Acid
25. Eicosatrienoic Acid
20. Arachidonic Acid
21. Eicosapentaenoic Acid
27. Heneicosanoic Acid
31. Behenic Acid
30. Erucic Acid
29. Docosadienoic Acid
28. Docosahexaenoic Acid
32. Tricosanoic Acid Methyl Ester (C23:0)
34. Lignoceric Acid Methyl Ester

Figure 2.5 a) A GC-FID graph showing a profile of 37component FAME mix on HP-5 column using the modified method.

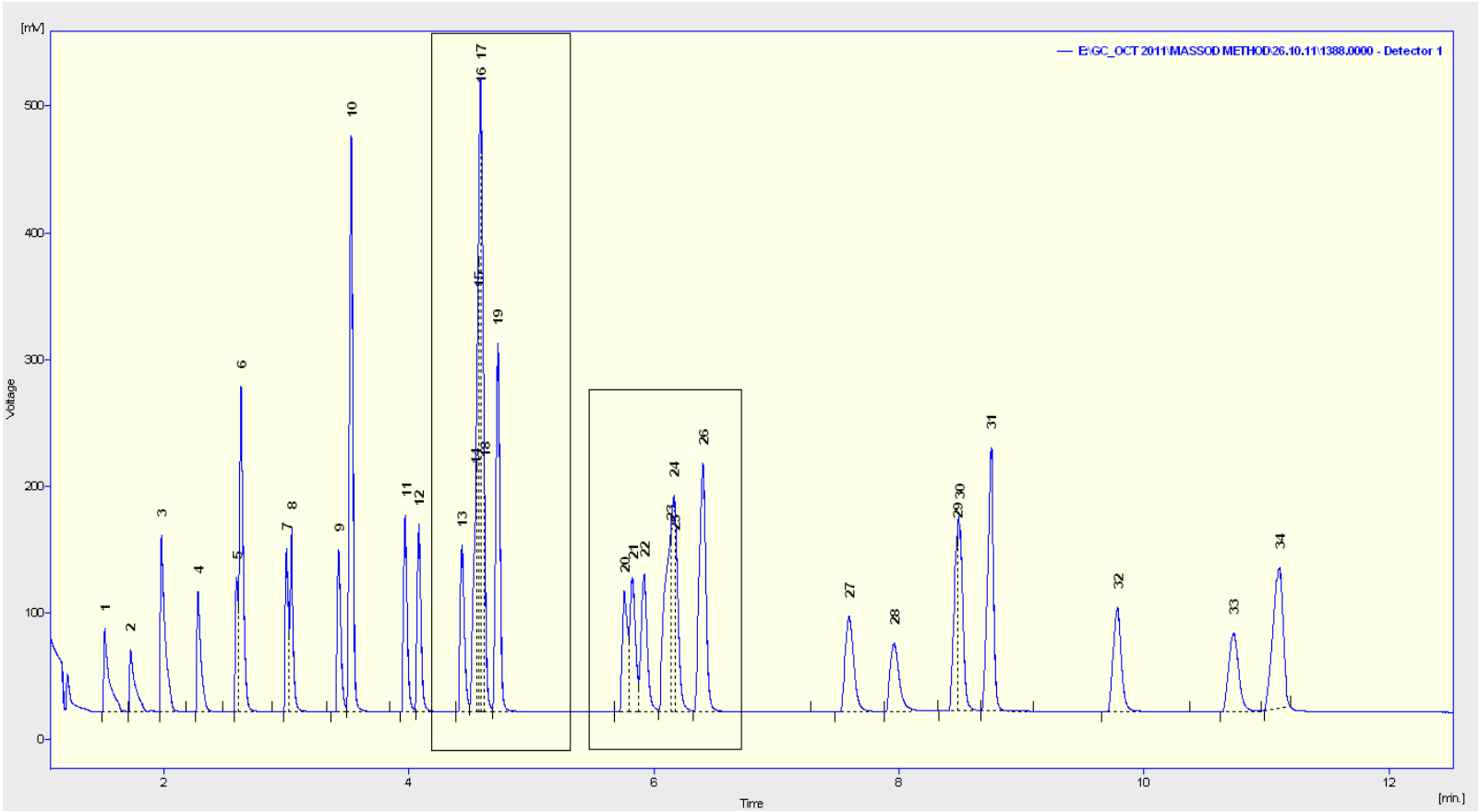
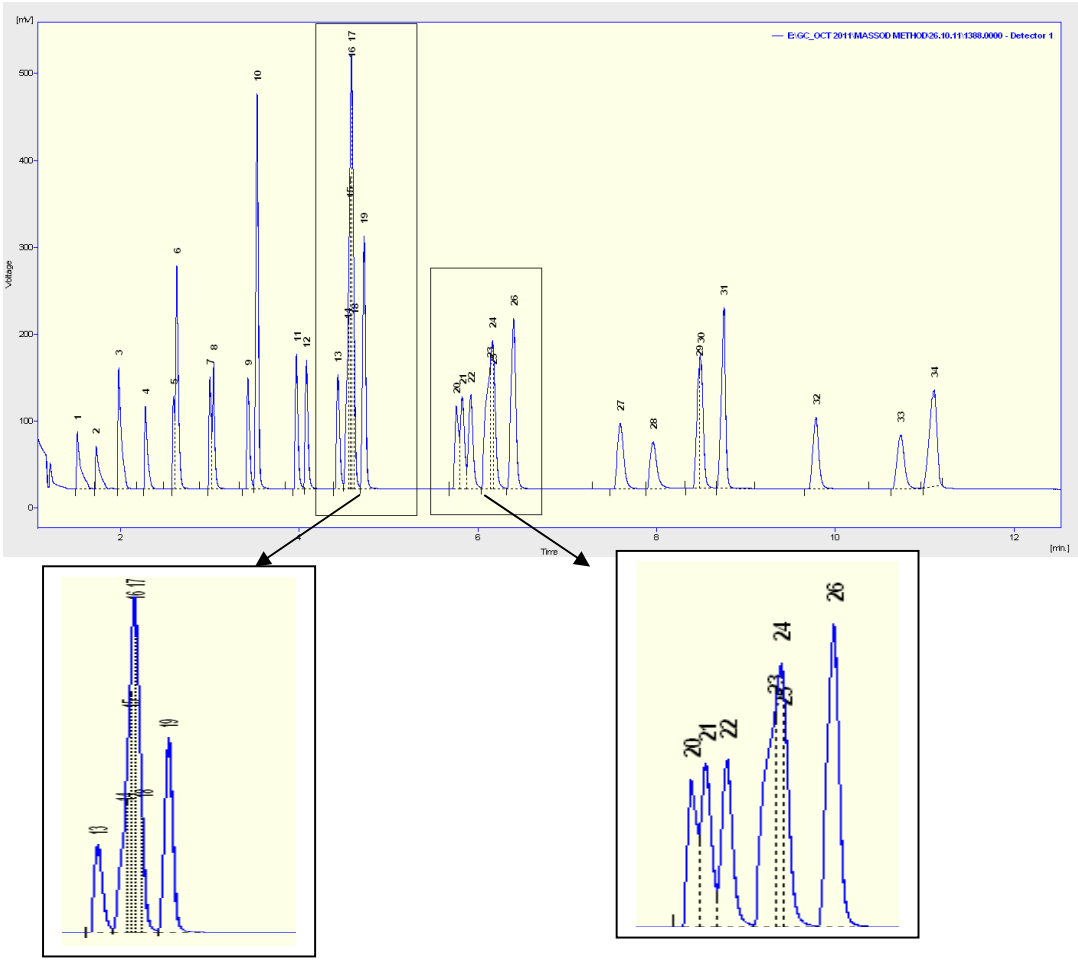


Figure 2.6 A GC-FID graph showing a profile of 37 component FAME mix on HP-5 using the Masood et al (2005) method. Key to the profile is shown on next page.



Key

3. Lauric Acid
4. Tridecanoic Acid
5. Myristic Acid
6. Myristoleic Acid
7. Pentadecanoic Acid
8. cis-10-Pentadecenoic Acid
10. Palmitic Acid
9. Palmitoleic Acid
11. Heptadecanoic Acid
12. cis-10-Heptadecenoic Acid
19. Stearic Acid
17. Oleic Acid
13. Linoleic Acid
14 α-Linolenic Acid
15. Linolelaidic Acid
16. γ-Linolenic Acid
18. Elaidic Acid
26. Arachidic Acid
24. Eicosenoic Acid
23. Eicosadienoic Acid
22. Eicosatrienoic Acid
25 Eicosatrienoic Acid
32. Tricosanoic Acid
33. Nervonic Acid
34. Lignoceric Acid
20. Arachidonic Acid
21. Eicosapentaenoic Acid
27. Heneicosanoic Acid
31. Behenic Acid
30. Erucic Acid
29. Docosadienoic Acid
28. Docosahexaenoic Acid

2.6 a) A GC-FID graph showing a profile of 37 component FAME mix on HP-5 using the Masood et al., (2005) method.

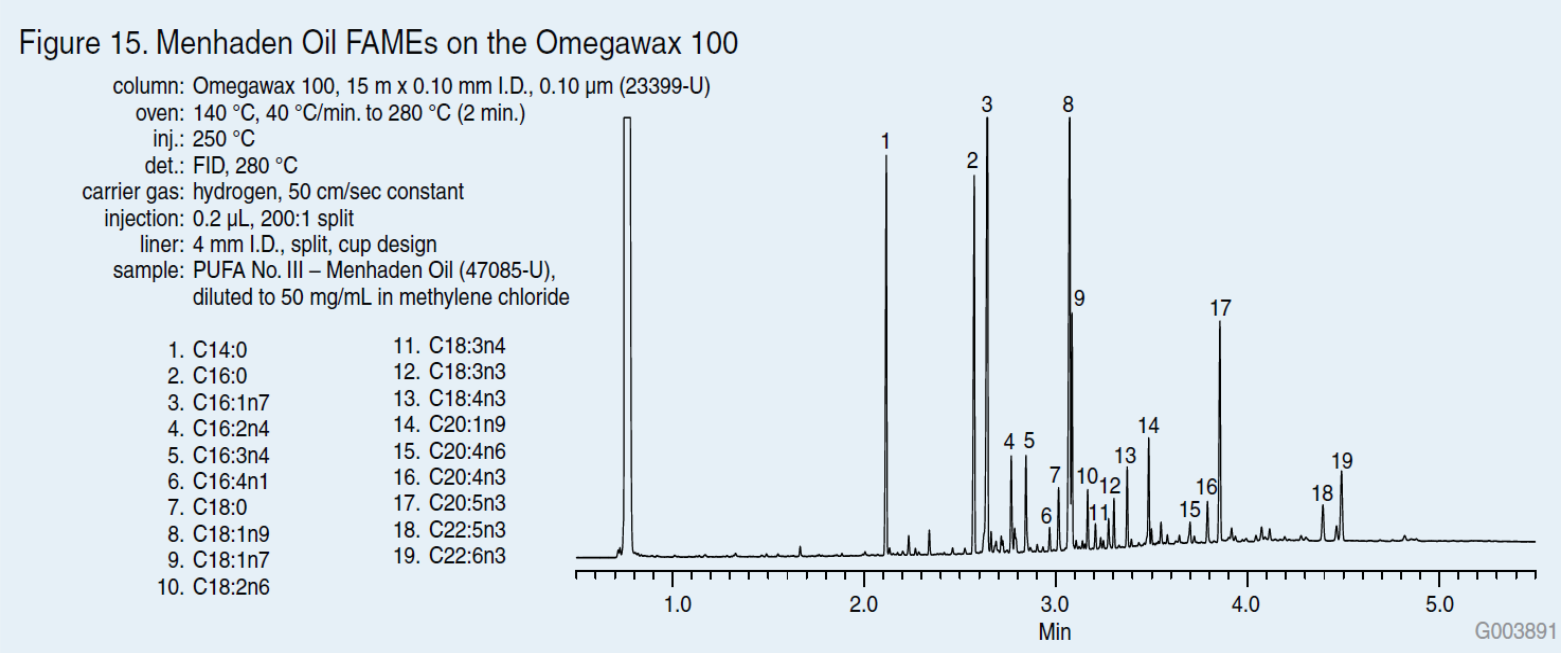
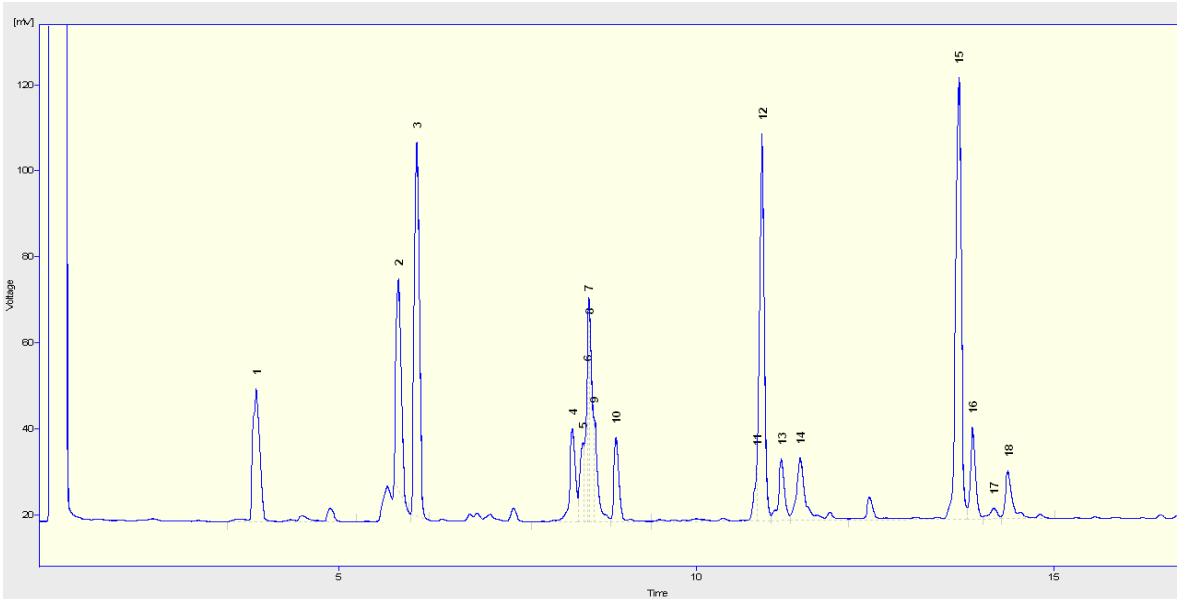
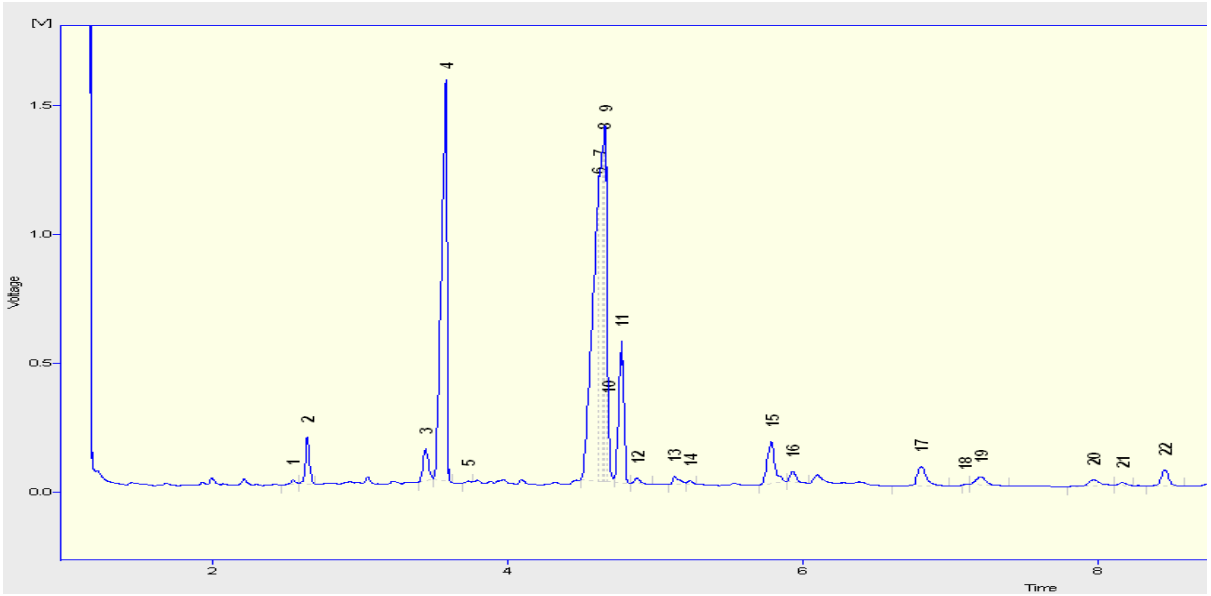


Figure 2.7 A GC-FID graph showing a profile of menhaden fish oil FAMEs on Omegawax 100 column (there were no comparable traces available from the manufacturers on HP-5 or related column).



Key
1. Myristic Acid
2. Palmitoleic Acid
3. Palmitic Acid
4. Linoleic Acid
5. α-Linolenic Acid
7. Oleic Acid
10. Stearic Acid
12. Eicosapentaenoic Acid
15. Docosahexaenoic Acid

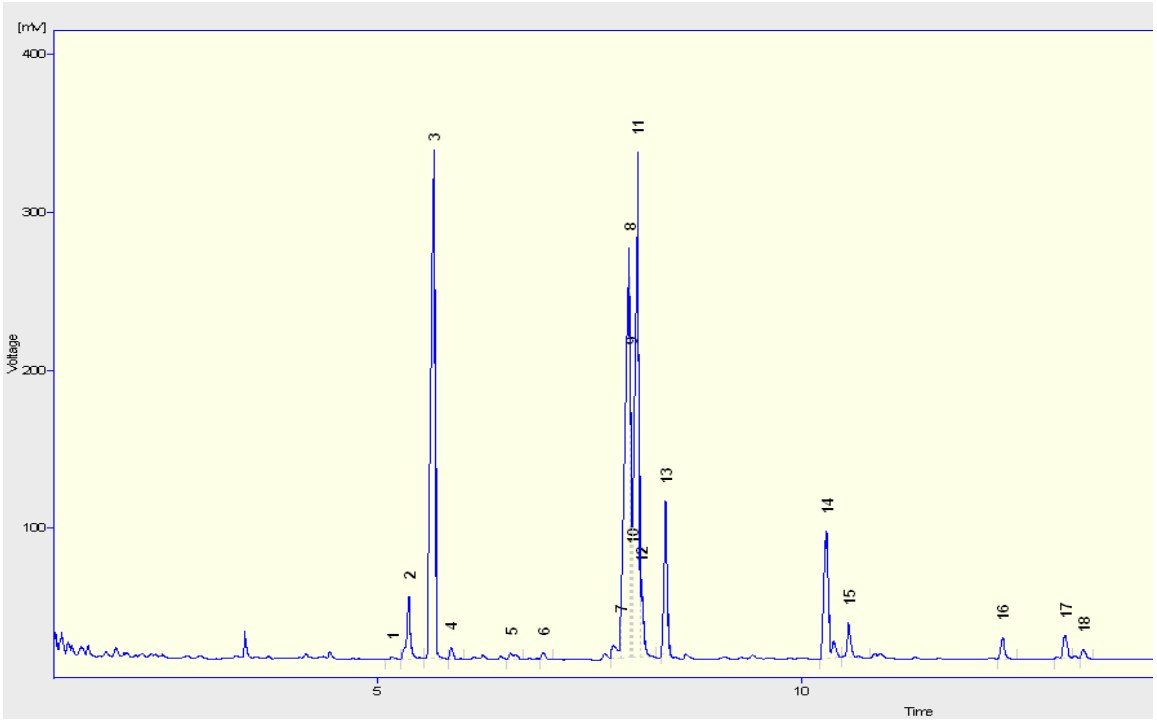
Figure 2.8 A GC-FID graph showing a profile Menhaden fish oil FAMEs on HP-5 column using the modified method



Key

2. Myristic Acid
3. Pentadecanoic Acid
4. Palmitic Acid
6. Heptadecanoic Acid
5. Linoleic Acid
7. α-Linolenic Acid
9. Oleic Acid
11. Stearic Acid
15. Arachidonic Acid
16. Eicosapentaenoic Acid
19. Docosahexaenoic Acid

Figure 2.9 A representative GC-FID graph showing a total fatty acid profile for PBMC on HP-5 colum using Masood et al (2005) method.



Key

2. Myristic Acid
3. Palmitic Acid
5. Pentadecanoic Acid
6. Heptadecanoic
7. Linoleic Acid
8. α-Linolenic Acid
11. Oleic Acid
13. Stearic Acid
14. Arachidonic Acid
15. Eicosapentaenoic Acid
17. Docosahexaenoic Acid

Figure 2.10 A representative GC-FID graph showing a total fatty acid profile for PBMC on HP-5 column using the modified method.

2.6.9 Limitations of the modified method

The modified method was fast and economical providing a reliable and accurate identification of the fatty acids. However, there were certain limitations that need to be highlighted. There was co-elution of some peaks and to address the issues of column temperature and operating conditions, the temperature programme was adjusted (Ackman, 1988, 2006). These modifications to the GC-FID parameters showed no improvement in peak co-elution and increased the retention time. The reference graph provided by the manufacturer (on Equity 1, a fused silica column) showed similar co-elution of the C18/C20 fatty acids requiring manual integration of peaks (figure 2.4).The original aim of the study was to report relative ratios of EPA, DHA and AA, therefore the 37- component FAME mix was considered a suitable standard. Due to limitation of co elution, although approximately 70% of peaks were identified (based on their retention times) it was not possible to measure the area under the curve for all of these fatty acids. Consequently, only selected fatty acids are reported and these include (saturated fatty acids – mysteric acid, pentadecanoic acid, palmitic acid, heptadecanoic acid and steric acid; monounsaturated fatty acids – plamitoleic acid and oleic acid; polyunsaturated fatty acids – LA, ALA, AA, EPA and DHA).

Although the method produced reproducible results, it is important to evaluate the PBMC fatty acid composition in relation to other studies. Within literature, total PBMC total lipid composition is found to made up of palmitic acid (~20%), stearic acid (<20%), oleic acid (~15-20%) linoleic acid (~10%) and alpha linoleic acid (<1%) per 100% of fatty acids. In addition, high levels of AA (15-20%) and low levels of EPA (<1) and DHA (<3%) are usually found in PBMC total lipid composition (Damsgaard et al., 2008; Kew et al., 2004; Yaqoob et al., 2000). In this thesis, using the modified method the following composition was found for total PBMC lipid: palmitic acid (17%), stearic acid (7%), oleic acid (16%), linoleic acid (9%), AA (5%), EPA (1.6%) and DHA (0.8%). Since immune function cells have been demonstrated to have high levels of AA (~20%) these low levels measured in this study suggest a technical limitation of GC-FID method which could affect the interpretation of our results. In addition, docosapentanoic acid (DPA) is another n-3 PUFA which has been shown to be present in PBMC, however this fatty acid was not present in

the 37-component FAME mix or in any of the biological standards tested; consequently it was not possible to identify this fatty acid in our samples.

Losses of long chain n-3 PUFA (EPA & DHA) due to high temperature (>180°C) of GC columns has been reported. This may result in a number of small minor peaks that separate from the main peaks and could account for the small peak area for DHA in our findings (Ackman., 1988, 2006; AOCS., 1997; Fournier et al., .2006). Similarly, there may be thermal degradation of AA methyl esters as AA is a long chain PUFA with 20 carbons. To overcome this issue of loss of PUFA in future, it may be possible to use empirical correction factors (chemical/instrumental deviations, determined by means of calibration with pools of standards/internal standard) which could then be adapted for accurate FAME analyses conducted at temperatures causing thermal losses (Ackman, 2006; Aued-Pimentel et al., 2010). Despite the limitations of co-elution and lower recovery of long chain n-6 and n-3 PUFAs, the method demonstrated reproducibility and it was expected that similar analytical effect would be observed at baseline and after supplementation with EPA/DHA; therefore the findings of this thesis will be consistent across all phases of the study. However, the interpretation of fatty acid composition requires a great deal of caution when comparing it with other published studies.

2.7 Measurement of circulating inflammatory cytokines by ELISA

In chapter 3, 4 and 5, inflammatory cytokines including IL-6, TNF-α and IL-10 were measured in serum samples using the commercially available ELISA kits as described in the sections below. ELISA is a fundamental, validated technique used for the measurement of a wide range of cytokines in blood or urine samples. The method is based on the principle of antibody-antibody interaction. For sample preparation whole blood was collected in a serum tube (with no anti-coagulant) and allowed to coagulate for up to an hour. The tubes were centrifuged at 1300 x g for 10 minutes. Serum was separated and aliquots were made and stored at -20ºC until further analysis. To minimise the effect of inter-plate variation, all the samples from each individual collected at different time points of the study were analysed on the same ELISA plate.

2.7.1 Determination of serum TNF-α concentrations

The TNF-α assay was used to determine the differences in serum TNF- α concentration between asthmatics and healthy individuals (chapter 3). In the supplementation study, (Chapter 4/5) this method was used to measure circulating levels of serum TNF- α in phases of pre- and post-supplementation with Omega 3. The materials and reagents required for this kit are listed in appendix 1. Human TNF-α Quantikine ELISA Kit (cat. no # HSTA00D, R & D systems) and ELISA MAX™ Deluxe Set (BioLegend, cat. no. 430204) were used for quantitative determination of TNF-α in the serum samples. The lowest detectable limit of the Quantikine ELISA kit was 0.106 pg/mL (range 0.11- 32 pg/mL) and the manufacturers guaranteed no cross-reactivity or interference in the assay. The recovery for the assay was suggested to be between 80-120%. The Biolegend kit was reported to provide a sensitivity of less than 2 pg/mL and was guaranteed to have no cross –reactivity or interference. The samples were run in duplicate on two plates for reproducibility and the intra-assay variation (CV%) was calculated as <10%, which was acceptable as per manufacturers guidelines.

Assay procedure

Quantikine ELISA Kit: All reagents and samples were brought to room temperature before use. All samples, standards, and controls were assayed in duplicate to assess the reproducibility of the assay. All reagents, samples, and working standards were prepared as directed in the table shown in appendix 1. Assay diluent RD1F (50 µl) was added to each well followed by the addition of 200 µl of standard, sample, or control per well. The plate was covered with an adhesive tape and incubated for 3 hours at room temperature. All liquid from the wells was removed by aspirating or inverting the plate and decanting the contents. Excess liquid was removed by grasping the plate firmly and smartly tapping the inverted plate on a clean paper towel at least 5 times. Each well was filled with 400 µl of wash buffer using a squirt bottle. Liquid was removed from the wells by aspirating or inverting the plate and decanting the contents. The wash was repeated 5 times, for a total of 6 washes. After the last wash, the inverted plate was tapped on to a clean paper towel at least 10 times to remove excess wash buffer. TNF-α HS Conjugate (200 µl) was added to each well; the plate was covered and incubated for further 2 hours at room temperature. After incubation, the wash step was repeated. In the next step, 50 µl of Substrate Solution was added to each well and the plates were covered and incubated for 1 hour at room

temperature. The plate was not washed after this step. Finally, 50 µl of amplifier solution was added to each well, the plate was covered and incubated for 30 minutes at room temperature. This leads to colour development. At the end of incubation, 50 µl of Stop Solution was added to each well. The optical density of each well was measured with a microplate plate reader set at 490 nm within 30 minutes. Since wavelength correction was not available, readings were subtracted at 650 nm or 690 nm from the readings at 490 nm. This subtraction was undertaken to correct for optical imperfections in the plate.

BioLegend's ELISA MAX™ Deluxe Set: This method required an overnight coating of the plate (96 well) with human TNF-α specific monoclonal antibody. Standards and samples were added to wells in the plate (100µl) the next day and TNF-α bound to the immobilized capture antibody. Next, a biotinylated anti-human TNF-α detection antibody (100µl) was added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidise (100µl) was subsequently added, followed by TMB Substrate Solution, producing a blue colour in proportion to the concentration of TNF-α present in the sample. Finally, a stop solution was added which changed the colour from blue to yellow, and the microwell absorbance was read at 450 nm with a microplate reader.

The two kits performed up to the recommended specifications provided by the manufacturers determined by a linear standard curve with correlation coefficient (r) $r \geq 0.99$ (for Quantikine ELISA Kit) and 0.98 (for BioLegend's ELISA MAX™ Deluxe Set), respectively. When multiple plates were run, the standard curves were compared to assess intra assay variations. The BioLegend's ELISA MAX™ Deluxe Set provided a good correlation for standard curves generated on different plates ($r \geq 0.95$).

2.7.2 Determination of serum IL-6 concentrations

A Quantikine HS Human IL-6 Immunoassay (R &D systems, cat. No. HS600B) was used for determination of IL-6 levels in serum samples for distinguishing differences between asthmatics and controls (chapter 3).Human IL-6 High Sensitivity ELISA (Diaclone cat no. 950 035 096) was used for assessing changes in levels following n-3 supplementation (chapter 4 and 5). The sensitivity of the Quantikine ELISA kit was 0.04 pg/mL (range 0.04-10 pg/mL), and the manufacturers guaranteed no cross-reactivity or interference in the assay. The recovery for the assay was suggested to be between 80-120%. The sensitivity

of the Human IL-6 High Sensitivity ELISA (diaclone) was 0.8 pg/mL (range 0.8-50 pg/mL), and the manufacturers guaranteed no cross-reactivity or interference in the assay. The mean recovery for the assay was 115%.The samples were run in duplicate on two plates for reproducibility and the intra-assay variation (CV %) was calculated as 10%, which was acceptable as per manufacturer's guidelines.

Assay procedure

Quantikine HS Human IL-6 Immunoassay: An antibody specific for IL-6 was pre-coated onto a microplate, standards and samples were subsequently pipetted into the wells and any non-specific IL-6 present was bound by the immobilised antibody. All reagents and samples were brought to room temperature before use. All samples, standards, and controls were assayed in duplicate to assess the reproducibility of the assay. All reagents and working standards were prepared as per the manufacturers guidelines. Assay Diluent -75 (100 µl) was added to each well followed by the addition of 100 µl of standard or sample per well. The plate was sealed with the adhesive strip provided and incubated for 2 hours at room temperature on an orbital shaker (speed 4). All liquid was removed from the wells by inverting the plate and decanting the contents. Excess liquid was removed by grasping the plate firmly and smartly rapping the plate inverted on a clean paper towel at least 5 times. Each well was filled with 400 µl of Wash Buffer using a squirt bottle. Excess liquid from the wells was removed by inverting the plate and decanting the contents, a total of 6 washes were done and after the last wash, the inverted plate was rapped on a clean paper towel at least 10 times to remove excess wash Buffer. After the wash, 200µl of IL-6 conjugate was added to each well. The plate was covered with a fresh adhesive seal and incubated for 2 hours at room temperature on the shaker. Washing was done 6 times as described earlier. Substrate solution (50µl) was added to each well; the plate was covered and incubated for 60 minutes at room temperature on the benchtop. After the incubation, 50 µl of amplifier Solution was added to each well. The plate was covered and incubated for 30 minutes at room temperature on the benchtop. Addition of the amplifier Solution initiated colour development. Finally, 50 µl of stop solution was added to each well. Addition of stop solution did not affect colour in the wells. The optical density of each well was measured within 30 minutes using a microplate reader set to 490 nm. Since wavelength correction was not available, readings were subtracted at 650 nm or 690 nm

from the readings at 490 nm. This subtraction was done to correct for optical imperfections in the plate as per manufacturer's guidance.

Human IL-6 High Sensitivity ELISA (Diaclone): An appropriate standard (100 µl) provided in the kit was added to the standard wells to create a standard curve. A monoclonal antibody specific for IL-6 was coated onto the wells of the microtiter strips provided. During the first incubation, IL-6 present in the sample or standard and a monoclonal IL-6 antibody conjugated to biotin were simultaneously incubated (50 µl of diluted biotinylated anti IL-6 and 100 µl of sample). Following incubation, unbound biotinylated anti-IL-6 was removed during a wash step. Streptavidin-HRP (100 µl) was added and bound to the biotinylated anti IL-6. After incubation and a wash step, a substrate solution reactive with HRP (100 µl) was added to the wells. A coloured product was formed in proportion to the amount of IL-6 present in the sample. The reaction was terminated by addition of stop solution and absorbance was measured at 450 nm.

The two kits performed up to the recommended specifications provided by the manufacturers, determined by a linear standard curve with correlation coefficient (r) $r \geq 0.99$ for the two kits. When multiple plates were run, the standard curves were compared to assess intra assay variations. The Human IL-6 High Sensitivity ELISA (Diaclone) showed a good correlation between standard curves generated on different plates on the same day ($r \geq 0.90$).

2.7.3 Determination of serum IL-10 concentrations

Human IL-10 ELISA MAX™ Deluxe Sets (cat. no. 430604, Biolegend UK) were used for assessing changes in serum IL-10 levels following n-3 supplementation (chapter 4 and 5). The sensitivity of the Quantikine ELISA kit was 0.04pg/mL, (range 0.04 - 10pg/mL) and the manufacturers guaranteed no cross-reactivity or interference in the assay.

Assay procedure

A human IL-10 specific monoclonal antibody was first coated on to a 96-well plate. Standards and samples (100µl) were added to the wells, and IL-10 bound to the immobilized capture antibody. Next, a biotinylated anti-human IL-10 detection antibody was added producing an antibody-antigen-antibody "sandwich". Avidin-horseradish

peroxidase was subsequently added, followed by TMB Substrate Solution, producing a blue colour in proportion to the concentration of IL-10 present in the sample. Finally, a Stop Solution changed the reaction colour from blue to yellow, and the microwell absorbance was read at 450 nm with a microplate reader.

The kits performed up to the recommended specifications provided by the manufacturers and determined by a linear standard curve with correlation coefficient (r) $r \geq 0.98$. When multiple plates were run, the standard curves were compared to assess intra assay variations and the assay showed a good correlation between standard curves generated on different plates on the same day ($r \geq 0.90$).

2.8 Design for the supplementation studies

For the omega 3 intervention study, a double blinded, randomised cross over study design was used (figure 2.11). Different study designs were considered, such as parallel study designs, randomised cross over studies with single blinding, and time point studies. In a cross over trial, each experimental participant receives two or more different treatments. The order in which each participant receives a treatment is dependent on the particular design chosen for the trial. For this study, a standard 2X2 crossover design was used as explained by Kenward & Jones (1996) who explain the advantages of comparing 'within-participant' differences (each participant serves as their control and provides direct comparison of treatments). Within-participant difference was also considered for the comparison of two different treatment periods. The cross over study design considers period differences when estimating the treatment difference, based on the fact that half of the participants received treatment in the order AB while the others received it in the order BA. The minimisation of the effect of any period and treatment differences within participants is an advantage of the cross over trials.

To achieve the cross over design, each subject acted as their own control and underwent both of the supplementation phases. A power analysis was performed to approximate the appropriate sample size using pulmonary function as an outcome based on previous studies showing the effect of n-3 on pulmonary function in EIB subjects (Mickleborough et

al., 2003, 2006). Based on findings of Mickleborough et al., (2006), our study was designed to detect a minimum change of at least 0.3 litres in post exercise FEV_1 when participants were supplemented with n-3 PUFA for 3 weeks. The significant level was set at alpha = 0.05 and the power at 0.80. A sample size of eight participants was appropriate to identify a difference of this magnitude. To allow for fluctuations in participant compliance, withdrawal and unsuitable data, the target sample size was set at 12 participants. Considering the sample size was small, the participants were randomly allocated in minimisation scheme (Altman & Bland, 2005), whereby next allocation of supplementation capsules was dependent on the supplementation provided to the previous participant. This scheme of randomisation has been found to be appropriate to achieve balance between treatment groups.

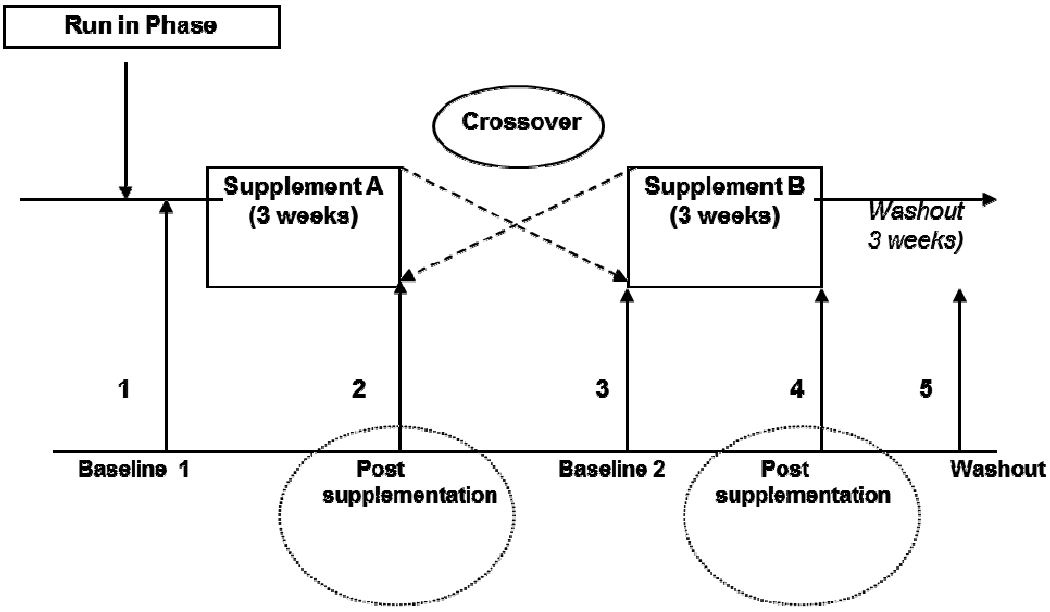


Figure 2.11 Crossover design used in the supplementation study

The participants, investigator (I) and outcome assessors (supervisors), were blinded to the study. The randomisation patterns were only known to the technician in charge of randomisation. All participants entered the study on their normal diet (phase 1/baseline1), after which they were randomly allocated to receive soft gelatin capsules: either 10 capsules per day of n-3 (3.2 gram EPA and 2.2 grams DHA, chapter 4) or Resolvin E1 (100 mg Resolvinol and 0.5% Vitamin E, chapter 5), or a placebo (olive oil and 0.5% Vitamin E) [phase 2]. There was a 3-week washout phase following the first

supplementation (normal diet; phase 3, baseline 2). Thereafter, the participants switched over to alternate supplementation for the remaining 3 weeks (Phase4). The participants reported to the lab at the end of each phase and completed the physiological and biochemical tests.

There were enough provisions in this method to ensure that whenever possible important comparisons of interest were estimated using the differences between the within-participant measurements. This is contrasted with another popular 'parallel study design' where each participant would have received only one type of treatment and to evaluate the differences in measurements, between-participant comparisons would be required. 'Wash-out' periods between the two periods were used to avoid 'carry over' effects. A 3-week 'washout' period was used between the treatment periods for this study. This duration was based on previous results from a similar n-3 intervention study conducted by Mickleborough et al. (2003, 2006). The measurements at the washout were compared with the measures taken at the beginning of the treatments (baseline data), and will be considered during analysis to assess if the participants had returned to their original physiological and biochemical parameters.

A 'run-in' period was used to familiarise the participants with procedures that they would follow during the study. The study was double-blinded, and a number of measurements were taken pre and post supplementation and during washout phases (figure 2.12). Each of the total five visits to the lab at each of the testing phases lasted for up to 2 hours. The measurements taken at each visit are described in the figure 2.12. These methods will be discussed in further detail in respective chapters for clarity. The n-3 and placebo capsules were received free of charge from Biocare and Bionovate. The details of the capsules are provided in table 2.6 as per manufacturers' guidance. The capsules were made of gelatin and were not suitable for vegetarians or individuals allergic to fish or fish by-products. Subsequently participants in the supplementation study were made aware of the composition of capsules and were screened out if they were any dietary concerns.

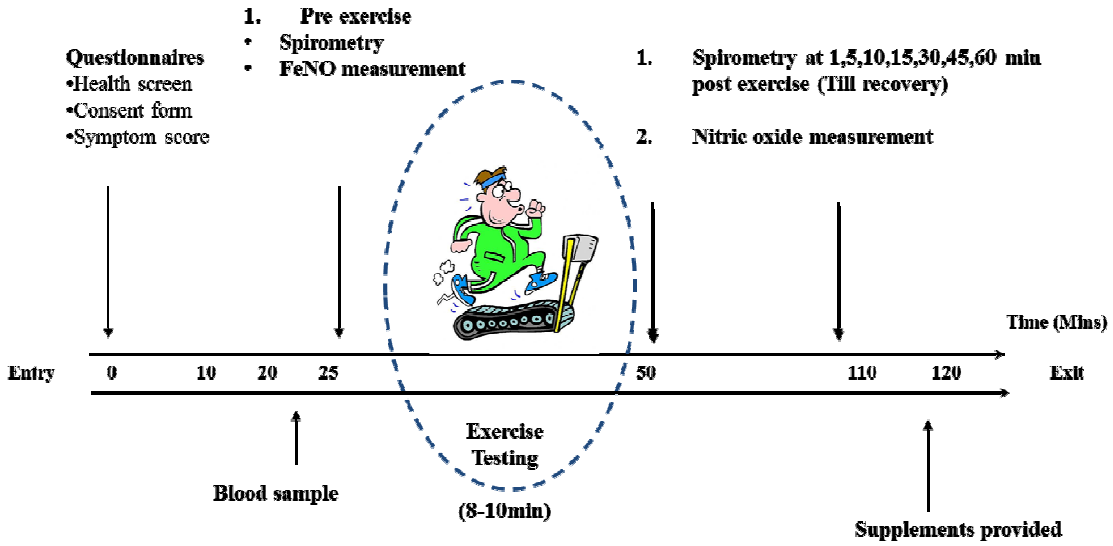


Figure 2.12 Study visit design

2.9 Statistical Analysis

Data was analysed using statistical software (SPSS, version 19; SPSS; Chicago, IL). Unpaired two tailed t-tests at a 95% level of significance were carried out to compare differences between asthmatics and controls (chapter 3) using the different characterisation techniques (fixed cut off values and LLN). A frequency count was carried for dyspnoea assessment and a chi square test was conducted to compare the differences between the two groups.

Analysis for n-3 and resolvin supplementation studies

The double blinded, placebo controlled, and crossover trial was designed to assess the effects of 3 weeks of supplementation with n-3 (EPA/DHA) on post exercise FEV_1 in EIB participants. A two-way repeated measures analysis of variance was used to analyse the data, with both treatment and time as “within-subject” effects. Mauchly’s test was conducted to determine whether sphericity was violated. In the cases where sphericity was violated, the repeated measures analysis of variance was corrected using the Greenhouse–Geiser correction factor.

Order effect was assessed by using the order as a variable during analysis. The relationship of oral contraceptive pill use with lung function improvements was assessed by using contraceptive pill usage as a variable for RM ANOVA. Student's paired t-tests were conducted to compare the physiological and biochemical outcomes of the group using contraceptives and the group without it.

Chapter 3

Classification of mild/moderate asthmatics

3 Classification of mild/moderate asthmatics

3.1 Abstract

Asthma is a reversible obstructive disease characterised by inflammation. Various guidelines suggest the use of fixed criteria and predicated equations derived from a reference population for the clinical diagnosis of asthma. The predicted equations are found to over or under-classify asthmatics due to factors such as stature, body mass, age and ethnicity; thus the measured values for pulmonary function provide a more reliable diagnosis. In addition, fixed ratios (80% ratio for FEV_1/FVC) have limited reliability, as there can be poor diagnosis of borderline asthmatics (those with mild-moderate symptoms). A lower limit of normality (LLN) derived from a reference population has been suggested to be used alongside 80% ratio for diagnosis of asthma. Though there are no guidelines so far for the usage of LLN in clinical settings, the LLN criteria has been found to be specific and reliable for asthma diagnosis. In this study, 16 male asthmatic and 21 healthy normal controls were recruited for pulmonary function testing, estimation of dyspnoea (symptoms) and measurement of venous blood based inflammatory mediators. Using the fixed criteria (80% ratio), the 16 asthmatics were demonstrated to have mild - moderate severity with all individuals showing a FEV_1/FVC ratio to be below the 80% ratio. When the control population (FEV_1/FVC = 89%) were used to calculate the LNN then a value of 82% was indicated which meant that all 16 individuals previously characterised as asthmatics were confirmed as mild to moderate asthmatics with the newer approach to asthma characterisation. The asthmatics showed worse dyspnoea and significantly elevated levels for and IL-6 and TNF-α than controls. The characteristics of mild-moderate asthmatics including pulmonary function, dyspnoea and levels of inflammatory cytokines confirm airflow obstruction and increased inflammation in asthma. The LLN criteria for asthma characterisation is comparable with standard fixed criteria for young males with mild-moderate asthmatics. Therefore, LLN can be used alongside fixed criteria for reliable characterisation of asthma for this population.

3.2 Background

The WHO estimates that respiratory diseases are the third major cause of mortality in the world and accounts for approximately 4.2 million deaths annually (WHO, 2010). Asthma is an obstructive respiratory disease characterised by airway obstruction. Other types of obstructive diseases include bronchiectasis, bronchitis and chronic obstructive pulmonary disease (COPD). Asthma is primarily characterised by acute eosinophilic and mast cell inflammation, mucus production and bronchoconstriction (Barnes, 2011; Bradding et al., 2006; Jeffery, 2000). The presence or absence of irreversible airflow is a major distinction between asthma and COPD with asthma showing a reversible airflow feature (Bereznicki & Bereznicki, 2009; Cazzola et al, 2008). In clinical settings airway obstruction is routinely assessed by symptoms (dyspnoea) and pulmonary function tests (PFTs) with the use of a spirometer (ATS, 1999; GINA, 2012; Miller et al, 2005a; NHLBI, 2007). The main PFT parameters are FEV_1, FVC, PEF, FEV_1/FVC ratio and FEF25-75%; details regarding these parameters are described in chapter 1, page 11.

3.2.1 Interpretation of pulmonary function results

In clinical setup, the PFT results or the *'measured data'* are interpreted based on comparison with a '*reference or predicted value'* based on healthy participants. Predicted values must be obtained from data from ''normal'' or ''healthy individuals'' with '*same'* anthropometric characteristics such as age, sex and height and where possible relevant ethnic characteristics of patients being tested (Pellegrino et al., 2005). The reference values are normally calculated with equations derived from measurements observed in matched or representative healthy volunteers from the general population. The ATS/ERS have included detailed guidelines on the strategies for interpretation of PFTs that have been widely used in the clinical and research settings (Miller et al., 2005a,b). The currently available electronic spirometers such as Superspiro (Micro Medical; Rochester, Kent, UK) are integrated with the choice of predicted values as per the ATS/ERS guidelines. This allows a very easy comparison of the *'measured data'* with the 'predicted values'.
The ATS/ERS have issued specific recommendations for selecting predicting values to be used, and these include matching age-range, anthropometric, race/ethnic, socio-economic and environmental characteristics between subjects tested by the laboratory

and the reference population from which the prediction has been derived (Wanger et al., 2005). A reference equation developed by Hankinson et al., (1999) is one of the widely used equations for predicted values and are specific for Caucasians, African-Americans, and Mexican-Americans (8 to 80 years of age, n = 7,429 asymptomatic, lifelong non-smoking participants, derived from the third National Health and Nutrition Examination Survey (NHANES III)). These reference values are specific for only three ethnicities; however, correction factors are available for other ethnicities.

3.2.2 Limitations of the predicted values for mild-moderate asthma

The ATS/ERS recommends the use of predicted values for characterisation of asthma severity with mild-moderate asthmatics 60-80% of predicted forced expiratory volume in 1 sec/forced vital capacity (FEV_1/ FVC) (Miller et al., 2005a, b). Values higher than 100% of predicted are sometimes seen in 'healthy individuals' and this may be due to dysanaptic growth or unequal growth of airways and lung parenchyma (Hyatt et al., 1986). This pattern is labelled as a potential 'normal physiologic variant' and is suggested as being not unusual among physically fit non-smoking individuals (Pellegrino et al., 2005). In a study by Mickleborough et al., (2006) recreationally active mild-moderate mixed group of young asthmatics with EIB showed predicted values in the range of 83 - 94% (mean = 90%). Stanford et al., (2006), reported mean predicted values of 104% (st dev = 3.9) in female athletes with atopic asthma. In another mixed group of mild-moderate elite athletes mean values of 92.4% (stdev = 3.6) was reported (Mickleborough et al., 2003). These high-predicted values in mild-moderate asthmatics are because of difference in the anthropometric characteristics of the reference equations used for the calculation of predicted values.

There are ethnic differences in lung volumes however, these differences are not clearly established (Cotes, 1993; Yang et al., 1991). These differences could be due to factors such as differences in trunk length relative to stature; differences in fat-free mass, chest dimensions and strength of respiratory muscles. In the current predictive equations, correction factors for African Americans and Asians have been recommended (Pellegrino et al., 2005; Stocks & Quanjer, 1995), however due to lack of studies pertaining to relationships between ethnicity and lung volumes these corrections may not be accurate. The ATS/ERS guidelines recommend that the predicted values must be obtained from

''normal'' or ''healthy individuals'' with '*same'* anthropometric characteristics such as age, sex and height and where relevant ethnic characteristics of patients being tested (Pellegrino et al., 2005). However, it is not possible to have '*same'* anthropometric and ethnic characteristics in a reference equation; therefore, predicted equations do not provide reliable values.

As a result, it is more reliable to use the *'measured values'* for FEV_1/FVC instead of predicted values; subsequently, in this thesis, '*measured values'* were used for asthma characterisation.

3.2.3 Current diagnostic criteria for asthma

Fixed criteria (80% ratio)

The current diagnostic ATS/ERS guidelines for asthma use a fixed criteria based on 80% FEV_1/FVC ratio; any individuals below the 80% ratio are characterised as asthmatics (Pellegrino et al., 2005). For this thesis the 80% FEV_1/FVC ratio will be used to characterise asthmatics and this will be referred as **'80% ratio'**.

There are other criteria for asthma characterisation that include Global Initiative for Asthma (GINA) criteria which states that the normal FEV_1/FVC ratio is 75-80% in adults and lower values are indicative of airflow obstruction (GINA, 2011). In several studies, fixed criteria (70% FEV_1/FVC) suggested by the guidelines from the Global Initiative for Chronic Obstructive Lung Disease (GOLD) have also been applied to asthmatic subjects (Roberts et al., 2006; Vollmer et al., 2009). *In the USA, UK and the rest of Europe, ATS/ERS **80% ratio** is widely used for diagnosis/characterisation of asthma, however; the validity of this 80% ratio in determining airway obstruction is questioned.* Cerveri and co-workers evaluated the pulmonary function characteristics and longitudinal outcomes of subjects aged 20–44 years from in the European Community Respiratory Health Survey dataset (ECRHS, n=6249) and found that an 80% ratio overestimates and conversely fixed criteria (70%) underestimates airflow obstruction in young adults (Cerveri et al., 2008). The use of these generalised fixed criteria have been acknowledged as source of misinterpretations and over/under diagnosis of asthma in different age groups (Hansen et al., 2007; Roberts et al., 2006; Swanney et al., 2008).

Lower limit of normality (LLN) criteria for asthma classification

The ATS/ERS have acknowledged these issues and have issued position statements for the use of a different characterisation approach using a 'lower limit of normal' (LLN) which is a value for a diagnostic value for the FEV_1/FVC ratio, derived from a matched healthy population (Miller et al., 2011; Pellegrino et al., 2005). ***A given PFT is said to be abnormal when the measured value is below the lower limit of normality (LLN), defined as 1.645 standard deviations FEV_1/FVC ratio (Miller et al., 2009*)**.

3.2.4 Fixed vs. LLN criteria

The sensitivity and specificity of fixed criteria have been calculated for ECRHS data set using the LLN as the gold standard for defining airflow obstruction (Cerveri et al., 2009). The authors suggest that a fixed criteria (70%) demonstrates low sensitivity and specificity compared to 75% criteria (100.0% sensitivity and 92.4% specificity), while 80% ratio provides 100% sensitivity but 58% specificity in males and similar patterns are observed in females. Though the importance of using LLN alongside fixed criteria, especially in the extremes of a population (i.e. age, stature) has been identified, there is currently no consensus on the use of LLN for diagnosing obstructive diseases (Celli et al., 2003; Margolis et al., 1997; Miller et al., 2009). In addition, *different sub groups of asthma such as mild-moderate/EIB show variability in symptom presentation; subsequently classification based on fixed criteria may lack sensitivity and under classify these individuals*. In the absence of asthma diagnosis, these sub groups of asthmatics may experience loss in the quality of life. For example, the DH recommends physical activity across the population (DH, 2011); in the absence of timely asthma diagnosis and relevant treatment, those susceptible to EIB (EIB-prone) will not be able to engage in physical activity

The use of LLN alongside the 80% ratio has the potential to overcome these issues, however the LLN is dependent on a comparable control population (matched for age, sex and ethnicity) to achieve specificity and sensitivity. This is a limiting factor for the application of LLN in diagnostic purposes and so far, the LLN criteria has only been used for research purposes. Thus, application of LLN is complicated in a clinical sector to quickly interpret if a patient's results are abnormal, however LLN has been widely applied in research for characterisation of asthma (Mannino, 2008; Miller et al., 2009).

In view of these arguments, this study classified asthmatics based on both, the 80% ratio and LLN.

3.2.5 Airway inflammation in asthma

Airway inflammation is a persistent feature in asthma pathophysiology and involves a number of inflammatory cells and their products; details on the pathophysiology of asthma are discussed in detail the literature review, chapter 1, page 15. A range of LTs (LTC_4, LTD_4 and LTE_4) are potent vasocontstrictors of human airways; these mediators affect microvascular and bronchial dilation, increase AHR and have been implicated in the pathogenesis of asthma (Barnes et al., 2006; Broughton et al., 1997; De Caterina & Basta, 2001). The 2 series PGs and 4 series LTs derived from AA (20:4, n-6) exert strong effects on airway function and increase airway inflammation (Barnes, 2008; Bloemen et al., 2007; Matsuoka et al., 2000; Ricciotti & FitzGerald, 2011; Swedin et al., 2009). In contrast, the 3 series PG_S and TXs and 5 series LTs derived from EPA (20: 5, n-3) have a reduced biological activity (Bagga et al., 2003; Calder, 2010a; Robinson & Stone, 2006). The reduced biological potency of EPA generated eicosanoids is because the eicosanoid receptors typically have a lower affinity to EPA derived mediators than those generated by AA (Tull et al., 2009; Wada et al., 2007). Overall the n-3 PUFA can exhibit a range of anti-inflammatory and pro-resolution effects and these are discussed in chapter 1 (page 28).

While inflammatory mediators such as LTs and histamine are suggested to be involved in acute and sub-acute inflammatory responses and in exacerbations, cytokines are important in maintaining chronic inflammation in allergic diseases. Many inflammatory cells (macrophages, mast cells, eosinophils and peripheral blood mononuclear cells, PBMC) have the ability to synthesise and these cytokines. Th1 derived cytokines including interleukin-2 (IL-2) and interferon-γ (IFN-γ), induce macrophage activation and are very effective in controlling infection with intracellular pathogens (Holgate., 2009; Bloemen et al., 2007). Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13 as primary cytokines and also help B cells in producing antibodies. Other cytokines such as IL-1ß, TNF-α and GM-CSF are released from different types of cells (e.g. macrophages and epithelial cells), and are considered important in increasing the inflammatory response, and prolong eosinophil survival in airways. TNF-α can cause an increase of histamine in allergic asthma causing vasoconstriction by secondary production of endothelin-1 (a peptide mediator) (Barnes, 2011; Chalmers et al., 1997, Goldie & Henry, 1999). An increase in the activity of Th2 cells

in asthma may be due to the reduction in regulatory T cells, which normally inhibit Th2 cells and increase in the natural killer (NK) T cells (Akbari et al., 2006; Larche et al., 2003). In this thesis the differences in the levels of inflammatory cytokines (IL-6 and TNF-α) will be measured in asthmatics and controls

3.2.6 Aims and hypothesis

The aim of this chapter was to characterise mild-moderate asthmatics based on 80% ratio and then separately characterise the same individuals using the lower limit of normality (LLN) from a control population.

Once the asthmatic population was identified, it was hypothesised that there would be a statistical difference between the dyspnoea score and circulating levels of inflammatory cytokines (IL-6 and TNF-α) of asthmatics and controls.

3.3 Methods

This section gives a brief description of the methods used in this study, for details refer to chapter 2.

3.3.1 Study protocol

The study was approved by Loughborough University's ethics committee; generic protocols were used for venepuncture (G03-P8) and specific protocols were used for pulmonary function testing (GO9-P8). The study was advertised in accordance with the University's policies through flyers, emails, meetings, presentations and personal contact. All participants gave full written consent for the study. Asthmatic and control participants were recruited based on previous clinical diagnoses and history of asthma (figure 3.1). Participants visited the laboratory on one occasion, lasting up to 1-2 hours, where they were briefed on the study procedure and were given an opportunity to familiarise themselves with the testing procedures; specifically spirometry. Anthropometric measurements (stature and body mass) were collected. Spirometry was conducted in accordance with ATS/ERS guidelines (Miller et al., 2005a,b). Venous blood was drawn from the antecubital vein for the analysis of inflammatory markers (IL-6 and TNF-α).

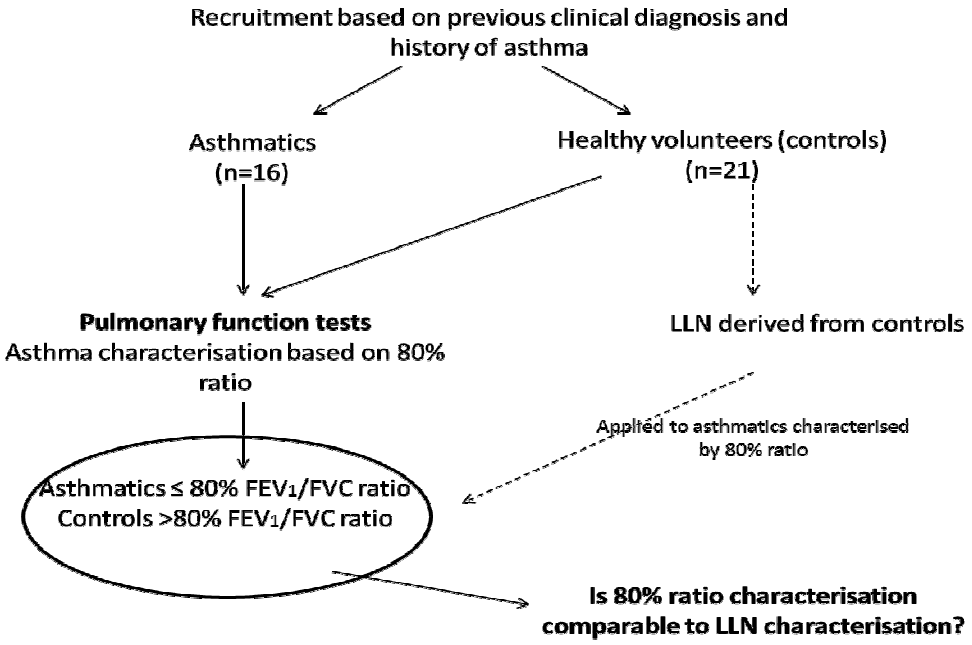


Figure 3.1 Study design to compare the validity of fixed cut off values and LLN

3.3.2 Participants

Thirty one males were recruited from Loughborough University and the local community. All participants in the asthmatic group had previously been clinically diagnosed as asthmatic and a history of shortness of breath, chest tightness and wheezing at some time on a weekly basis. The asthmatics had prescribed medication (n= 3 on bronchodilator and preventative; n=13 on bronchodilator only); there were 5 asthmatics out of the 13 (on bronchodilators) who were not regularly using their prescribed medication. Bronchodilator use was reported and testing was rescheduled if inhaler medication was used within the 48 hours preceding the testing. Additionally, the recruitment was completed within a short 3-month period to avoid the effect of seasonal variation. For the inclusion criteria, participants were recruited if they were between 18-30 years, had not taken any medication. The study design was such that an age range of 18-30 years was chosen to maintain consistent pulmonary function tests keeping in view maturation and ageing of lungs. Maturation of lungs is reached by 18 years and ageing of lungs begins after 30 years of age (Sharma & Goodwin, 2006). Participants were excluded if they had any respiratory infection within the past 8 weeks. The controls were healthy volunteers with no history of asthma and the two groups of participants demonstrated matching demographic and anthropometric characteristics. Participants were asked to refrain from coffee/alcohol and physical exercise 8 hours prior to testing to avoid any misinterpretation of the results (Rundell & Sue, 2010). Recruitment flyers and participant information sheets are attached in appendix 1.

3.3.3 Pulmonary function tests (PFTs)

Spirometry was performed by all participants in a seated position using a calibrated computerised spirometer (Superspiro; Micro Medical; Rochester, Kent, UK) following ATS/ERS guidelines (Miller et al., 2005a,b). Participants were required to perform three acceptable FVC manoeuvres with no greater than 5% variation for reproducibility and details of the technique are provided in chapter 2 (page 70). FEV_1 is an important pulmonary function value that is critically significant in the diagnosis of obstructive and restrictive diseases such as asthma, similarly PEF is a useful measure to assess the full potential of lungs. FEF 25-75% is an important measurement of airway function that describes the amount of air expelled from the lungs during the middle half of the FVC test.

Asthmatics have a reduced FEV_1, PEF and FEF 25-75% due to airflow obstruction because of inflammation, mucus and bronchoconstriction. The parameters recorded in this study were FEV_1, FVC, PEF and FEF 25-75%.

Characterisation based on 80% ratio

According to established guidelines for asthma severity, a fixed criteria of ≤ 80% for FEV_1/FVC ratio is representative of mild-moderate airflow obstruction (Miller et al., 2005a,b). Consequently, a fixed criterion of ≤ 80% was chosen in this study to characterise asthmatics. Any participants who exhibited an FEV_1/FVC ratio > 80% were characterised as controls.

Characterisation based on LLN

To confirm the characterisation with fixed criteria, a LLN was calculated from the measured values (litres) for FEV_1/FVC ratio of the control population based on ATS/ERS suggestions. All participants in the study groups were between 18-30 years of age; subsequently the calculated LLN was age specific representing age group of 18-30 years. The standard deviation of the FEV_1/FVC ratio of the control group was used in the calculation of LLN to identify the 5% of the ''normal'' subjects with the lowest values in the control population (Pellegrino et al., 2005). The equation used is shown below:

$$\mathbf{LLN = (FEV_1/FVC) - 1.645*standard\ deviation\ of\ the\ control\ population}$$

…….....equation 2

3.3.4 Dyspnoea

A Borg analogue scale was used by the participants to report symptoms before a PFT. The participants selected a number from 0-10 to report their perception of breathlessness (dyspnoea) at that time. A frequency count was carried out to compare dyspnoea between the asthmatics and controls.

3.3.5 Cytokine measurement

Cytokine levels in serum were measured using Standard ELISA techniques as described in chapter 2, page 106. IL-6 and TNF-α were determined using high-sensitivity ELISA (R& D Systems Europe Ltd, Abingdon, U.K., catalogue number HS600B and HSTA00D). Whole blood was collected in a serum tube and allowed to coagulate for up to an hour following which the tubes were centrifuged at 1300 x g for 10 minutes. Serum was separated and aliquots were made and stored at -20ºC until further analysis.

3.3.6 Data analysis

Differences between asthmatics and controls were analysed using unpaired two-tailed t-tests at a significance level of $p<0.05$ with equal variances assumed (Levine's test). All statistical analysis was undertaken using SPSS version 19.0 (SPSS Inc.; Chicago, IL). A chi-squared test was used to assess the difference in reported dyspnoea score between asthmatics and controls at significance level of $p<0.05$.

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3.4 Results

The following section presents the main findings from this study. All results are reported as mean ± SEM unless stated otherwise.

3.4.1 Descriptive statistics

There was no difference between the asthmatics and controls in terms of age, stature, body mass, or BMI (table 3.1). The PFTs showed significant differences in lung function between the two groups. The asthmatics showed significantly lower values for FEV_1 (asthmatics = 3.79 ± 0.16 litres, controls = 4.45 ± 0.11 litres, $p < 0.05$), PEF (asthmatics = 533 ± 13 litres/min, controls = 575 ± 8 litres/min; $p=0.06$) and FEF 25-75% (asthmatics = 4.18 ± 0.15 l/sec, controls = 4.61 ± 0.10 l/sec) than controls. The asthmatics also showed a lower FEV_1/FVC ratio compared to controls (asthmatics = 71.23 ± 1.17%; controls = 88.74 ± 0.95%). FVC represents the vital capability of the lungs and as expected there were no significant differences in FVC for the two groups (asthmatics = 5.32 ± 0.20 litres, controls = 5.02 ± 0.14 litres; $p=0.21$). These data demonstrate that the study population comprised of individuals matched for age and body size with significant differences in pulmonary function. The results clearly indicate the presence of airway obstruction in asthmatics.

Table 3.1 Participant characteristics

	Controls (n=21)	Asthmatics (n=16)
Age (years)	21.67 ± 1.68	25.81 ± 1.07
Stature (metres)	1.78 ± 0.02	1.76 ± 0.02
Body mass (kg)	74.92 ± 2.02	75.81 ± 1.78
BMI (kg/m^2)	23.64 ± 0.50	24.56 ± 0.38
FEV_1 (litres)	**4.45 ± 0.11**	**3.79 ± 0.16 ***
FVC (litres)	5.02 ± 0.14	5.32 ± 0.20
PEF (l/min)	**575 ± 8**	**533 ± 13 ***
FEV_1/FVC	**88.74 ± 0.95**	**71.23 ± 1.17***
FEF25-75%(l/sec)	4.61 ± 0.10	4.18 ± 0.15*

*statistically significant difference ($p<0.05$)

3.4.2 Justification for excluding predicted values for FEV_1/FVC ratio from the analysis

The mean predicted values of FEV_1/FVC ratio for controls was 106 (range 100 – 120) Values higher than 100% of predicted are sometimes seen in 'healthy individuals' and this may be due to dysanaptic growth or unequal growth of airways and lung parenchyma (Hyatt et al., 1986). This pattern is labelled as a potential 'normal physiologic variant'' and is suggested as being not unusual among physically fit non-smoking individuals (Pellegrino et al., 2005). Since the study population in this thesis comprised of young healthy individuals with mild-moderate asthma, higher predicted values than 100%, subsequently, under-classification can occur based on fixed criteria that have proven issues of under diagnosis in young adults. Thus, higher predicted values in the study population comprising of healthy males (106% for controls and 86% for asthmatics) was considered unreliable for analysis in this thesis, which could cause under diagnosis of asthma. Therefore, measured values were used for further characterisation and analysis

3.4.3 Characterisation based on 80% ratio

Using a fixed ratio, 21 participants were characterised to have a normal pulmonary function with a FEV_1/FVC ratio of >80 and constituted the control group (mean = 88.74 ± 0.95). A total of 16 participants showed ≤80% FEV_1/FVC ratio (mean = 71.23 ± 1.17%). The two groups were statistically different with asthmatics having a lower mean value of FEV_1/FVC ratio ($p<0.05$, figure 3.2). As expected, the asthmatics showed significantly lower FEV_1, PEF and FEF 25-75% as shown in table 3.1.

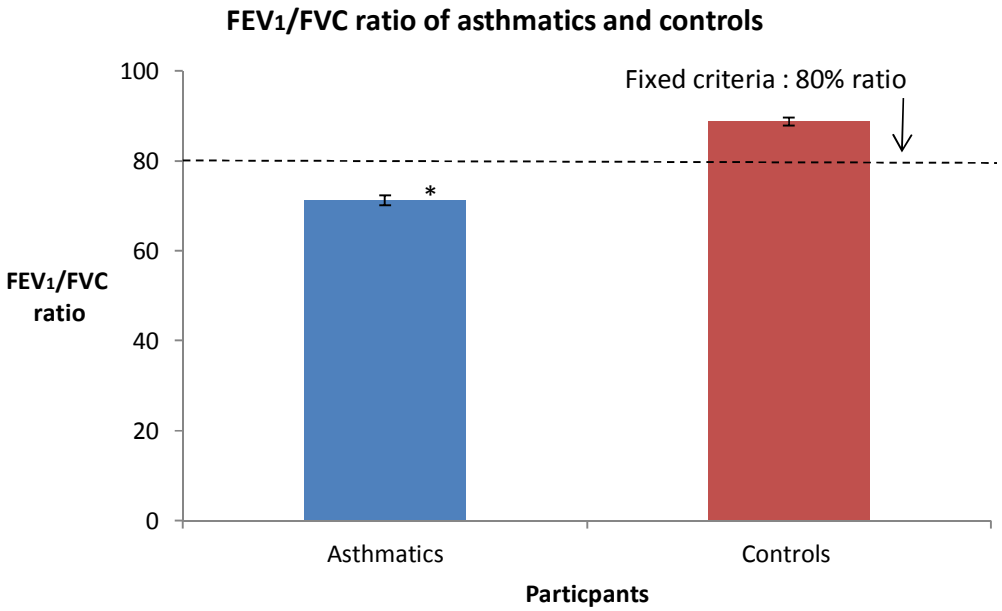

Figure 3.2 Graph showing differences in a FEV_1/FVC ratio of asthmatics and controls based on the fixed criteria of 80%
* Statistically significant difference in asthmatics compared to controls ($p<0.05$)

3.4.4 Characterisation based on LLN criteria

When the control population (FEV_1/FVC ratio = 88.74 ± 0.95) were used to calculate the LNN then a value of 82% was indicated which meant that all 16 individuals previously characterised as asthmatics were confirmed as mild to moderate asthmatics with the LLN criteria for asthma characterisation. The calculated LLN was specific to a 18-30 year age group and was derived from the control population comprising of healthy individuals with no history of asthma (n = 21).

The FEV_1/FVC ratio of all the participants previously characterised by fixed criteria were below the LLN of 82% (mean = 71% range 65-80%). Therefore all asthmatics within the age group of 18-30 years previously characterised by fixed criteria were confirmed as asthmatics as their FEV_1/FVC ratio fell below the LLN criteria (n=16).

3.4.5 Dyspnoea score

The distribution of the dyspnoea score for the two groups on the day of testing, prior to the PFTs is shown in figure 3.3. There were 57% participants in the control group with a score of 0 suggesting no breathlessness compared to only 6% asthmatics reporting the 0 score. There were 33% of controls reporting very very slight breathless compared to 25% of asthmatics (score = 0.5). While 10% of controls reported very slight breathlessness, there were 19% of asthmatics reporting the same score (score =1). There were 44% of asthmatics with slight breathlessness (score =2) and 6% with moderate breathlessness (score = 3). None of the control participants reported scores greater than 1. The most frequently reported score for asthmatics was 2 representing slight breathless while the most frequent score for controls was 0 representing no breathlessness. Score of 2 and 3 were combined for analysis as only 12% of the expected values fell within these ranges, based on the chi square analysis there was a significant difference between the dyspnoea scores of the two groups ($\chi^2 = 18$, df = 3, $p < 0.001$)

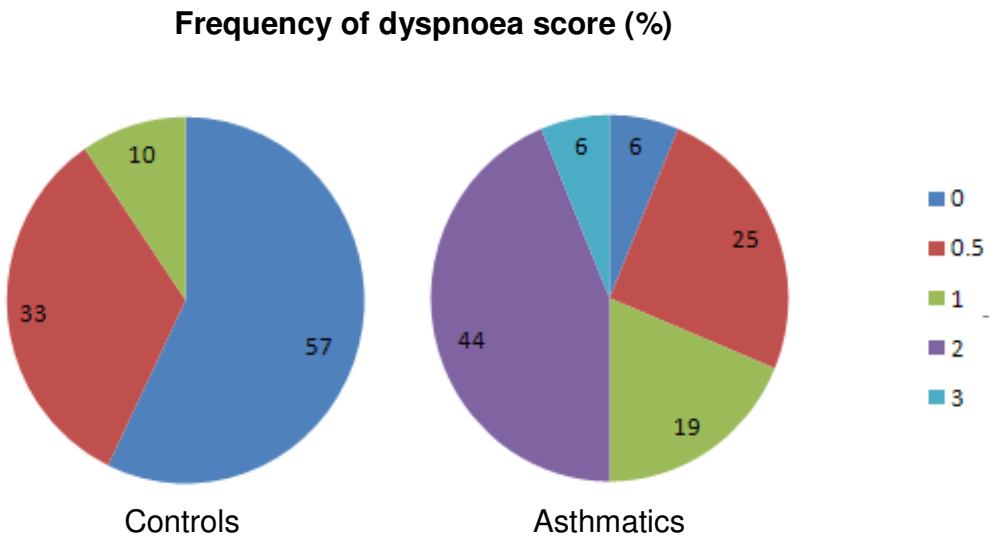

Figure 3.3 Pie chart showing the frequency of dyspnoea score for the two groups, the numbers inside the chart represents the percentage of participants showing a score.

3.4.6 Serum cytokine levels

As expected, the asthmatics showed a significantly higher level for IL-6 (mean = 1.66 ± 0.08 pg/mL) than controls (mean = 1.38 ± 0.06 pg/mL) ($p<0.05$). Similarly asthmatics had elevated levels for TNF-α than controls (asthmatics, mean = 4.28 ± 0.25pg/mL, controls, mean = 3.35 ± 0.26) (<0.05). Mean data is displayed in figure 3.4.

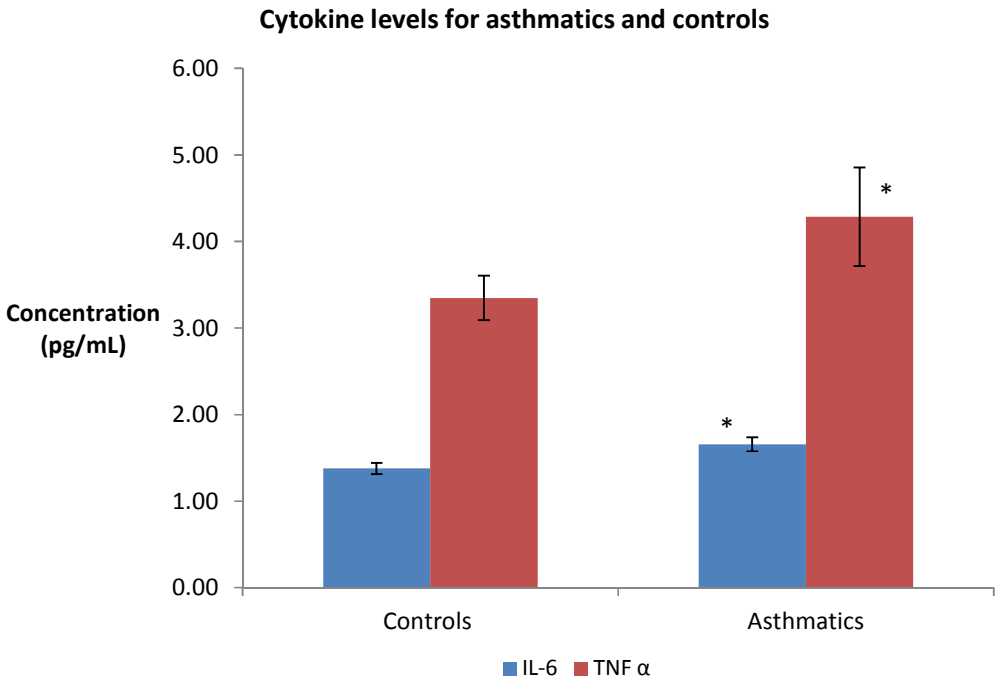

Figure 3.4 Differences in serum cytokine levels for asthmatics and controls based on the fixed cut off value of 80%; respectively
* statistically significant difference between controls and asthmatics ($p<0.05$)

3.5 Discussion

Asthma is an obstructive lung disease that is routinely assessed by symptoms (dyspnoea) and pulmonary function tests. Fixed criteria (80% ratio) are conventionally used in the clinical set up for diagnosis of asthma. Mild-moderate asthma and EIB are subtypes of asthma that represents a large proportion of asthmatics. EIB affects approximately 90% of asthmatics and 10% of healthy population. The public health guidelines from Department of Health (UK) acknowledge the relationship between lack of physical activity and chronic diseases including asthma; consequently physical activity is encouraged across the population. Accurate and timely diagnosis of asthma and other respiratory diseases are vital to ensure appropriate therapies are provided. Symptoms and PFTs alone do not provide a correct diagnosis of the varied and complex respiratory conditions such as mild-moderate asthma and EIB. Furthermore, there are issues of non-specificity associated with 80% ratio and ATS/ERS have issued position statements to address this. Most recently, LLN derived from a control population had been suggested as an 'alternative criteria' to be used alongside 80% ratio for assessment for respiratory diseases (Miller et al., 2009; Pellegrino et al., 2005). A LLN can be derived from a matched control population that allows for a reliable diagnosis of asthma. In this study mild-moderate asthmatics were characterised by an 80% ratio with asthmatics showing a significantly lower FEV_1/FVC ratio (mean = 71% , range: 65-80%) than controls. In addition, asthmatics showed lower PEF, FEV_1 and FEF 25-75% which confirmed airflow obstruction. A LLN was derived using the control population included in this study and was specific for 18-30 year old males. All the participants in this study were recruited in a short 3 month period to avoid the effect of seasonal variation. In this study, the 16 asthmatics classified using 80% ratio were confirmed by LLN and this suggests that both, the fixed criteria and the LLN provide comparable diagnosis of mild-moderate asthma.

To address the limitations of predicted values derived from reference population, these values were excluded from analysis and only measured values were used. In this study though the 16 asthmatics classified using fixed criteria were confirmed by LLN, a number of studies have shown under or over diagnosis based on fixed criteria. Since the predicted FEV_1/FVC ratios are derived from reference population (comprising of healthy individuals) and is dependent on stature and age, subsequently the validity of the fixed criteria has been questioned (Cerveri et al., 2009; Miller et al., 2009; Roberts et al.2006). Mild-moderate asthmatics have been suggested to have a predicted FEV_1/FVC ratio of 61-69%

(moderate asthma) and >70% (mild asthma). Since predicted values are dependent on age and stature, there is a likelihood that underestimation of airway obstruction can occur for young adults (18-30 years) when using a fixed criteria based on predicted values (Cerveri et al., 2009). Based on these issues, predicted equations were not measured in our study.

If the dyspnoea scores recorded in this study were matched to the FEV_1/FVC ratio, individuals with a poor FEV_1/FVC ratio (<70%) show worst symptoms and lower PEF (518 l/min) than those who have a relatively better FEV_1/FVC ratio (>72%, PEF = 558l/min). This suggests that possible mischaracterisation could occur if fixed criteria were used using the GOLD fixed criteria (70%). Participants with FEV_1/FVC ratio (72-80%) representing mild asthma may not be accurately classified as the symptoms of these participants are minimal, subsequently there is a likelihood of "under called" obstruction leading to false negatives. This pattern is in line with the findings that suggest an 80% ratio overestimates and conversely fixed criteria (70%) underestimates airflow obstruction in young adults (Cerveri et al., 2008). Additionally, Hansen and co-workers have shown using a GOLD fixed criteria (70%) results in high prevalence of airway obstruction in older adults (>70 years of age). The authors have demonstrated that the fixed criteria has a strong negative age dependency, and the frequently used fixed criteria (70%) may not be attained till 50 years of age in males and later in females (Hansen et al., 2007). This suggests that airway obstruction in younger adults may be missed if a fixed criteria (70%) is used. In our study, the mean FEV_1/FVC ratio of the asthmatic group was 71.23 ± 1.17% and had the GOLD criteria been used, a number of individuals would have been under characterised.

Similarly, for other obstructive diseases such as COPD, Miller and co-workers., (2011) have reported significant discrepancy rates in PFT interpretation between the fixed criteria (70%) and LLN methods in populations from the UK, New Zealand, and the USA (Miller et al., 2011). Approximately 24% of the older participants from these countries were misclassified and the analysis showed that about 10% of younger patients with normal lung function were falsely characterised for airway obstruction and were subsequently included in the disease category. Therefore, there are proven risks of over diagnosis in younger adults using fixed criteria values and the ATS/ERS statements (Miler et al., 2009) have acknowledged these issues; it is likely that future guidelines will address this topic.

Thus, use of these generalised fixed criteria has been rightly identified to cause misinterpretations and over/under diagnosis of asthma in different age groups.

To evaluate the discrepancies related to predicted values, the LLN from this study can be compared with similar studies. The LLN derived from predicted equations have varied widely between studies but consistently dropped with age. In an analysis based on 57 studies on healthy males and females worldwide between the ages 20-80 years, the LLN has been found to be lower than 70% at various ages in a number of studies (Swanney et al., 2008). In addition, for younger age group (20-30 years) the LLN was found in the range of approximately 68 - 87% (Swanney et al., 2008). In another study, Cerveri and co-workers calculated a LLN of approximately 76% for males aged 20-30 years from a large European cohort using very rigid inclusion criteria for obstructive lung diseases, smoking history, breathlessness, cough and wheeze (Cerveri et al., 2008). This clearly demonstrates the variation in LLN across different studies for the same age group. These differences could be attributed due to the heterogeneity in height, sex and ethnicity in the 'predicted equations' used in different studies (Collen et al., 2008; Miler et al., 2011).

Based on the discrepancies in asthma characterisation, symptoms and spirometry are not sufficient to appropriately diagnose different sub types of asthma, thus additional markers of airway inflammation and symptoms can support the diagnosis of asthma. Asthma is now characterised to exhibit localised as well as systemic inflammation. The strength of our study was that both the 80% ratio and LLN were used to characterise asthmatics, in addition, the circulating levels of inflammatory cytokines were measured and asthmatics showed elevated levels of inflammatory cytokines (TNF-α, and IL-6) than controls (IL-6, asthmatics = 1.66 ± 0.08 pg/mL; controls = 1.38 ± 0.06 pg/mL; TNF-α (asthmatics = 4.28 ± 0.25pg/mL, controls = 3.35 ± 0.26). These values demonstrate that asthmatics have a higher level of inflammation, which underlines the pathophysiology of asthma.

In a recent study by Subrrahmanyam et al., (2011), levels of inflammatory cytokines including IL-6 and TNF-α were compared in atopic and non-atopic adult asthmatics; the non-atopic asthmatics showed serum levels of 0.81 ± 0.47pg/mL and 5.16-± 4.40pg/mL for IL-6 and TNF-α respectively. In another case control study asthmatics have been shown to have approximately 7 fold higher value for serum IL-6 than controls (asthmatics =15.49 ± 1.65pg/ml, controls = 2.10 ± 0.45pg/ml) (Canöz et al., 2008). In our study, the observed

value for IL-6 and TNF-α is representative of normal healthy participants when compared to other studies. Kim et al., (2011) have reported values of 2.91 ± 6.45 pg/mL and 3.21 ± 4.04 pg/mL for IL-6 and TNF-α respectively in healthy males (<45 years), which is comparable to the values measured to our study. For TNF-α, a recent study comparing the levels in asthma and controls have shown a slightly elevated level in asthmatics (asthmatics = 5.7(4-8) pg/mL, controls = 4.9(4-8) pg/mL) (Pukelsheim et al., 2010). Another study evaluating the role of airway hyperreactivity has reported values of 4.12 ± 0.43pg/mL in asthmatics (Halasz et al., 2002). Therefore, the values measured in this study represent normal values for mild-moderate asthmatics that have been reported within the literature.

Depending on the severity of asthma, differences in the levels of these circulating markers have been observed. In COPD, asthmatics have shown to have serum IL-6 and TNF-α values in the range of 1.77-11.26 pg/mL and 3.62 - 7.84 pg/mL respectively and this was found to be dependent upon the stage of COPD (Samy et al., 2010). Similarly for lung cancer risk, a case–control study within the prospective Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening trial reported serum IL-6 levels of 3.7 pg/mL(2.3-7.2pg/mL) in asthmatics and suggested an association between elevated serum IL-6 levels and lung cancer (Pine et al., 2011). Several reports have shown that elevated IL-6 levels are found in plasma, bronchoalveolar lavage, and lung epithelial cells of asthmatics (Kicic et al., 2006; Marini et al., 1992; Neveu et al., 2010). There is some evidence from severe and mild-moderate asthmatics that there is an inverse association between induced sputum IL-6 levels and pulmonary function outcomes including FEV_1/FVC ratio or PEF (Dixon et al., 2006,2008; Neveu et al., 2010), however further studies are required to assess to confirm associations between circulating levels of inflammatory cytokines and pulmonary function outcomes. Thus, measurement of inflammatory cytokines may be important measures for assessment of asthma severity and could be conducted parallel with physiological assessment (dyspnoea and spirometry). Though systemic markers have gained interest in present times there are limitations which need to be addressed. These systemic markers may provide the information about the general inflammatory state; however more specific localised markers derived from localised cells and tissues (such as alveolar macrophages) provide a detail insight into any structural and functional abnormities of the pulmonary system. However in non-clinical research setting, access to airway cells and tissues may be limited, subsequently systemic markers can be

considered. Besides, circulating biomarkers are associated with asthma onset and are considered good candidates as predictors for rapid decline of FEV_1 in obstructive lung diseases (Pukelsheim et al 2010; Samy et al., 2010); subsequently monitoring changes in the cytokine levels during intervention trials can be considered a useful indicator of response to treatment. Recently indirect estimation of inflammation in the lung via exhaled breath nitric oxide (FeNO) has been suggested to be used for characterising asthma (ATS/ERS, 2005; Dweik et al., 2011; Zitt et al., 2005). This measurement can now be conducted by using a portable device in clinical settings or in patient's home and has been shown to be accurate for monitoring changes to therapy (Chen et al., 2007; Kim et al., 2012). If this study was repeated then FeNO measurements could be obtained to characterise mild-moderate asthmatics and distinguish them from the healthy non-asthmatics. FeNO measurements were conducted in the supplementation studies presented in this thesis (chapter 4 and 5) to evaluate the changes related to inflammation.

Currently the clinical application of the LLN criteria is limited for diagnosis as there are no guidelines provided by the regulatory authorities such at ATS/ERS, BTS and GOLD (Miller et al., 2009). However, the application of LLN is widely recognised and applied in research settings. When LLN are used along with standard reference equations for predicted values, there is a much reliable diagnosis of airway obstruction. The strength of this study was a characterisation of the asthmatics based on 80% ratio and LLN derived from a healthy control population (matched for age). In addition, this characterisation included participants aged 18-30 years to allow for an age specific characterisation. This study has formed the basis of classification for the next chapter in this thesis (chapter 4) which was aimed to assess the effect of n-3 supplementation in mild to moderate EIB - prone asthmatic males. The LLN derived from the population of healthy males in our study can only be used for assessment of airflow obstruction in males and cannot be applied to females due to differences in stature and physiology (chapter 5).

3.6 Conclusions

Both the 80% ratio and LLN criteria were found to be comparable methods for assessing airflow obstruction in young adults with mild-moderate asthma. Therefore, LLN can be used alongside fixed criteria for reliable characterisation of asthma for this population. To support the physiological assessment, circulating levels of IL-6 and TNF-α provide an indication of inflammation; asthmatics demonstrate significantly elevated levels of these cytokines compared to controls. The LLN derived from a healthy control population in this chapter was used to recruit and characterise asthmatics for the next study (chapter 4) to assess the effect of n-3 supplementation in asthmatics. In addition, LLN derived from this study is specific for males only (aged 18-30 years) and should not be used for females.

Chapter Four

Effect of a three-week dietary supplementation with EPA/DHA on exercise induced bronchoconstriction in mild-moderate asthmatic males

4 Effect of a three-week dietary supplementation with EPA/DHA on exercise induced bronchoconstriction in mild-moderate asthmatic males

4.1 Abstract

There is limited evidence suggesting the beneficial effects of two n-3 PUFAs namely EPA and DHA on EIB. EIB affects approximately 90% of asthmatics and approximately 10% of healthy individuals with no history of asthma, consequently this limits the ability of a large proportion of the population to meet physical activity guidelines. Due to health side effects related to pharmacological medication, the use of complementary therapies including omega 3 supplementation are gaining impetus. This study was designed to assess the impact of dietary supplementation with EPA/DHA on EIB participants to evaluate changes in their post exercise pulmonary function. Participants were included based on lower limit of normal (LLN) derived from a control population used to confirm abnormal spirometry (chapter 3), a drop of >10% in FEV_1 following exercise challenge tests, and elevated levels of FeNO (>40ppb). A randomised crossover trial (RCT) design with three weeks of supplementation (3.2 grams of EPA and 2.2 grams of DHA or placebo) with a three week of wash phase was used. Post exercise changes in pulmonary function were measured at baseline and after n-3, placebo and wash phases to assess changes after supplementation. Secondary markers of pulmonary function, namely FeNO and dyspnoea, were measured to monitor any changes across all phases. Venous blood samples were collected at all time-points to estimate changes in concentration of n-3 and n-6 PUFAs of interest (LA, ALA, AA, EPA and DHA) within the PBMC total lipids. Additionally, serum levels of inflammatory markers (IL-6, TNF-α and IL-10) were measured. Results showed a significant improvement in post exercise pulmonary function in participants with EIB after EPA/DHA supplementation, which was supported by a significant reduction in serum IL-6 levels. There were no significant changes to total lipid composition of PBMC for key fatty acids assessed in this thesis; however, there was a non-significant trend showing increases in total PBMC EPA and DHA with a reduction in AA after supplementation. There was no significant improvement in exhaled breath nitric oxide or serum levels of TNF α and IL-10. Three weeks of dietary n-3 supplementation showed a beneficial impact on pulmonary function for EIB participants.

4.2 Introduction

Exercise-induced bronchoconstriction (EIB) is characterised by transient narrowing of airways after exercise (Anderson et al., 1975; McFadden & Gilbert, 1994). Occasionally the narrowing of airways can occur during exercise (Beck et al., 1994; Rundell et al., 2003; Suman et al., 1999). For individuals with EIB a brief period of exercise or hyperventilation triggers airflow obstruction, which lasts for 30-90 minutes in the absence of treatment (Anderson, 2010; Hallstrand, 2012). Up to 90% of people with clinically diagnosed asthma experience EIB at some point during their lives. The majority of those affected by EIB consider exercising to be a major precipitant of their symptoms and as a result, their involvement in physical activity is affected. Approximately 10% of the healthy population with no history of asthma also show symptoms of EIB at some point during their lives following exercise or hyperventilation (ATS/ACCP, 2003; Anderson & Kippelen, 2005, Rundell & Jenkinson, 2002). EIB is primarily identified by recording a post exercise reduction in forced expiratory volume in 1 second (FEV_1) of 10% to 15% of the pre exercise value (Haby et al., 1994; Sterk et al., 1993). The value for FEV_1 may start falling during exercise; however, the lowest value is usually measured 5-12 minutes after the end of exercise.

EIB results from the loss of water by evaporation from the airway surface while inhaling large volumes of air in a short period of time. Detailed mechanisms underlying the pathophysiology of EIB are described in chapter 1(page 24) (Anderson, 1984; Anderson & Daviskas, 1992; Anderson & Kippelen, 2010; Carlsen et al., 2008; Deal et al., 1979). There are a number of lipid mediators (derived from AA, 20:4, n-6 PUFA) in the cell membranes which an act on bronchial smooth muscle to cause contraction and narrowing of airways. In the airway epithelium of asthmatics, there are large numbers of mast cells, epithelial cells, glandular cells and sensory nerves that are affected by airway dehydration and osmotic changes. Increased levels of PGD_2 and PGF_2a have been found in urine and sputum samples of EIB subjects suggesting a key role of these mediators in the severity of EIB symptoms (Haverkamp et al., 2005; Kippelen et al., 2010; Pavord et al.1993, 1995; Sastre & Pozo, 2012). In addition, neutrophils, monocytes and macrophages produce LTB_4, while LTC_4, LTD_4, and LTE_4 are produced by mast cells, basophils, and eosinophils. LTB_4 is a strong leukocyte chemotactic agent and an activator of neutrophils, which induces release of ROS and lysosymal enzyme. In addition LTB_4 promotes secretion of

inflammatory cytokine such as TNF-α, IL-1(ß) and IL-6 by macrophages (Calder, 2012; Tilley et al., 2001). LTC_4, D_4 and E_4 increase vascular permeability, increase mucous secretion, increase hypersensitivity and cause bronchoconstriction. Thus, LTs, histamine and PGs help determine the severity of loss in FEV_1 after exercise and are important in the persistence of EIB symptoms (Duong et al., 2008; Gulliksson et al., 2006; Moloney et al., 2003). The modifying effects of specific antagonists and COX inhibitors on EIB suggest a potential role of these mediators in EIB severity and incidence. An increased concentration of AA derived mediators is found in arterial plasma, induced sputum, and urine following exercise (Anderson & Kippelen, 2010).

The Department of Health (DH) guidelines have suggested a role for diet and physical activity in maintaining health. Those with asthma and EIB often do not achieve the recommended targets for daily physical activity and this has implications for quality of life. Current therapies for asthma and EIB include short acting bronchodilators (including short acting β agonists) and long-term control medication (such as corticosteroids, LT modifiers, long acting β agonists). Though these therapies are effective in the short term, issues such as tachyphylaxis have complicated usage of β agonists; in addition, serious systemic and local side effects of inhaled corticosteroids include conditions such as osteoporosis and glaucoma. Another issue related to current pharmacological therapies is that these medications are broad spectrum and not specific, thus, there is an impetus towards specific therapies that target a single inflammatory mediator and are less likely to have major health side effects (Barnes, 2002, 2010; MacRedmond & Dorscheid, 2011). Many asthmatics are not satisfied with the long-term side effects of these pharmacological therapies and are resorting to alternative therapies (GINA, 2011; Mickleborough et al., 2008).

Evidence from observational studies have suggested a beneficial role of fish oil (omega -3 fatty acids) in asthma. There are no major health side effects associated with the short term or long-term administration of fish oil, subsequently the use of fish oil as a complementary therapy for asthma is becoming popular. Omega 3 (n-3) PUFAs form the structural components of cells and have been shown to exert a range of anti-inflammatory and pro resolution effects following different mechanisms as described in the literature review. There are only a limited number studies investigating the effect of n-3 (EPA/DHA) on EIB subjects and relevant trials are described in the literature review, (page 45). Lung

function measurements have been the primary outcome of asthma supplementation trials and are assessed by variables such as FEV_1, FVC and PEF. While some studies have shown improvement in pulmonary function outcomes, these results are not consistent. In two studies reported by Mickleborough and co-workers, (2003, 2006), no changes in pulmonary function were reported after 3 weeks of n-3 supplementation (3.2 gram EPA and 2.2 gram DHA/day) in asthmatics with EIB. However, these studies have shown significant improvement in post exercise FEV_1 in EIB athletes and recreationally active subjects (Mickleborough et al., 2003, 2006).

FeNO is considered a non-invasive marker for airway inflammation and is increasingly becoming an adjunct diagnostic technique used alongside spirometry. Schubert and co-workers (2009) (n=23, atopic asthma) have shown that dietary supplementation with n–3 PUFA-enriched fat blend (0.69 g/day, n=12 participants) for 5 weeks was effective in reducing FeNO levels within 3 weeks of n-3 supplementation. Though the levels of FeNO increased during allergen exposure in both groups (n-3 and placebo), the mean increase was significantly lower in the n–3 PUFA group. In another double blind, placebo-controlled pilot study, a shorter duration of supplementation was used (n=20; dose 0.9 g EPA and 0.65 g DHA/day) for 2 weeks (Moreira et al., 2008). The study showed no changes to FeNO levels, FEV_1 or self-reported asthma quality of life questionnaire scores. However, this study was not a well-controlled trial and the participants were on their regular medication of inhaled corticosteroids. In addition, the dose and duration of supplementation was not sufficient to compare this study to other studies.

These prior studies, and others reviewed in the literature review suggest that dietary supplementation with n-3 PUFA may lead to some improvement in asthma symptoms (PEF and FEV_1) and other markers of inflammation (FeNO). These studies are particularly suggestive of the protective effects in asthmatics with EIB symptoms. The inconsistencies in findings can likely be attributed to heterogeneity among the studies. These inconsistencies in the findings of the studies are possibly due to the different dosages, duration of the studies, mixed groups (males/females) and the sub group of asthmatics studied.

An obvious gap in the current literature is a lack of studies investigating the effects of n-3 supplementation in EIB individuals with a non-athletic background who represent a large proportion of the asthmatic population and a significant amount of the healthy population. Therefore, this study was designed to assess the effect of a 3 week EPA/DHA supplementation in recreationally active EIB prone males.

4.2.1 Aims and hypothesis

The aim of this study was to assess the effect of EPA/DHA supplementation on post exercise FEV_1 in mild – moderate EIB prone males.

It was hypothesised that there would be a statistically significant improvement in post exercise FEV_1 after a three-week EPA/DHA supplementation.

To support this study, EIB prone mild – moderate asthmatic males were recruited using the previously derived LLN. To evaluate the effects of EPA/DHA supplementation on pulmonary function in EIB-prone males, dyspnoea score and FeNO levels were simultaneously measured. Additionally, the effect of EPA/DHA supplementation on the total lipid composition in PBMC was assessed. Circulating levels of IL-6, IL-10 and TNF-α were measured to evaluate the changes in cytokines with supplementation.

4.3 Methods

This section contains a brief description of the methods used during this study and will refer to chapter 2 for further particulars.

4.3.1 Sample size calculation

A double blinded, placebo controlled, randomised, crossover trial was carried out to assess the effects of a 3 week supplementation with n-3 (EPA/DHA) on EIB participants. The method for randomisation by minimisation and double blinding is described in chapter 2; page 111. A power analysis was completed to approximate the appropriate sample size based on previous studies showing the effect of n-3 PUFAs on pulmonary function in EIB subjects (Mickleborough et al,. 2003, 2006). The design aimed to observe a minimum detectable difference or change of 0.3 litres in FEV_1 after exercise with the supplementation. The significance level was set at alpha = 0.05 and the power at 0.80. Using these data, a sample size of 8 participants was necessary to detect a difference of this magnitude. To allow for fluctuations in participant compliance, withdrawals and incomplete data, a target sample size was set at 12 participants. Considering the sample size was small, the participants were randomly allocated using a minimisation scheme (Altman & Bland, 2005), whereby next allocation of supplementation capsules was dependent on the supplementation provided to the previous participant. This scheme of randomisation has been found to be appropriate to achieve balance between the treatment groups. The participants, investigator (I) and outcome assessors (supervisors) were blinded to the study; the randomisation pattern was kept with the technician in charge of randomisation.

4.3.2 Study Design

The placebo-controlled crossover trial was conducted over 12 consecutive weeks wherein each subject acted as their own control and underwent both of the supplementation phases (figure 4.1). All participants entered the study on their normal diet (phase 1/baseline1), after which they were randomly allocated to receive 10 soft gelatin capsules per day containing either n-3 (3.2 grams of EPA, 2.2 grams of DHA and tocopherol (Biocare, UK) or Placebo (olive oil, Bionovate, UK) [phase 2]) with a 3 week washout phase in between (normal diet; phase 3, baseline 2). Thereafter the participants switched

over to the alternate supplementation for the remaining 3 weeks (phase 4). A 3 week end of study washout followed the second supplementation phase (normal diet; phase 5). The study design is shown in Figure 4.1. The participants were screened for EIB (≥ 10% drop in post exercise FEV1) and high FeNO (≥ 40ppb) at baseline 1. The participants reported to the lab for physiological testing at the two baselines, after the completion of supplementation phases and after washout. PFTs were assessed pre-exercise and post exercise at 1, 5, 10, 15, 30 and 45 minutes or until recovery (whichever was earlier).

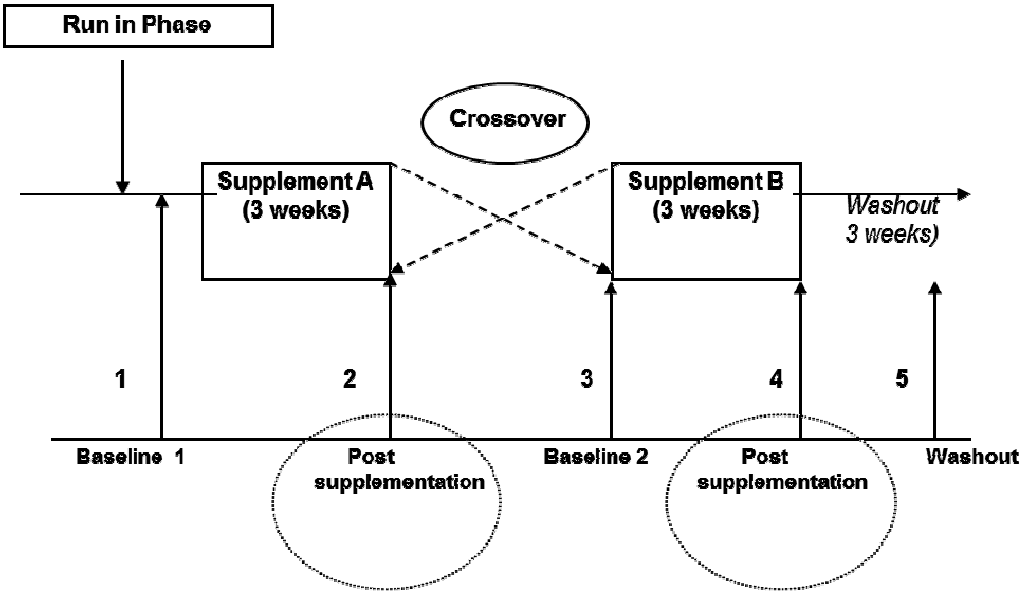

Figure 4.1 Study design for n-3 supplementation

At each visit, blood was drawn before exercise by a certified phlebotomist from the antecubital vein for PBMC extraction and measurement of serum cytokines, namely TNF-α, IL-6 and IL-10. All subjects recorded bronchodilator use during the supplementation phases (Phase 2 and 4). Additional samples were collected at the end of washout phases to establish that individuals had returned to their baseline level for all the physiological parameters.

Justification for dose and duration of study

A dose of between 0.5 to 3.5 grams of EPA/DHA per day has been shown to improve pulmonary function, inhibit CystLT production by neutrophils and polymorphonuclear cells *in vitro* and decrease inflammatory cytokine production (Arm et al., 1989; Emelyanov et al., 2002; Hodge et al., 1998; Mickleborough et al., 2003, 2006; Surette et al., 2008; Schubert et al., 2009). In rheumatoid arthritis a dose of approximately 3.5 grams of EPA+DHA per day has been shown to have anti-inflammatory effects via decreased IL-1 production by monocytes, decreased plasma IL-1β concentrations and decreased serum TNF-a concentrations (Esperson et al., 1992; Kolahi et al., 2010; Peter et al., 1999). In a dose response study by Rees et al. (2006) using healthy participants supplementing with a ''high dose'' of 2.7 gram/day for EPA resulted in reduced ex vivo PGE_2 production by stimulated mononuclear cells compared to a ''low dose'' of 1.35 grams of EPA/day. A number of studies looking at inflammation have suggested a threshold for an anti-inflammatory effect to be exhibited by n-3 supplementation and this is expected to be in range of 1.3 -2.7 grams EPA per day (Calder, 2006, 2012; Dignass et al., 2004; Kelley et al., 1999; Kew et al., 2004; Grimble et al., 2002). In athletes with EIB, a dose of 3.2 grams EPA and 2.2 grams DHA has been shown to be effective in improving post exercise pulmonary function (Mickleborough et al., 2003, 2006). Therefore, a dose of 3.2 grams EPA + 2.2 gram DHA was used in this thesis in an attempt to reduce the severity of EIB.

The major supplementation studies in asthma (as outlined in chapter 1) have used supplementation duration ranging from 3 weeks up to 9 months. There is a significant incorporation of EPA and DHA in inflammatory cell phospholipid, plasma, erythrocyte membrane content with 3 , 4 and 5 weeks of supplementation (Mickleborough et al., 2006; Schubert et al., 2009; Surette et al., 2008). Since the biological properties of n-3 PUFA are mediated by the incorporation of n-3 PUFA in the phospholipids of the cell membranes, beneficial effects are observed within a 3-4 weeks of supplementation. In a randomised cross over trial with EIB prone individuals Mickleborough et al., (2006) reported an improvement in post exercise FEV_1, reduction in bronchodilator use, improvement in symptoms with reduction in sputum pro-inflammatory mediator concentration (LTC_4,LTE_4, PGD_2, IL-1β, and TNF-α) after three weeks of supplementation. In another trial by Schubert et al. (2009) on allergic asthma, significant improvement in FeNO levels and a reduction in eosinophilic cationic protein and *in vitro* CystLTs release were observed by

the end of 3 weeks of supplementation. Therefore, a duration of 3 weeks was chosen for EPA/DHA supplementation in this thesis.

For a cross over study design in intervention studies, washout phases are important and must be sufficiently long enough for complete reversibility of the supplementation effect. Washout periods ranging from 2-16 weeks have been used in supplementation studies. There is, however, a progressive decline in the levels of EPA and DHA after the completion of supplementation period with a steep decline within the first 2 weeks (in plasma phospholipids and RBC membranes) (Cao et al., 2006). Mickleborough et al. (2006) demonstrated a washout of n-3 PUFA in neutrophils after 2 weeks following supplementation in EIB prone males. The washout of n-3 PUFA from plasma and cell membranes of platelets and leukocytes after parenteral supplementation of n-3 is found to be ≥5–7 days (Roulet et al., 1997; Senkal et al., 2007) and rapid washout may be attributed to disease severity (van der et al, 2011). Furthermore, in a cross over trial, due to the added length of the study, participant drop out is an important issue (due to changes in disease severity and voluntary withdrawals), compared to parallel group studies. A shorter duration of intervention and washout was implemented in light of the previous studies and to maximise participant retention (Max et al., 1991).

4.3.3 Outcome measures

The primary outcomes for this study were changes in post exercise FEV_1 and pre exercise pulmonary function measures (FEV_1, FVC, FEV_1 /FVC ratio and PEF) following a 3-week supplementation with EPA and DHA. In addition, pre exercise symptom scores (dyspnoea), bronchodilator use (during each supplementation and washout phase), pre and post exercise FeNO, levels of inflammatory cytokines and PBMC lipid composition were analysed.

4.4 Participants

All participants completed a health screen questionnaire and gave written informed consent before participating in the study (appendix 1). Loughborough University's ethics committee approved the protocol for the study. The recruitment flyer, health screen questionnaire, participant information sheet and consent form are attached in appendix 1.Twelve male participants with physician diagnosed asthma and a history of EIB were recruited from Loughborough University, East Midlands, UK. Participants were included if they were 18-30 years old, had physician diagnosed mild-moderate asthma, had no respiratory infection within the past 8 weeks and had not taken steroids in the last 4 weeks of any kind, Hay-fever or other allergy medication in the past 7 days, and dietary n-3 supplementation in the last year. Twelve participants were recruited, however, only nine participants successfully completed the entire protocol (mean age 21 ± 2.7 years, mean height 1.8 ± 0.1 meters). The basic characteristics of the participants are listed in table 4.1. One participant did not complete the study due to injury; another participant was excluded because his asthma symptoms worsened (self-reported) for unexplained reasons during the study; a recommendation was made to this participant to discuss his situation with his clinician. Another participant was affected by seasonal changes and withdrew from the study on personal grounds. All participants showed abnormal lung function (FEV_1/FVC ratio of ≤ 80%) and fell below the LLN (82%) derived from a population of healthy individuals, and were characterised as asthmatic in accordance with the latest ERS/ATS suggestions (Miller et al., 2009). All participants tested positive for EIB (≥ 10% drop in post exercise FEV_1) and had high FeNO concentrations (≥ 40ppb).

At recruitment all participants reported accounts of shortness of breath, chest tightness, and intermittent wheezing post exercise on a regular basis, which was normally relieved by administration of a bronchodilator. There were 4 participants currently reliant on bronchodilators (β2 agonist), and 5 participants were using a preventative medication and bronchodilators (β2 agonist and short acting steroids). Participants refrained from their medication 48 hours (for short acting β2 agonists) prior to testing and were advised to discuss the situation with their medical practitioner prior to the participation in the study. Testing was rescheduled if participants used medication 48 hours prior to testing and a log of medicine usage was maintained until the participant was tested. Participants were also asked to refrain from caffeine intake (for 8 hours) and physical activity (for 24 hours) prior to the exercise challenge test following the rationale listed in chapter 2, page 73. The

research design for this study was such that the blood samples were collected at the same time of the day at each collection point (±30 minutes) and the sample analysis was conducted within 2 hours of collection. There were no dietary restrictions during the study other than personal n-3 supplementation.

The participants in this study were comparable for age, stature, body mass and pulmonary function parameters (FEV_1/FVC, PEF) with the asthmatics from chapter 3 and no differences were found in the between the asthmatics from both the studies. .

Table 4.1 Baseline characteristics of Participants, FEV_1 = Forced Expiratory Volume in 1 sec, FVC = Forced Vital Capacity, PFT = Peak Expiratory Flow, FEF 25-75% = Forced Expiratory Flow (showing 25% and 75 % of FVC). Values are shown as mean ± SEM and were recorded before exercise challenge test

Characteristics	Measurement (n =9)
Stature (m)	1.8 ± 0.1
Age (years)	21.0 ± 0.9
Weight (kg)	74.4 ± 2.0
FEV_1/FVC	**77** ± 1.4
% predicted FEV_1/FVC	**101.8** ± 3.4
Predicted FEV_1	84.8 ± 1.2
FEF 25-75 (l/second)	4.9 ± 0.1
PEF (l/minute)	574 ± 28.7
FeNO (ppb)	**52.1** ± 3.7

4.4.1 Physiological tests

Exercise challenge test

Each participant ran on a motorized treadmill until they achieved 80-90% of their estimated maximum heart rate for up to 6 minutes and the total testing time was between 8-10 minutes (Miller et al., 2005a,b). Participants wore a heart rate monitor (Polar F4 Heart Rate Monitor Watch and strap, Polar Ltd, USA), and their heart rate was constantly monitored during the test following ATS/ERS safety guidelines on exercise testing (Crapo et al., 2000; Sterk et al., 1993). Full details of this procedure are given in chapter 2 (page 70).

Pulmonary function test

Spirometry was performed using a calibrated spirometer (Superspiro; Micro Medical; Rochester, Kent, UK), and all participants remained in an upright seated position during the procedure wearing a nose clip. These devices were calibrated as 'gold standard' providing reproducible volume measurements for asthma patients and they comply with ATS/ERS guidelines (for details refer to general methods, page 70. Participants were required to perform three acceptable FVC manoeuvres according to ATS/ERS recommendations (Miller et al., 2005a,b). The percentage drop in post exercise FEV_1 compared to pre exercise values was calculated based on the following formula as described by Mickleborough and co-workers, (2006):

$$\frac{(\text{pre-exercise } FEV_1 - \text{lowest postexercise } FEV_1)}{(\text{Pre-exercise } FEV_1)} \times 100 \quad \textit{Equation 1}$$

Fraction of exhaled breath nitric oxide (FeNO) measurement

FeNO measurements were conducted using a portable NIOX MINO analyzer ((Aerocrine Inc., Solna, Sweden) according to ATS/ERS guidelines (ATS, 2005). Each participant sat on a chair in an upright position, wearing a nose clip to minimise any nasal NO contamination. The participants completed a prolonged inspiration of NO free air (NIOX MINO removes ambient NO, which ensures accuracy and follows the ATS recommendations) followed by expiration into the NO analyser against an expiratory resistance achieving the recommended expiratory flow rate of approximately 0.05 l/second. Three acceptable manoeuvres were performed to control intra subject variability based on ATS/ERS recommendations (Miller et al., 2005b). The values were expressed in ppb (for details of the procedure and quality control, refer to chapter 2, page 70.

Perception of breathlessness (dyspnoea)

A standard Borg analogue scale was used to record the asthma symptoms of the participants before and after exercise (Borg, 1982). Each participant gave a score from 0 (no symptoms) to 10 (being worst asthma symptoms).

4.4.2 Biochemical analysis

PBMC total lipid analysis

PBMC total lipid analysis was carried out at all time-points using venous blood. PBMC were extracted using a density centrifugation method and esterified to FAMEs and analysed by GC-FID (method chapter, page 76). A 37-component standard (Supelco, catalogue number 47885-U) was used as reference. The area under the curve was measured for the samples and the results were expressed as percentage of each fatty acid content relative to total fatty acid content.

Serum cytokine analysis for IL-6, TNF α and IL-10

Venous blood was collected in serum gel-tubes and the blood was allowed to clot for 45-90 minutes before separation of serum following centrifugation. Serum levels for TNF-α and IL-10 were measured using an ELISA kit (Biolegend, UK) while IL-6 was measured using a high sensitivity ELISA kit (Tepnel/Diaclone), UK). For details of procedures, please see methods chapter (page 106).

4.4.3 Compliance monitoring

The participants were provided with an approximate number of capsules necessary to complete the supplementation phases and were asked to return the remaining capsules after the n-3 supplementation and placebo phase. The remaining capsules were counted to estimate compliance.

4.4.4 Statistical Analysis

Data were analysed using SPSS version 19 statistical software (SPSS Inc., Chicago, IL). A two-way repeated measures analysis of variance (RM ANOVA) was used to measure changes in physiological and biochemical parameters within the same individuals as a result of n-3/placebo supplementation over a period of time (within-subject effects). Mauchly's test was conducted to determine whether sphericity was violated. In the cases where sphericity was violated, the repeated measures analysis of variance was corrected using the Greenhouse–Geiser correction factor. Order effect was assessed by using the order as a variable in the analysis.

4.5 Results

Out of the twelve participants recruited, nine participants successfully completed the entire study protocol. The participants were given clear instructions about the supplementation (n-3 and placebo) and were provided with necessary number of capsules in a pot. Additionally, the participants were sent reminders at the end of each week of supplementation and the remaining capsules in the returned pots were counted to estimate compliance. All participants had less than 5 capsules returned, which was considered an acceptable marker of compliance. In addition, there was no effect of order in which supplementation was provided ($p>0.05$). All results reported in this section are expressed as mean ± SEM unless stated otherwise

4.5.1 Primary outcomes

4.5.2 Pulmonary function Test and Exercise challenge

No Carry over effects

Reduction in FEV_1 in excess of 10% post exercise represents abnormal pulmonary function. Mean percentage change from pre exercise to post exercise FEV_1 was calculated, and the change in FEV_1 after n-3 supplementation is shown in figure 4.3. There were no differences in either the pre exercise (rest) or post exercise FEV_1 between the two baselines based on RM ANOVA, demonstrating no carry over effects as shown in figure 4.2 ($p>0.05$). Subsequently, all comparisons after supplementation were made with baseline1. There was also no differences between the two baselines, placebo and the end of study washout ($p>0.05$, figure 4.2)

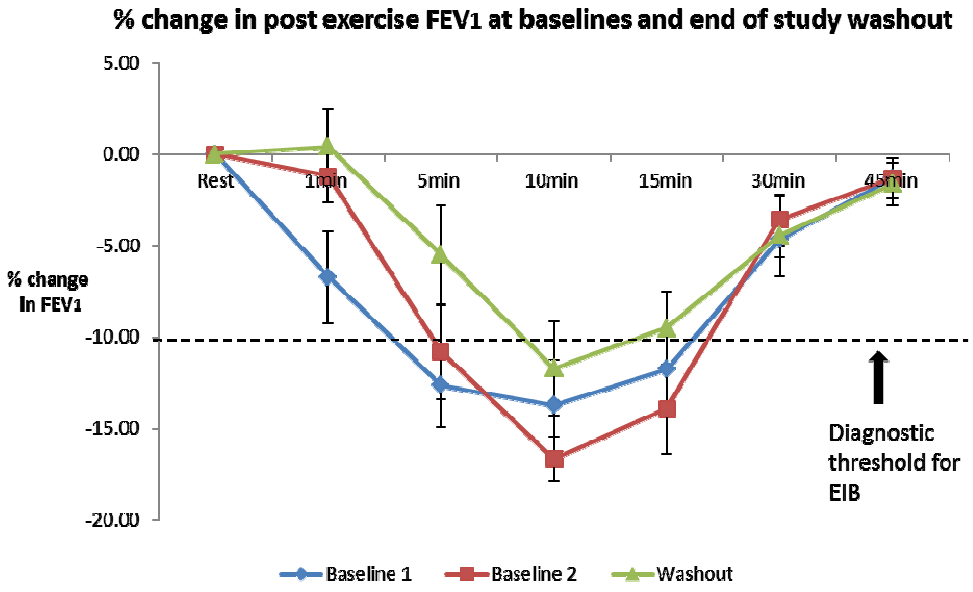

Figure 4.2. No carry over effects: There were no significant differences observed for % change in FEV_1 after exercise at baseline1, baseline 2, and end of washout phase for all participants using RM ANOVA. Reduction in FEV_1>10% shows a positive diagnosis for EIB.

Improvement in post exercise FEV_1 with EPA/DHA supplementation

With the existence of abnormal pulmonary function, the decline in FEV_1 is usually maintained over two time points post exercise. All participants had a >10% drop for post exercise FEV_1 at two time points (between 5-15 minutes) at the baselines and placebo, and the mean of the two time points where the percentage dropped the lowest (post exercise FEV_1) were used for further analysis. All participants met the diagnostic criteria for EIB at baseline (mean drop = 14.8%) and after the placebo phase (mean drop = 12.4%; figure 4.2). There was a significant improvement in post exercise FEV_1 at 5, 10 and 15 minutes post exercise after the EPA/DHA supplementation phase compared to baseline1 for all participants ($p<0.05$, figure 4.3), individual data is shown in appendix 3. The mean percentage drop in FEV_1 post n-3 intervention was 6.6%, which is indicative of attenuated EIB response.

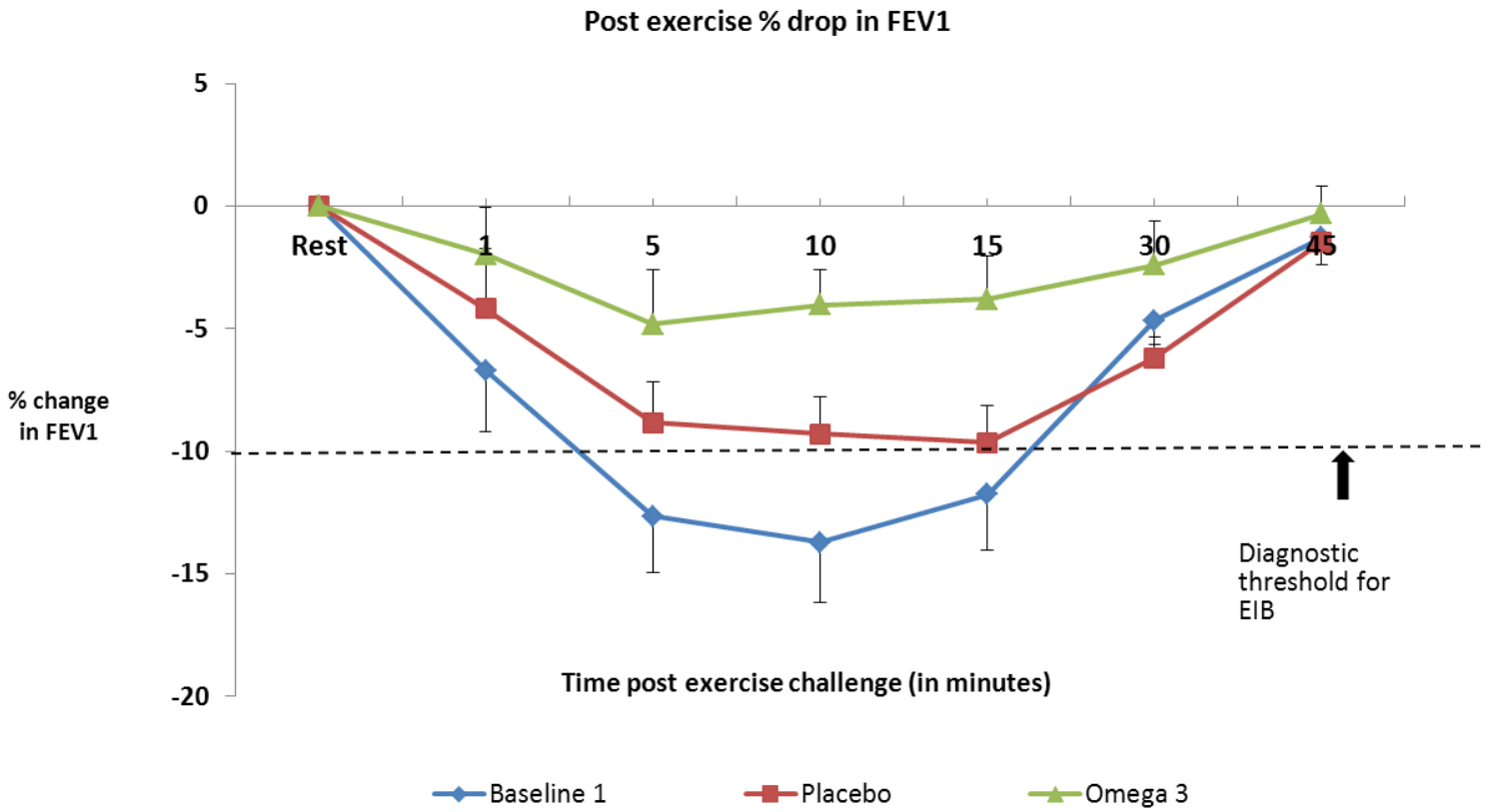

Figure 4.3 Mean percentage change in FEV_1 before and after exercise at baseline1, placebo and omega – 3 (EPA/DHA) phase for all participants. Reduction in FEV_1>10% shows a positive diagnosis for EIB
*shows significant improvement from baseline ($p<0.05$). There were no significant differences between the baseline 1 and baseline2 ($p>0.05$). All comparisons were made with baseline 1.

Pulmonary function tests (pre exercise)

No changes in FEV_1/FVC ratio with supplementation

FEV_1 and PEF are measures to assess the full potential of the lungs. There was no significant change in mean FEV_1 and PEF across all time points based on RM ANOVA ($p>0.05$). FVC represents the vital capability of the lungs and there was no change observed in the FVC value at any time point ($p>0.05$). FEV_1/FVC ratio is an important parameter for asthma diagnosis and no changes were observed in the FEV1/FVC ratio across all time-points ($p>0.05$, figure 4.5). The FEV_1/FVC ratio fell below the LLN criteria (82%) derived from a matched reference population in chapter 3 and this clearly demonstrated that the participants showed airway obstruction all time points of the study. Overall, there was no effect of n-3 supplementation on the pulmonary function outcomes; however, there was substantial improvement in post exercise FEV_1 as presented in the last section (figure 4.4).

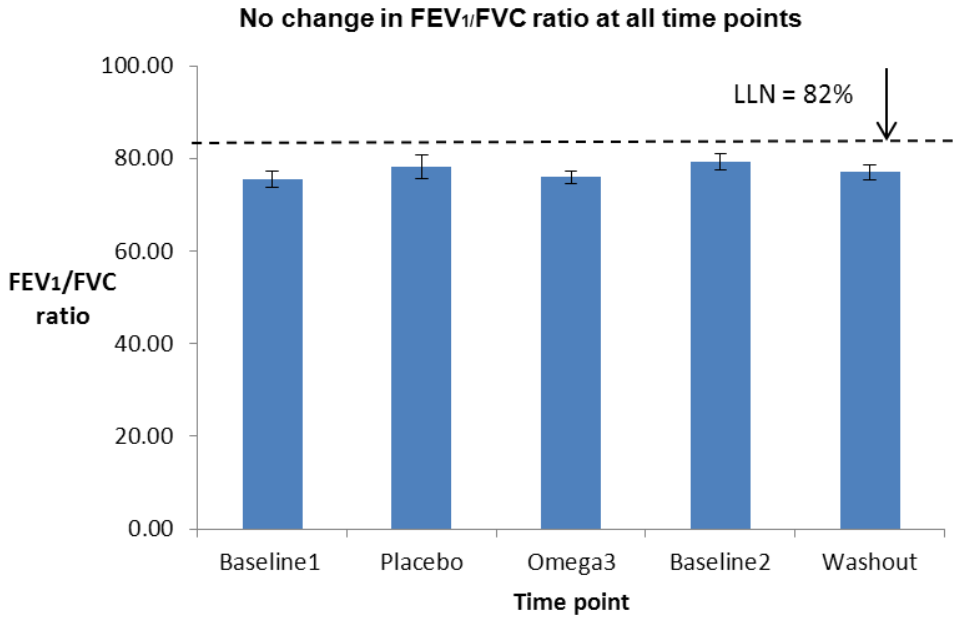

Figure 4.4 Changes in FEV_1/FVC ratio all time points, LLN = lower limit of normality

4.5.3 No change in dyspnoea but reduced bronchodilator usage after EPA/DHA supplementation

There was no improvement in dyspnoea score with n-3 supplementation (mean score = 1) compared to baseline, placebo or other wash phases (mean score =1) ($p>0.05$). Thus, all participants reported very slight breathlessness before the exercise test at each time of the study.

Participants recorded bronchodilator usage during the supplementation phases and each usage accounted two puffs on average. There was no change in the bronchodilator usage between placebo, baseline 2 and the washout ($p>0.05$, figure 4.6). There was no data collected for baseline 1 measurement. The total bronchodilator usage during the three week n-3 supplementation phase (4 times, 8 puffs was lower than the total usage during placebo phase (7 times, 14 puffs). Significant reduction (50%) in bronchodilator use was observed during the last week of n-3 supplementation (1 times, 2 puffs) compared to placebo phase (2 times, 4 puffs) ($p<0.05$). Mean bronchodilator use across all time points is shown in figure 4.6.

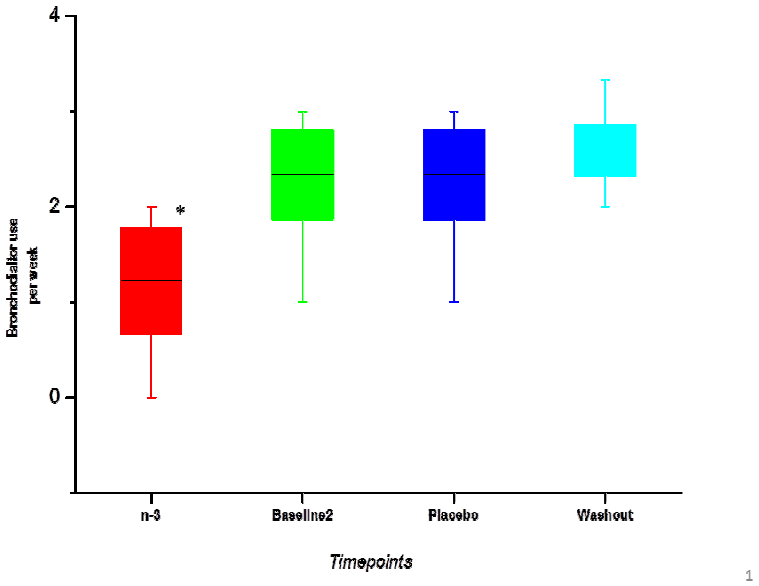

Figure 4.5 Bronchodilator use at different time points

* Significant reduction in bronchodilator usage with n-3 supplementation compared to baseline 2, washout and placebo phases ($p<0.05$).

4.5.4 Secondary outcomes

No change in FeNO with EPA/DHA supplementation

All participants had elevated levels of resting FeNO compared to upper threshold of healthy individuals as per ATS/ERS guidelines (upper threshold = 25ppb; ATS, 2005) before the start of supplementation. Measurements of FeNO were collected at rest and post exercise challenge test at all-time points.

At rest, the values measured for baseline (52 ± 3.7 ppb) was comparable to values measured after n-3 supplementation (57 ± 9.3 ppb) or placebo (47.0 ± 8.0 ppb) and there were no significant differences between the two baselines and washout ($p>0.05$) . Similarly, there was no change in the post exercise FeNO levels measured at each time point ($p>0.05$). There was no overall reduction in FeNO levels with a 3-week EPA/DHA supplementation.

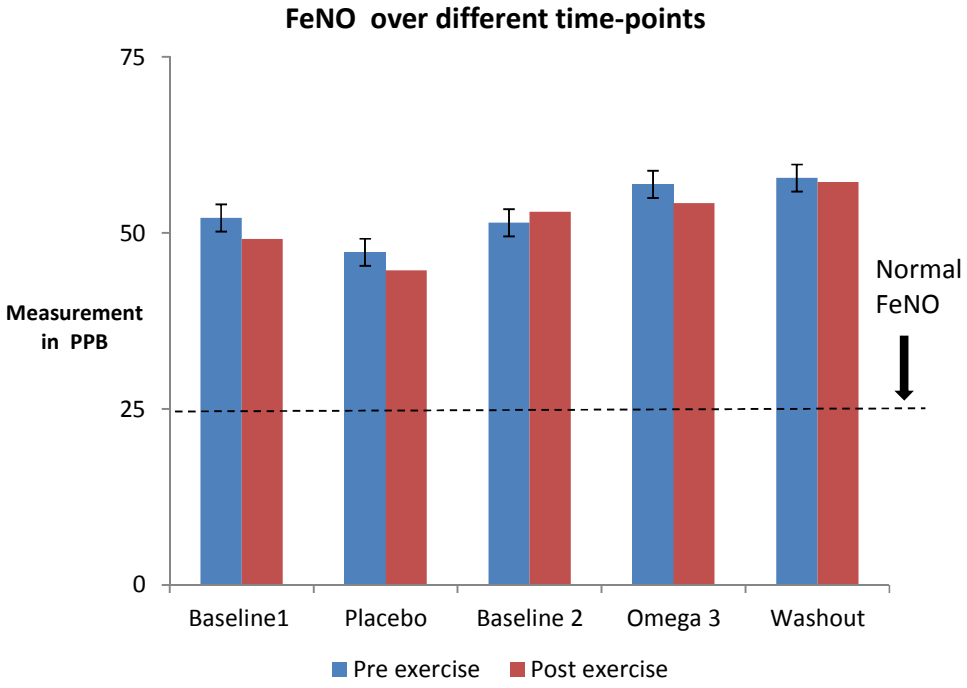

Figure 4.6 FeNO levels at all-time points

PBMC lipid analysis

Changes in EPA, DHA and AA

PBMC levels for all fatty acids were expressed as percentage (%) of total fatty acids across all time-points in the study and figure 4.8 shows content of EPA, DHA and AA at all time-points. The mean CV% for all PBMC samples analysed was found to be <10% on the same day. Example profiles are shown in appendix 3.

Although there was an increase in overall EPA and DHA levels after supplementation with EPA/DHA, the mean EPA and DHA levels didn't differ statistically between the time points ($p>0.05$,figure 4.8). There was a reduction in AA levels post n-3 supplementation, however this change was also not statistically significant ($p>0.05$).

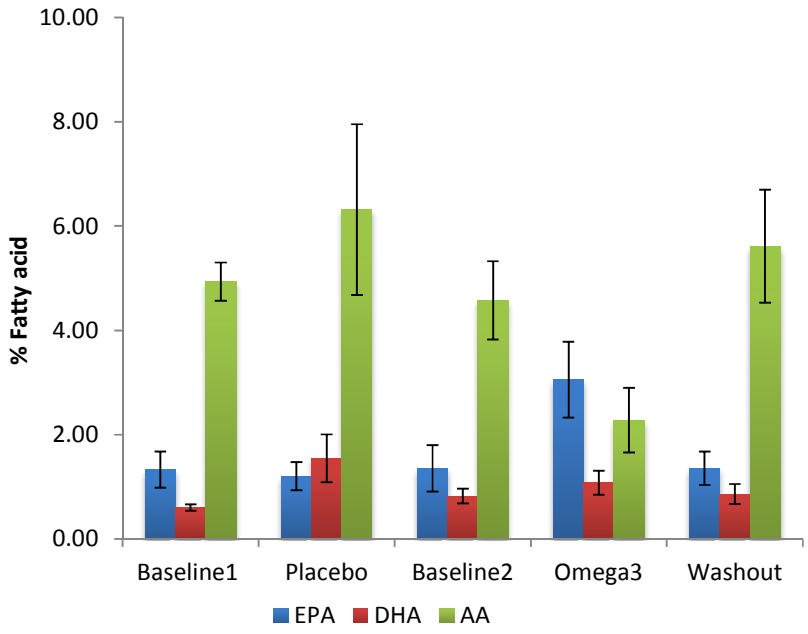

Figure 4.7 Fatty acid content for EPA, DHA and AA expressed as a percentage (%) of total fatty acid content

Changes in ALA and LA

Mean ALA levels did not change significantly across the time points during the study ($p>0.05$) (figure 4.9). In addition, there was no change in LA levels during the study ($p>0.05$)

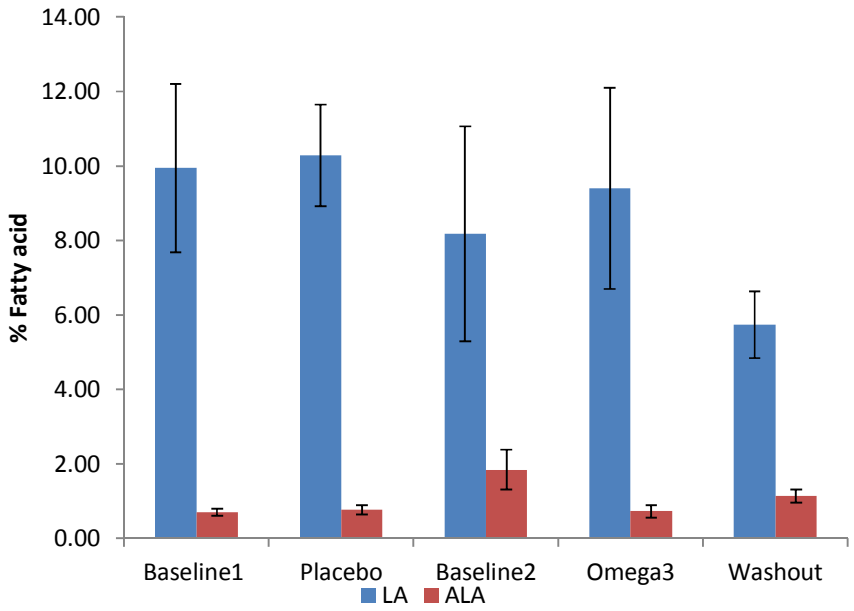

Figure 4.8 Fatty acid content for ALA and LA expressed as a percentage (%) of total fatty acid content

Changes in the levels of other fatty acids

No significant differences were observed for the proportions of lauric acid, myristic acid, pentadecanoic acid, palmitic acid, stearic acid or palmitoleic acid. There were marginally significant changes observed in oleic acid levels between baseline1 and baseline2 ($p=0.047$) possibly due to some technical error in measurement. The mean data is shown in appendix 3.

Serum cytokine levels

Figure 4.10 shows serum levels of IL-6, IL-10 and TNF-α at different time points of study, hence all the comparisons were made with the baseline. The IL-10 and TNF-α levels remain unchanged ($p>0.05$) across all time points as shown in the figure 4.10. There was a statistically significant reduction in IL-6 levels after n-3 supplementation (37% reduction) compared to baseline ($p<0.05$).

The IL-6 estimates in this chapter are higher than those obtained in chapter 3 for a matched mild-moderate asthma population, however, these results are still within the range for asthmatics. The differences are possibly due to the differences in the kits. In this study and the next study (chapter 5) the TNF-α and IL-10 were measured using an ELISA kit (Biolegend, UK) while IL-6 was measured using a high sensitivity ELISA kit (Tepnel, UK), which provided consistency within all phases of the supplementation studies.

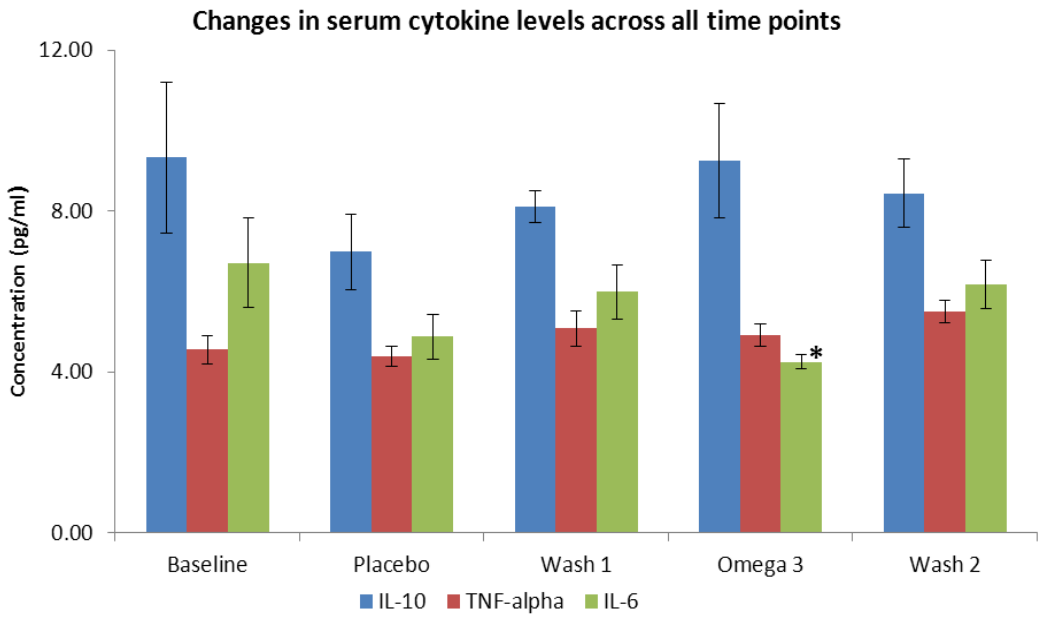

Figure 4.9 Serum levels of IL-6, IL-10 and TNF-α at different time points of the study
* $p<0.05$, significant difference in IL-6 level after n-3 supplementation compared to baseline

4.6 Discussion

This thesis presents a dietary supplementation study with EPA/DHA in mild-moderate EIB males. There are few studies conducted to assess the efficacy of n-3 in EIB prone participants and most prior studies have focussed on athletes. The primary reason for the EPA/DHA supplementation was to determine the improvement in post exercise pulmonary function (FEV_1), so as to test the hypothesis that three-weeks of EPA/DHA supplementation would result in a significant improvement in post exercise FEV_1. The study has demonstrated that a supplementation with n-3 (3.2 grams of EPA and 2.2 grams of DHA) for three weeks reduces the severity of EIB in participants with mild-moderate asthma. The supplementation improved pulmonary function post exercise to below the diagnostic threshold value for EIB, i.e. 10% fall in post exercise FEV_1. There was approximately 71% improvement in FEV_1 at 15 minutes after exercise. The mean level for the two time points where the percentage dropped the lowest in FEV_1 after exercise at post n-3 supplementation was 6.6%, compared to a drop of 14.8% at baseline when participants were on their regular diet. This improvement in post exercise FEV_1 is accompanied by >60% reduction in bronchodilator use. This trend in improvement in post exercise FEV_1 is consistent with the findings of Mickleborough and colleagues where 3 weeks of fish oil supplementation improved pulmonary function to below the EIB threshold of 10% in athletes (Mickleborough et al., 2003, 2006).

There was no change in the pulmonary function outcomes for FEV_1, FVC and PEF before exercise (at rest) after three-week supplementation with n-3. FVC represents the vital capability of the lungs and asthmatics with obstructive disease (asthma) do not usually have reduced FVC. Consequently, it was not expected that a change would be observed in FVC with any treatment or supplementation. If changes are observed it is possible that measurement errors have occurred. Recommendations for standardisation have been published and it has been determined that spirometry is reproducible, but is effort dependent (ATS, 1999; GINA, 2011; Miller et al., 2005; Pellegrino et al., 2005). In the present study, the participants were given guidance and practice prior to the PFTs; repeat measures were collected and the best out of the three measures were used analysis as per ATS/ERS guidelines. The lack of variation in FVC observed here suggests that measurements of pulmonary function carried out in this study were accurate.

Asthmatics have a reduced FEV_1 and PEF due to airflow obstruction as confirmed from the data in chapter 3 where asthmatics were compared to matched healthy controls. Thus, improvements in these parameters of the pulmonary function are important while assessing the benefits of EPA/DHA intervention. The adult n-3 intervention trials in asthma have provided inclusive information with regard FEV_1 or PEF. In a number of trials with varying dose and duration of n-3 supplementation, no significant changes have been observed for FEV_1 or PEF (Hodge et al., 1998; Kirsch et al., 1988; McDonald, 1990; Mickleborough et al., 2003, 2006; Stenius-Aarniala, 1989). There are some studies that have shown positive outcomes; Emelynov et al., (2002) showed a significant increase in morning PEF with 8-week supplementation with a low dose of n-3 (50mg EPA+ DHA per day) in mild-moderate atopic asthmatics. Surette et al., (2008) reported a significant improvement in quality of life and asthma management scores (including symptoms) assessed by questionnaires after 3 weeks of supplementation (0.5 gram EPA + 0.75 gram DHA per day). Mickleborough et al., (2003, 2006) have reported significant improvement in post exercise FEV_1 after 3-week supplementation with a dose similar to the present study (chapter 4); however no changes were observed for resting pulmonary function values for FEV_1 and PEF reported by Mickleborough et al., which was confirmed in the present study (chapter 4). Although some literature and our knowledge of the physiological action of n-3 fatty acids suggest that we should potentially see effects of supplementation on lung function there is no consistent effect of n-3 supplementation on FEV_1 or PEF reported. This could be attributed to the heterogeneity between the studies. These studies have used a range of doses, as low as 50 mg to > 3grams of EPA/DHA per day and have studied different sub group of asthmatics such as mild-moderate, EIB and atopic populations from 3 weeks to months.

Symptoms of dyspnoea are an important diagnostic marker and a number of clinicians rely on this outcome along with spirometry for the diagnosis of asthma and other respiratory diseases. In previous studies, n-3 supplementation has been accompanied by some improvement in symptoms assessed by reduction in daytime wheezing and quality of life questionnaires. Two studies have shown an improvement in mean daytime wheeze, morning PEF and improvement in quality of life (Emelyanov et al., 2002; Surette et al., 2008). The study by Emelyanov et al, (2002), the study comprised of a group of mild-moderate atopic asthmatics (18-56 years, n=46) supplemented with green lipped mussel extract with 50mg EPA+DHA /day for 8 weeks. Despite the relatively lower dose of EPA

and DHA used by Emelyanov et al., (2002) the longer duration of the study could be a reason why beneficial effects were seen in mean daytime wheeze and morning PEF. Surette et al., (2008) had a 3 week supplementation period with 0.75 gram GLA+ 0.5gram EPA or 1.3 gram GLA + 0.75 gram EPA in mild-moderate asthmatics (n=65) and showed improvement in self-reported status and medication use, and based on quality of life questionnaires there was an improvement in symptoms. The major limitation for this study was that no PFT measurements were taken and the results are based on self-reported outcomes. Since symptoms (or perception) have been suggested to be not a true indicator of the status of pulmonary function, the results from the Surette et al, study can be considered to have serious limitations. Furthermore, the lack of improvement in PFT outcomes in the n-3 supplementation studies conducted in the last two decades could be due to the nature of asthmatics used. These studies have been conducted mainly on mild-moderate (Mickleborough et al., 2003,2006; Surette et al., 2008) or atopic/allergic (Arm et al., 1989; Emelyanov et al., 2002; Schubert et al., 2009; Thien et al., 1993) participants. As discussed in the previous chapter (chapter 3) there are issues relating to the diagnosis of asthma using a fixed criteria or 80% ratio. It is likely that the individuals in these prior studies were mild-moderate asthmatics or not asthmatic at all. If incorrect characterisation was the case, then it is not likely to see a major change in the PFT values. To see substantial changes in PFT outcomes, a larger sample size with participants with severe airflow obstruction are required to assess the effect of EPA/DHA on PFT outcomes. The study conducted in this chapter involved characterisation of asthmatics based on LLN, in addition, the inclusion criteria included EIB (with exercise testing) and high FeNO levels (>40ppb). Thus, the asthmatics recruited in the study were representative of a mild-moderate asthmatic young population (18-30 years) with EIB being the major characteristic. Therefore, improvement in post exercise FEV_1 is indicative of the beneficial effect of n-3 supplementation.

Although the supplementation studies have not shown major improvement in PFT values, substantial improvement in reduced medication usage has been observed. Hodge et al.(1998) showed a significant reduction in medication use in asthmatic children after 6 month supplementation (1.2gram EPA per day) while Mickleborough and co-workers, (2006) reported that bronchodilator use was significantly reduced during the last 2 weeks of a fish oil supplementation compared to baseline for EIB using a dose similar to the present study. However, some of the earlier intervention trials have not shown any

significant changes to medication (Arm et al., 1989; McDonald et al., 1999; Thien et al. 1993). These inconsistencies once again highlight the differences among these studies relating to dose, duration and nature of asthmatics studies, as discussed above. In this thesis by the third week of n-3 supplementation there was approximately 50% reduction in bronchodilator usage compared to placebo phase that support the previous findings that n-3 supplementation exerts bronchodilatory effects within 2-3 weeks of supplementation.

In this chapter, no changes were observed in symptoms after supplementation and the nature of the asthma experienced by the participants could explain why no change was observed in the reported dyspnoea score. The average dyspnoea score reported in this chapter at baseline was already very low. All participants displayed a score between 0-3, which corresponds to no symptoms to mild symptoms of perceived breathlessness (the mean was 1 on a 0-10 scale). This fits well with mild-moderate asthma status of the individuals. The likelihood of improvement in the mean symptom score from this already very low value to reporting no symptoms on average was unlikely. All participants had mild-moderate asthma and had been living with and managing the condition for several years. Because of this, the perception of symptoms was also possibly under reported by the participants. Though self-reported symptoms are widely used in asthma diagnosis it is suggested that symptom score cannot be independently used to assess the severity of asthma, thus these need to be used in conjunction with standard pulmonary function tests (ATS, 1999, Parshall et al., 2012). Despite no changes in symptoms, this reduced bronchodilator usage with n-3 supplementation suggests a possibility of reducing the reliance on pharmacological medications which has proven issues with health side effects. The improvement in post exercise FEV_1 in EIB prone individuals is suggestive of the possibility of EPA/DHA supplementation being used as a treatment for EIB, which would significantly improve the quality of life and prove beneficial in physical activity, particularly to help achieve the recommended levels proposed by the Department of Health for maintaining a healthy lifestyle.

FeNO is now becoming accepted as a non-invasive marker of airway inflammation and is recommended by the National Institutes of Health as a supplemental outcome for the characterisation of study populations, for prospective clinical trials, and for observational studies, and there are standard testing guidelines available (ATS/ERS, 1999, 2005; Dweik et al., 2011; Szefler et al., 2012). Low sputum eosinophilia and low FeNO are strongly

linked however, FeNO and sputum eoisinophila are not duplicate outcomes. FeNO and eosinophil levels are significantly reduced with corticosteroid treatment, unlike anti–IL-5 and anti-IgE therapies, which reduce sputum eosinophilia without affecting FeNO (Berry et al., 2005; Brightling et al., 2003). There are issues related to tachyphylaxis and side effects due to prolonged usage of corticosteroids, which is making non –pharmacological interventions attractive to users. Thus, there is an interest in the potential role of dietary supplementation with EPA/DHA for reducing FeNO levels. In the present study, there were no changes in FeNO levels at any time point. The study population displayed FeNO levels higher than normal (>25ppb) and values remained constant across the 10-12 weeks of the study, and these results match with the lack of effect shown in the results by Moreira and colleagues with 2 week dietary n-3 supplementation (Moreira et al., 2007).

A previous parallel prospective study has shown a significant difference between the FeNO levels of placebo and n-3 group after a 3-week supplementation (450mg of EPA and 180mg DHA per day) (Schubert et al., 2009). Though there was an increase in FeNO levels during low dose allergen exposure after 3 weeks in both groups, the n-3 supplementation group displayed an attenuated response, which indicates a beneficial effect of n-3 PUFA on FeNO levels (Schubert et al., 2009). The positive results shown by Schubert et al., (2009) in three weeks with a relatively lower dose of n-3 could be due to the mild inflammation in the house dust allergic asthma study group (baseline FeNO = ~25ppb); on the contrary the present study included those with substantially elevated levels of FeNO (52 ± 3.7 ppb).

There is a lack of studies confirming the positive effects of n-3 supplementation on FeNO levels and further studies are required to address this. A recent cross sectional study by Barros et al., (2011) using validated FFQs it was reported that higher dietary intake of n-3 PUFA (0·94 g/day of EPA+DHA) and ALA (1·96 g/day) can reduce the odds of non-controlled asthma (unstable PFT and FeNO>35 ppb with asthma medication). The study has suggested that the prevalence of non-controlled asthma in their study was high; subsequently it is likely that the risk was overestimated (Barros et al., 2011). In clinical practice, it has been observed that long term medication use (corticosteroids) can stabilise FeNO levels (Dweik et al., 2011;Zitt et al., 2005). Subsequently it is likely that the effect of EPA/DHA supplementation if measured over a longer period may improve FeNo levels. In future, studies are required with long-term supplementation to explore this possibility to

elicit the role of n-3 PUFA as a complimentary therapy for asthma by reducing reliance on corticosteroids which have proven long term health side effects.

A possible limitation of this study was that the FeNO measurements in this thesis were conducted using a hand-held potable device that was available within the budget of this thesis research. The major advantage of these devices is that they can be used to monitor airway inflammation in clinics, in the field and in the patient's home (Kim et al., 2012). There are some concerns about the reliability of these devices due to their limited ability to control flow rates, and it is suggested that a stationary chemiluminescence device such as 'Sievers nitric oxide analyser' may be a more reliable method for assessment. While there are a number of studies showing a good association between FeNO values measured with the two devices, a few studies reported that there were differences in the FeNO values obtained with the hand-held NIOX MINO compared to those measured with stationary devices (Chen et al., 2007; Menzies et al., 2007; Schiller et al., 2009). In a recent study by Kim et al. (2012) it was demonstrated that there is a good correlation ($r = 0.876$, $P < 0.001$) but only moderate agreement between the FeNO values measured by the hand-held and stationary devices. The NIOX MINO values are significantly lower than stationary devices, thus it is important that results must be carefully interpreted (Kim et al., 2012). The mean value of the asthmatics in this thesis was >40 ppb and if these measurements were conducted using a stationary device, as Kim et al. suggests, it would have been relatively higher. This suggests high levels of inflammation in the study population (chapter 4) compared to healthy individuals (25 ppb). Consequently, the duration and the dose of supplementation given here may not have been sufficient to reduce FeNO values. Dose response and time course studies are required to evaluate the dose and duration required to reduce FeNO levels significantly in groups with high levels of inflammation.

When PBMC lipid composition was studied, no significant changes in EPA and DHA levels after n-3 supplementation were observed, however there was a trend toward an increase in EPA (3 fold) and DHA (2 fold), which is suggestive of changes in cellular total lipid composition. There was also a non-significant trend showing a reduction in AA (2 fold) levels with EPA/DHA supplementation. It is documented that different cells and tissues have their characteristic fatty acid composition and their composition is dependent on the availability of different fatty acids as well as the metabolic properties of the cells and tissues (Calder, 2012; Calder & Yaqoob, 2009). Dietary supplementation with fish oil can

alter cellular fatty acid profiles, and their incorporation is in a dose response manner. EPA and DHA have the ability to compete and replace AA and there is an increase in overall EPA and DHA in different cells and tissues (Blonk et al., 1990; Calder et al., 1991; Katan et al., 1997; Mickleborough et al., 2006; Sanders et al., 1983; Witte et al., 2010). The lack of significant change in EPA, DHA and AA could be attributed to measurement error, shorter duration of supplementation (than has been used in other studies with positive outcome) or small sample size and low power. It is demonstrated that incorporation of fatty acids in PBMC membrane reaches near maximum in 4 weeks (Rees., et al. 2006; Thies et al., 2001; Yaqoob et al., 2000), and so it was expected that after 3 weeks there would be a substantial incorporation of EPA and DHA into the PBMC. The results from this thesis do show a positive trend in the incorporation of EPA alongside a reduction in AA levels, demonstrating a positive direction in the study and the likelihood that with continued supplementation the findings would have reached significance. The absence of significant changes outcome could be attributed to possible limitations of the GC-FID method, particularly low recovery of AA and DHA due to possible loses in esterification and/or due to high temperature of GC parameters. In future studies, to address these issues longer duration of supplementation can be included, also an improvement in the GC-FID method can improve the findings.

The modification of cell and tissue fatty acid composition is suggested to influence cell behaviour, which can, affect their physiological responses. In a previous double blinded, cross over supplementation study in EIB individuals using the same dose as the present study, there was a significant increase in EPA and DHA content in neutrophil phospholipids with a 3-week supplementation accompanied by a reduction in LA and AA (Mickleborough et al., 2006). This study also showed improvement in post exercise FEV_1 and reduction in bronchodilator usage; there was a significant reduction in induced sputum differential cell count percentage and concentrations of inflammatory lipid mediators (LTC_4-LTE_4, PGD_2) and inflammatory cytokines (IL-1β, and TNF-α) before and following exercise on the n-3 diet. There was a reduction in AA derived LTB_4 along with an increase in LTB_5 generation from activated polymorphonuclear cells on the n-3 diet. Arm et al. (1989) reported a 10 fold increase in neutrophil phospholipid EPA content with supplementation with 50% attenuation of total LTB (LTB_4 and LTB_5) generation by stimulated neutrophils and suppression of neutrophil chemotaxis with n-3 supplementation using a dose similar to the present study. In another study, doses of <1 gram EPA/day were effective in reducing

stimulated whole blood LTB_4 biosynthesis after a 4-week supplementation; additionally, there was a significant incorporation of EPA in plasma and RBC phospholipids with 4-week supplementation (Surette et al., 2008).

In a prospective double blind, parallel study on atopic asthmatics with 5 week n-3 supplementation (fat blend - 450mg of EPA and 180mg DHA per day), there was a significant increase in EPA levels after 3 weeks of supplementation compared to the placebo group (Schubert et al., 2009). Furthermore, other fatty acids showed no trend in plasma lipid composition while there was an overall increase in DHA values after supplementation. There was an overall reduction in allergen-induced airway inflammation verified by lower levels of FeNO, serum and sputum eosinophils and suppression of the *in vitro* CystLTs release by leukocytes (Schubert et al., 2009). Thus, there is evidence to suggest that there is incorporation of EPA and DHA in the different types of cells and tissues following supplementation. Though our study failed to show significant differences in PBMC fatty acid composition, there is an encouraging trend to suggest that compliance to supplements was good. The improvement in post exercise pulmonary function, reduced reliance on bronchodilator usage and reduction in circulating IL-6 levels is encouraging and suggests a beneficial role of EPA and DHA in EIB. The present study was powered to detect differences in post exercise pulmonary function outcomes and has met the expected outcome. The insignificant PBMC fatty acid incorporation for EPA and DHA could be due to the small sample size of the study. The observed power based on post hoc analysis was found to be less than 0.4 for EPA and DHA; thus, a larger sample size would warranted to carry out a comprehensive study to illustrate changes in total lipid composition of PBMC.

Asthmatics have shown to have elevated levels of inflammatory cytokines such as IL-6 and TNF-α compared to healthy non-asthmatic controls and this was confirmed in the previous study (chapter 3). In addition, asthmatics have shown to have lower levels of IL-10, which is a regulatory cytokine. Therefore monitoring the changes in the levels of these cytokines is important when assessing the effect of any anti-inflammatory treatment. Our results show a significant reduction in the levels of IL-6 (approximately 37% reduction) with three week EPA/DHA supplementation. This study confirms the recent findings from Gray et al., (2012) where 6-week supplementation with n-3 (1.3 gram EPA + 0.3 gram DHA/ day) was shown to reduce circulating IL-6 level in young males before and after exercise.

Several supplementation studies with EPA and DHA in healthy volunteers have shown decreased production of TNF-α, of IL-1β and IL-6 by endotoxin-stimulated monocytes or mononuclear cells (Caughley et al., 1996; Endres et al., 1989; Trebble et al., 2004). Mickleborugh et al., (2003) reported no changes in the circulating levels of IL-6 with 3-week supplementation at a dose and duration similar to the present study however there was a significant reduction in TNF-α levels. In a type 2 diabetes n-3 supplementation (4 gram EPA or DHA/day) study, there was a significant decrease plasma TNF-α levels after six weeks (Mori et al., 2003). There are a number of studies which have shown no effect of EPA and DHA supplementation on the reduction of inflammatory cytokine production in humans (Deike et al., 2012; Wallace et al., 2003). Some of these studies have used low dose (<2gram EPA+DHA) (Deike et al., 2012; Kew et al., 2003; Schmidt et al., 1996; Wallace et al., 2003); however some studies have provided a higher dose (>2gram EPA+DHA per day) (Kew et al., 2004; Mickleborough et al., 2003).

The inconsistencies in these findings could be due to dose and duration of the study or could be due to technical errors in measurement (Calder, 2012). Additionally the relative effect of EPA and DHA is also considered an important factor in determining the effects associated with fish oil supplementation. In cell culture work by Mickleborough et al., (2009) has demonstrated that 120 mM pure EPA and EPA-rich media can significantly supress TNF-α and IL-1β mRNA expression and the production of LTB_4, PGD_2 and TNF-α and IL-1β in stimulated alveolar macrophages obtained from asthmatic patients. In the last decade, a number of studies have used multiplex approaches for gene expression to gain insight into the regulation of genes involved in the inflammatory process. In a study on fish oil supplementation 26 weeks supplementation (0.4 g EPA/DHA (n=36) and 1.8 g EPA/DHA (n=37)) it was demonstrated that approximately the expression of 1000 genes including TNF-α and IL-6 were down regulated (Bouwens et al., 2009). It is likely that the effect of n-3 PUFA on pro-inflammatory cytokines is independent of eicosanoid activity and is associated with regulation of signalling pathways (such as NFkB-mediated pathways) and transcription factors (e.g. peroxisome proliferator activated receptor (PPAR)-γ) (Afman & Muller, 2012; Michaud & Renier, 2001; Rudkowska et al., 2009; Tai et al., 2005).

In our study there were no changes observed in the level of IL-10. This Th2 cytokine is found to have a critical role in limiting the immune response to pathogens to prevent host damage; in addition, IL-10 is involved in regulation of the expression of a number of inflammatory cytokines (IL-1ß, TNF-α and GM-CSF) and inflammatory enzymes (prostaglandin desaturase and COX) (Baine et al., 2011; Larché., 2007; Holgate et al., 2010). In animal models of inflammatory bowel disease supplementation with fish oil has shown to increase levels of IL-10 (Barros et al., 2010) and reduce *ex vivo* production of TNF-α,IL-6 IL-10 and IFN-γ levels in plasma (Folador et al., 2009). These studies show that fish oil supplementation can affect lymphocyte function by affecting cytokine production. Using the whole genome and gene expression approaches the regulation/expression of IL-10 genes can be studied in relation to n-3 supplementation.

The findings from this chapter confirm previous findings relating to the beneficial effects of n-3 supplementation in asthma and EIB. Furthermore, this chapter presents data from the *first study* in *'mild-moderate asthmatic males with EIB'* where a 3 week EPA/DHA supplementation has shown to have beneficial effects EIB. The previous studies on EIB have been based on athletes and recreationally active mixed groups (males and females). The current pharmacological therapies for EIB include long and short acting β agonists and corticosteroids, which have proved effective in asthma management in the majority of patients; but they still have issues associated with their use. Complementary and alternative therapies are becoming popular in present times as they have the potential to reduce dosage and reliance on pharmacological therapies (Barnes, 2008; Mickleborough & Rundell, 2005). Thus, the results from this chapter provide evidence for the beneficial effects of EPA/DHA supplementation in EIB prone males; and suggest the use of EPA/DHA supplementation as a complimentary therapy in management of EIB symptoms.

4.7 Conclusions

Three weeks of dietary supplementation with EPA/DHA reduced severity of EIB in recreationally active male asthmatics, marked by an improvement in their post exercise FEV_1 and bronchodilator usage. The supplementation did not alter FeNO levels and cytokine profiles for TNF-α and IL-10; however, there was a reduction in IL-6 levels suggesting a potential role of EPA/DHA in Th-2 regulation. There was no conclusive evidence about changes in PBMC lipid composition for EPA/DHA, with a trend showing an

increase in EPA and a reduction in AA following supplementation. The improvement in pulmonary function demonstrates a benefit to mild-moderate asthmatics of EPA/DHA supplementation however, the lack of consistent inflammatory mediator data means that possible mechanisms are still not elucidated. To further assess the modification of PBMC total lipid composition by n-3 supplementation, a larger sample size and modified study methods are required.

Chapter Five

Effect of 3-week resolvin supplementation on exercise induced bronchoconstriction in asthmatic females

5 Effect of 3-week resolvin supplementation on exercise induced bronchoconstriction in asthmatic females

5.1 Abstract

Studies using animal models suggest that E series resolvins derived from EPA have anti-inflammatory effects in small quantities. There is limited evidence from human studies to confirm these protective effects. The study aimed to investigate the impact of resolvins on the pulmonary function of mild-moderate asthmatic females with EIB. Eight female participants were recruited based on exercise challenge tests, spirometry and exhaled breath nitric oxide (FeNO) values. The participants entered a randomised crossover trial (RCT) with three weeks of E series resolvin supplementation (100 mg Resolvinol and 0.5% Vitamin E) / or placebo containing olive oil per day) and they crossed over to placebo/Resolvinol with a 3 week washout phase in between. A record of menstrual cycle (normal/abnormal) and oral contraceptive pill usage was made prior to the supplementation. Additionally, venous blood samples were collected to measure circulating levels of inflammatory markers (IL-6, TNF-α and IL-10). Results show a significant improvement in post exercise FEV_1 for EIB after 3-weeks of resolvin supplementation. There was a significant reduction in serum IL-10 levels; but no overall change in serum TNF- α, IL-6 or FeNO levels. There was no impact of oral contraceptive pill usage on any of the study outcomes. The study demonstrates for the first time that a 3-week resolvin supplementation can improve post exercise pulmonary function in EIB females.

5.2 Introduction

Asthma is a complex disease characterised by airflow obstruction, airway hyperresponsiveness (AHR), mucous production and airway inflammation (Hisada et al., 2009: Miller et al., 2005a,b). Mild-moderate asthma symptoms result in temporary change in treatment, in an effort to prevent the condition from becoming severe (NHLBI, 2007; Reddel et al., 2009).This sub group of asthmatics show a reduced pulmonary function (FEV_1/FVC ratio= 60-80%) and their symptoms persist for >2 days per week and are attenuated by bronchodilator use. Exercise-induced bronchoconstriction (EIB) is a sub group of asthma in which there is transient narrowing of airways after exercise, occasionally this transient narrowing of airways can occur during exercise (Anderson & Kippelen, 2005; Beck et al., 1994; McFadden & Gilbert, 1994; Rundell et al., 2003; Suman et al., 1999). The eicosanoids (2 series PGs and 4 series LTs) derived from arachidonic acid (20:4, n-6 PUFA) which is present in the phospholipids of inflammatory cells are involved in the pathophyisiology of airway inflammation (Bradding et al., 2006; Carter & Bradding ,2011; Mickleborough & Rundell, 2005). A review of the pathophysiology of asthma and EIB is presented in chapter 1 (page 24). Elevated levels of CystLTs are detected in exhaled breath condensate (EBC), urine, blood and BAL fluid of asthmatics and those with EIB after an exercise challenge (Anderson & Kippelen, 2010; Gulliksson et al., 2006; Hallstrand et al., 2005. Consequently, change in CystLTs in EBC following an exercise challenge has been related to the severity of EIB (Bikov et al., 2010; Mickleborugh et al., 2006). Based on the involvement of AA derived lipid mediators in inflammation, it has been hypothesised that increased dietary intake of n-6 PUFA accompanied by a low intake of n-3 PUFA has played a causal role in increased asthma incidence in the last 30 to 40 years (Black & Sharpe, 1997; Hodge et al., 1994; Peroni et al., 2012).

Pharmacological therapies for asthma are associated with issues of tachyphylaxis and health side effects (Barnes, 2002; MacRedmond & Dorscheid, 2011). There is an increased public health interest in the benefits of dietary fish oil (n-3) and a number of supplementation studies have been conducted to understand the associations between n-3 and inflammation. Studies have reported anti-inflammatory effects of n-3 in asthmatics with decreased production of CystLTs and leuckocyte chemotaxis (Arm et al., 1989; Kirsch et al., 1988; Micklebough et al., 2006). Overall, the n-3 supplementation studies in the last

two decades have reported some beneficial effect on PEF, FEV_1, self-reported symptoms and quality of life (Emelynov et al., 2002; Dry et al., 1991; Hodge et al., 1998 Surette et al., 2008). However the findings of these studies are not consistent and some studies show no effect on pulmonary function outcomes (Arm et al. 1988, Kirsch et al. 1988, McDonald et al. 1990). Despite the inconsistencies in pulmonary function outcomes, a number of these n-3 supplementation studies have shown significant *in vitro* reduction *of* CystLT generation by stimulated neutrophils and polymorphonuclear cells, thereby confirming the anti-inflammatory effect of n-3 PUFA (Arm et al., 1989; Schubert et al., 2009; Mickleborough et al., 2006).

There are a very few studies to our knowledge which have evaluated the role of n-3 on pulmonary function in EIB prone females. In a cross over study with 10 asthmatics with EIB and 10 controls (male and female elite athletes; mean age of EIB group = 23.2 ± 1.9 years), three week supplementation with 3.2 gram EPA and 2.2 gram DHA per day improved post exercise pulmonary function (FEV_1) compared to placebo and baseline phases. There was a reduction in LTE_4, LTB_4, 9α 11β-PGF_2, TNF-α and IL-1β with supplementation (Mickleborough et al., 2003). In another study by the same group (n=16 recreationally active EIB participants, 10 males and 6 females, mean age 23 ± 1.6 years) their previous findings were confirmed and they reported a significant improvement in post exercise pulmonary function, following three week n-3 supplementation with the same dose. There was also a reduction in bronchodilator usage, induced sputum differential cell count percentage, concentrations of CystLTs and inflammatory cytokines (TNF-α and IL-1β). These studies suggest that dietary supplementation with n-3 PUFA has a beneficial effect in reducing EIB in males and females (Mickleborough et al., 2006). Although there are limited number of studies focussed on the effect of n-3 supplementation in females with asthma or EIB there are numerous studies describing variations in asthma during the menstrual cycle, figure 5.1 illustrates hormonal activity during menstrual cycle. Gibbs and co-workers have demonstrated that 30-40 % of asthmatic patients attending the outpatients department reported a pre-menstrual deterioration in their asthma symptoms (Gibbs et al., 1984). In a survey conducted by Eliasson & co-workers, (1986) it was found that 33% of women had significantly higher symptom scores during the premenstrual phase and the worsening of asthma symptoms were correlated with dysmenorrhea (menstrual pain) and premenstrual syndrome (Eliasson et al., 1986). In a recent study by Dratva et al., (2010), a large population-based sample of women with AHR was

investigated with reference to menstrual phase. It was found that women had high AHR in the pre-menstrual phase (mid-luteal phase) and cyclical variations in AHR were observed to be smaller among women taking oral contraceptives (Dratva et al., 2010). In another analysis conducted with a general population, fluctuations in pulmonary symptoms during the menstrual cycle were more severe during mid-luteal to mid-follicular stages, often with a dip near time of ovulation. It was suggested that the peaks varied between subgroups with different cyclical patterns according to asthma status, smoking status and BMI (Macsali et al., 2009). The mechanisms underlying the menstrual cycle, asthma and AHR are not clear and there appears to be reduced symptoms for those taking oral contraceptives; the two reproductive hormones oestrogen and progesterone are likely to have an effect (Lim & Kobzik., 2008; Stanford et al., 2006; Tan et al., 1997; Vrieze et al., 1997).

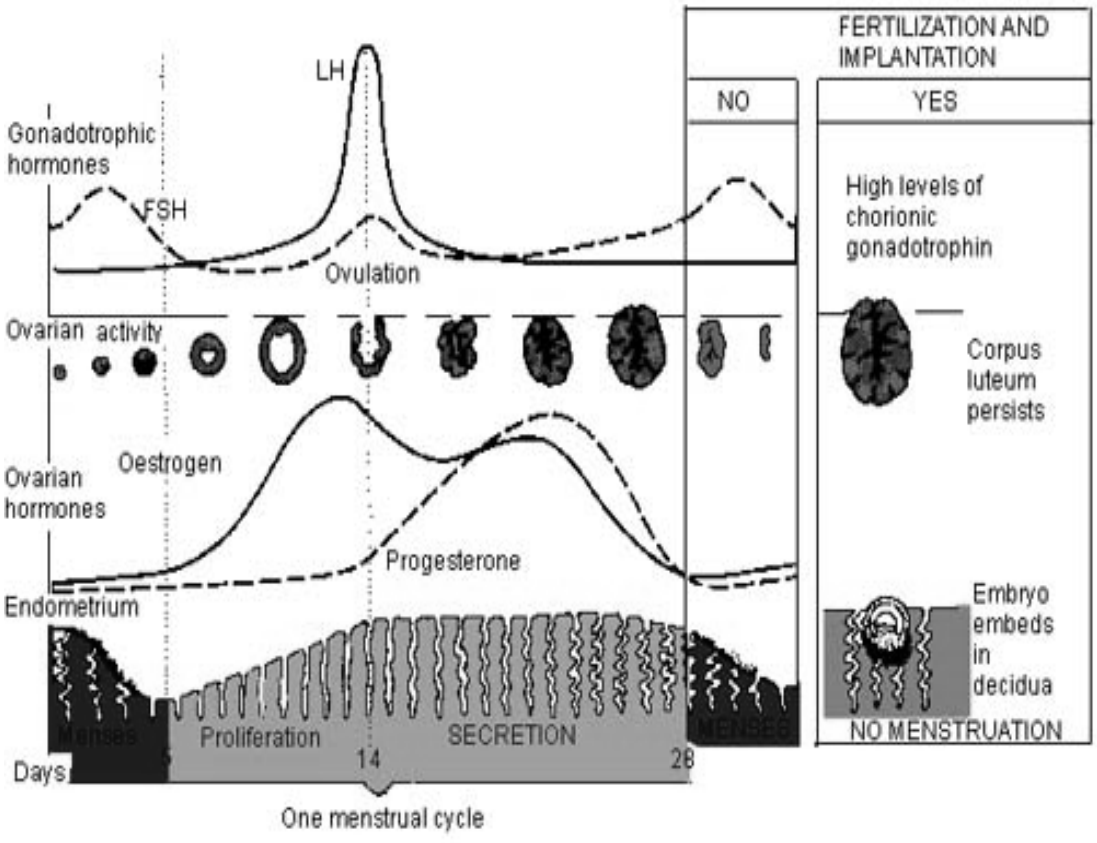

Figure 5.1: Hormonal activity during the menstrual cycle LH= luteinising hormone, FSH = follicle stimulating hormone.

Mandhane and co-workers (2009) have shown that elevated FeNO is a marker of airway inflammation and inversely related to oestrogen in the menstrual cycle, while the opposite was true for progesterone. Progesterone receptors found on the proximal region of the cilia of airway epithelia and when exposed to progesterone reduce cilia beating tendency. This reduced efficiency of cilia could impede airway clearance making individuals more susceptible to abnormal lung function and AHR (Jain et al., 2011). However, no consistent results have been reported when sex hormones are compared against various clinical and diagnostic outcomes of asthma (Macsali et al., 2012). Trials involving assessment of pulmonary function in females include monitoring and or adjustments based on menstrual cycle and may indirectly have implications for the study design. Potentially, this could be the reason behind the lack of intervention trials on females to assess the effect of n-3 on lung function in asthma. In this thesis, a record of menstruation (normal/abnormal) and use of oral contraceptive (pill) usage was collected to assess their effect on the study outcomes.

Increasing phospholipid EPA/AA ratios in inflammatory cells with dietary n-3 supplementation is likely to be one of the mechanisms that can potentially facilitate the production of weaker eicosanoids that may exhibit anti-inflammatory effects. Additionally fish oils may modulate T cell response and functions independently of eicosanoid production (Alexander et al., 1988; Jolly et al., 1997; Fan et al., 2003, 2004, Zeyda et al., 2002; Versleijen et al., 2012). The exposure to n-3 PUFA (with supplementation) has been reported to affect cell-signalling pathways by altering the expression and activity of membranes receptors. In addition these PUFAs can modify the expression of genes by the activation of transcription factors such as NFκB and PPAR-α (Calder, 2012; Rudkowska et al., 2009; Michaud & Renier, 2001; Mickleborough et al., 2008; Tai et al,. 2005).

In addition to the anti-inflammatory action of n-3 PUFAs via eicosanoid generation, in the last decade new families of lipid mediators produced from PUFAs have been discovered. These novel lipid mediators are endogenously generated during inflammation have potent anti-inflammatory effects; acting as specialised pro-resolving lipid mediators promoting resolution of inflammation (Hisada et al, 2009; Serhan., 2008, 2011). Serhan and collaborators identified, characterised, and elucidated families of novel pro-resolving lipid mediators from EPA/DHA (Bannenberg et al., 2010; Serhan et al., 2010). These include the E-series resolvins (RvE1 and RvE2) derived from EPA, D series resolvins

(RvD1,RvD2,RvD3,RvD4, RvD5, RvD6); protectins and the maresins which are all derived from DHA (Hong et al, 2003; Marcheselli et al., 2003; Serhan et al., 2002). There are additional series of lipid mediators generated in the presence of aspirin including the D-series Rvs, PDs and their aspirin-triggered forms. These mediators are synthesised from PUFA and via the aspirin mediated COX-2 pathway (Ariel & Serhan, 2007; Kohli & Levy, 2009; Serhan, 2011). In animal models, it has been shown that Rv synthesis is increased by feeding fish oil diets to rodents (Hudert et al., 2006). RvE1 can regulate IL- 23 and LxA_4 to promote resolution of allergic airway inflammation in a murine model of asthma (Haworth et al., 2008; Hisada et al., 2009). RvE1 can act on several phases of asthmatic inflammation and may have anti-inflammatory effects on various cell types such as lymphocytes, macrophages, eosinophils and neutrophils. Furthermore, it has been shown that RvE1 can bind to and block leukocyte B_4 receptor 1 and is involved in the dampening of airway inflammation and in the resolution of inflammation (Aoki et al., 2008; Haworth et al., 2008).

The biological characteristics of these new anti-inflammatory and pro-resolving mediators derived from n-3 PUFAs and the pathways that drive the formation and actions of these molecules have provided a new concept for treating airway inflammation. The majority of studies conducted have been in animal models including murine, feline and rabbits, with limited studies on humans. Following the identification of pro-resolving and anti-inflammatory effects of RvE1, a clinical trial is currently being conducted to assess the effect of RvE1 on dry eye. Dry eye is a common ocular surface disease, common in women and the elderly, with chronic symptoms of eye irritation and in severe cases, blurred vision; it is suggested that there is an inflammatory component underlying this disease (Li et al., 2011). The phase 2 results from the clinical trial provide an indication that the agonist Rv has the ability to produce a dose-dependent improvement in both the signs and symptoms of dry eye. Furthermore, no health side effects are reported in this trial (Brooks, 2009; Resolvyx, 2012: ClinicalTrials.gov identifier: NCT00799552). This first clinical study of the effect of Rv in humans will help improve the understanding of the agents that can stimulate the resolution mechanisms and resolve acute inflammation along with chronic inflammation to reduce human diseases where uncontrolled inflammation forms the basis of their pathophysiology (Serhan, 2011). Early phase trials are currently on going for natural and synthetic resolvins for various disease conditions such as asthma, inflammatory bowel disease and other related inflammatory diseases; however, no

information about the appropriate dosage of these compounds have been publicised. Based on animal model studies a dosage ranging from 10ng - 0.1mg/kg is found to have anti-inflammatory effects by reducing pain, mucus, AHR and leukocyte infiltration (Duffield et al., 2006; Haworth et al., 2008; Xu et al., 2010).

These results from animal model are encouraging and highlight the anti-inflammatory and pro resolution effects of Rvs and other lipid mediators. Additionally, these mediators have no proven health side effects making them attractive as alternative treatments for inflammation. Thus, well-designed studies are required to investigate the relationship of these new mediators with asthma in humans. However, due to lack of guidance on dosage, the preliminary findings from animal models are required for careful translation and application for research in humans.

Aims and hypothesis

The third aim of this chapter was to investigate the effect of EPA-derived bioactive mediators (E series Resolvins) on post exercise FEV_1 in mild – moderate EIB-prone females.

It was hypothesised that there would be a statistically significant improvement in post-exercise FEV_1 after three-weeks of resolvin supplementation. To support this study, EIB prone mild – moderate asthmatic females were recruited using fixed criteria (80% ratio for FEV_1/FVC). To evaluate the effects of resolvin supplementation dyspnoea score and FeNO levels were simultaneously measured. Circulating levels of IL-6, IL-10 and TNF-α were measured to evaluate the changes in cytokines with supplementation.

5.3 Methods

This section contains brief description of the methods used during this study and will refer to chapter 2 for further particulars.

5.3.1 Study Design and sample size estimation

A double blinded, placebo controlled, randomised, crossover trial was designed to assess the effects of a 3 week supplementation with E series resovins on EIB prone participants. The method for randomisation by minimisation and double blinding is described in chapter 2; page 111. A power analysis was completed to approximate the appropriate sample size based on previous studies showing the effect of n-3 PUFAs on pulmonary function in EIB subjects (Mickleborough et al., 2003, 2006). The design aimed to observe a minimum detectable difference or change of 0.3 litres in FEV_1 after exercise with the supplementation. The significance level was set at alpha = 0.05 and the power at 0.80. Using these data, a sample size of 8 participants necessary to detect a difference of this magnitude. To allow for fluctuations in participant compliance, withdrawals and incomplete data, a target sample size was set to 12 participants. Considering the sample size was small, the participants were randomly allocated using a minimisation scheme (Altman & Bland, 2005) whereby the next allocation of supplementation capsules were dependent on the supplementation provided to the previous participant. This scheme of randomisation has been found to be appropriate to achieve balance between the treatment groups. The participants, investigator (I) and outcome assessors (supervisors) were blinded to the study; the randomisation pattern was kept with the technician in charge of randomisation.

5.3.2 Dose and duration of supplementation

All participants entered the study on their normal diet (phase 1/baseline1), after which they were randomly allocated to receive soft gelatine capsules: either 10 capsules per day of Resolvin E1 (containing 100 mg Resolvinol and 0.5% Vitamin E) or Placebo (olive oil and 0.5% Vitamin E, Bionovate, UK) [phase 2]. After the initial supplementation, participants completed a 3 week washout phase [phase 3, baseline 2]. Thereafter the participants switched over to alternate supplementation for the remaining 3 weeks [Phase4] (figure 5.2). The dosage was determined by the preliminary findings (from animal models and

human studies) reported by Bionovate Inc. who supplied the supplements for the study. Another three-week end of study washout followed completion of the second supplementation phase (normal diet; phase 5). In a number of well-designed n-3 supplementation studies washout phases ranging from 2-10 weeks are used (McDonald et al., 1990; Mickleborough et al., 2003, 2006; Kirsch et al., 1988; Yaqoob et al., 2000). A shorter duration was chosen for participant retention as longer washout or intervention phases have a likelihood of drop out (Max et al., 1991).

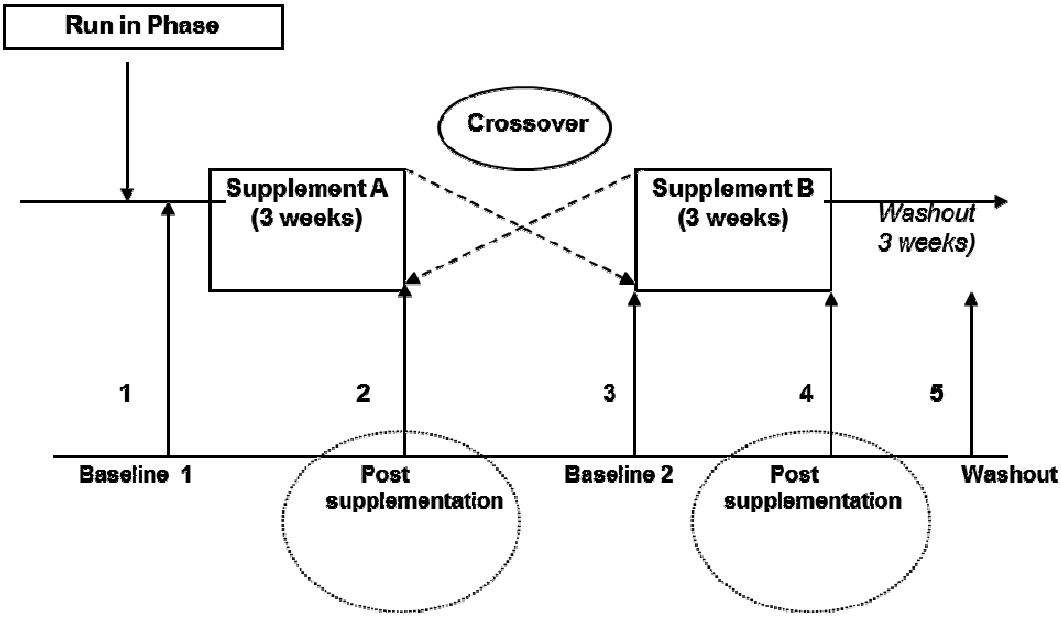

Figure 5.2 Study design for resolvin supplementation

5.3.3 Participants

Considering the issues of lung maturation and ageing, female participants were included if they were between 18-30 years of age to represent young adults. All participants had previous physician diagnosed mild-moderate asthma , had no respiratory infection within the past 8 weeks and had not taken any medication [steroids, of any kind in last four weeks, hay-fever or other allergy medication (in the past 7 days)] and dietary n-3 supplementation in last one year. There were no dietary restrictions during the study other than personal n-3 supplementation. All participants completed a health screen questionnaire and gave written informed consent before participating in the study (appendix 1). The information about menstrual cycle and oral contraceptive pill usage upon entry to the trial (at baseline) collected from the health screen questionnaire were

used for analysis. The Loughborough University's ethics committee approved the protocol for the study, recruitment flyer, health screen questionnaire, participant information sheet and consent form are attached in appendix 1. All the participants were screened for EIB (≥ 10% drop in post exercise FEV_1) and FeNO (≥ 40ppb) at baseline.

At the baseline and following completion of the supplementation and washout, the subjects reported to the lab for testing. PFTs were assessed pre exercise and post exercise (at 1, 5,10,15,30 and 45 minutes or until recovery (whichever was earlier). Blood was drawn before exercise by a certified phlebotomist from the antecubital vein for ELISA for measurement of serum cytokines (TNF-α, IL-6 and IL-10). Bronchodilator use was recorded by participants during all the study phases. The participants also reported to the lab at the end of washout and underwent all physiological tests; in addition blood samples were collected for measurement of serum cytokines. These tests were conducted to assess if individuals had returned to the baseline level for all the physiological parameters to determine any carry-over effects.

Nine female participants with mild-moderate asthma and a history of EIB were recruited from the Loughborough University, East Midlands, UK. One participant dropped out due to difficulties with swallowing of the capsules and dropped after 3 weeks. Eight participants (mean age 21.8 ± 1.14 years, and mean height 1.6 ± 0.01 meters successfully completed the entire protocol as they met all the inclusion criteria for the study. Relevant anthropometric and pulmonary function characteristics of participants are reported in table 5.1

Table 5.1 Anthropometric and pulmonary function characteristics of Participants, FEV_1 = Forced Expiratory Volume in 1 sec, FVC = Forced Vital Capacity, PFT = Peak Expiratory Flow, FEF 25-75% = Forced Expiratory Flow (showing 25% and 75 % of FVC). Values are reported in mean ± SEM

Characteristics	Measurement (n =8)
Stature (m)	1.6 ± 0.01
Age (years)	21.8 ± 1.14
Weight (kg)	64.3 ± 4.18
FEV_1/FVC	**75.27** ± 1.02
% predicted FEV_1/FVC	**85.8** ± 0.36
FEF 25-75 (l/second)	3.8 ± 0.10
PEF (l/minute)	393.0 ± 24.09
FeNO (ppb)	**46.5** ± 2.74
Medication usage	n = 4 on β2 agonist only; n = 4 on β2 agonist and short acting steroids

Since the LLN derived from a reference population in chapter 3 was specific to males only, therefore the LLN criteria was not used for asthma characterisation in this study. All participants were tested positive for asthma in the laboratory, with a FEV_1 of ≤ 80% in accordance with the latest ERS/ATS guidelines (2009), tested positive for EIB (≥ 10% drop in post exercise FEV_1) and showed high FeNO (≥ 40ppb). In addition, participants reported accounts of shortness of breath, chest tightness, and intermittent wheezing post exercise which was relieved by bronchodilator. Participants were advised to refrain from medication usage (for short acting β_2 agonists) for 48 hours prior to testing and their medication use was recorded. The participants were advised to discuss the situation with their medical practitioner before coming off medication. Testing was rescheduled if participants used medication 48 hours prior to testing and a log of medicine usage was maintained till the participant was tested. Participants were also asked to refrain from caffeine intake (8 hours) and physical activity (for 24 hours) prior to the exercise challenge test. The research design for the studies conducted in the thesis was such that the physiological tests were conducted at similar times and blood samples were collected at the same time of the day at each collection point (±30 minutes); and the sample analysis was conducted within 2 hours of collection.

5.3.4 Outcome measures

The primary outcomes for this study were changes in baseline pulmonary function measures (FEV_1, FVC, and PEF) and post exercise change in FEV_1 following 3 weeks of supplementation with RvE1. In addition, symptom scores, bronchodilator use, fraction of exhaled breath nitric oxide (FeNO) and levels of cytokines were measured. Additionally, the relationship of oral contraceptive pill usage and its potential effect on the study outcomes were also assessed.

5.3.5 Physiological and biochemical measurements

Physiological measurements including the exercise challenge test, pulmonary function tests, FeNO test and perceived symptoms score (dyspnoea) were completed following procedures outlined in chapter 2, page 72-76. Serum cytokine analysis for IL-6, TNF α and IL-10 and compliance monitoring were conducted following procedures outlined in chapter 4, page 152. Information about oral contraceptive (pill) usage was collected from the health screen questionnaires collected prior to the study.

5.3.6 Statistical Analysis

Data was analysed using SPSS version 19 statistical software (SPSS Inc., Chicago, IL). A two-way repeated measures analysis of variance (RM ANOVA) was used to measure changes in physiological and biochemical parameters within the same individuals as a result of resolvin/placebo supplementation over a period of time (within-subject effects). Mauchly's test was conducted to determine whether sphericity was violated. In the cases where sphericity was violated, the repeated measures analysis of variance was corrected using the Greenhouse–Geiser correction factor. Owing to the small sample size of the test, the least significant difference was applied for adjustment during pair-wise comparison of data using estimated marginal mean. Order effect was assessed by using the order as a variable during analysis. The relationship of oral contraceptive pill use to lung function improvements was assessed by using contraceptive pill usage as a variable for RM ANOVA. Student's paired t test were conducted to compare the physiological and biochemical outcomes of the group using contraceptive and the group without it.

5.4 Results

Out of the nine participants recruited, eight participants successfully completed the entire study protocol. The participants were given clear instructions about the supplementation (resolvin and placebo) and were provided with approximate number of capsules in a pot. Additionally, the participants were sent reminders by end of each week of supplementation and the remaining capsules in the returned pots were counted to estimate compliance. All participants had less than 5 capsules returned which was considered acceptable. Also, there was no effect of order in which supplementation was provided ($p>0.05$). All results reported in this section are expressed as mean ± SEM unless stated otherwise. The following section describes the findings from this study

5.4.1 Primary outcomes

5.4.2 Pulmonary function test and exercise challenge

No Carry over effects

Reduction in FEV1 in excess of 10% post exercise represents abnormal pulmonary function. Furthermore, two time-points showing >10% drop in FEV_1 are considered indicative of EIB and are reliable for EIB diagnosis. Mean percentage change from pre exercise to post exercise FEV_1 was calculated, and the change in FEV_1 after resolvin supplementation is shown in figure 5.4. There were no differences in either the pre exercise (rest) or post exercise FEV1 between the two baselines based on RM ANOVA, demonstrating no carry over effects as shown in figure 5.3($p>0.05$). Subsequently, all comparisons after supplementation were made with baseline1. There was also no differences between the two baselines, placebo and the end of study washout ($p>0.05$, figure 5.3)

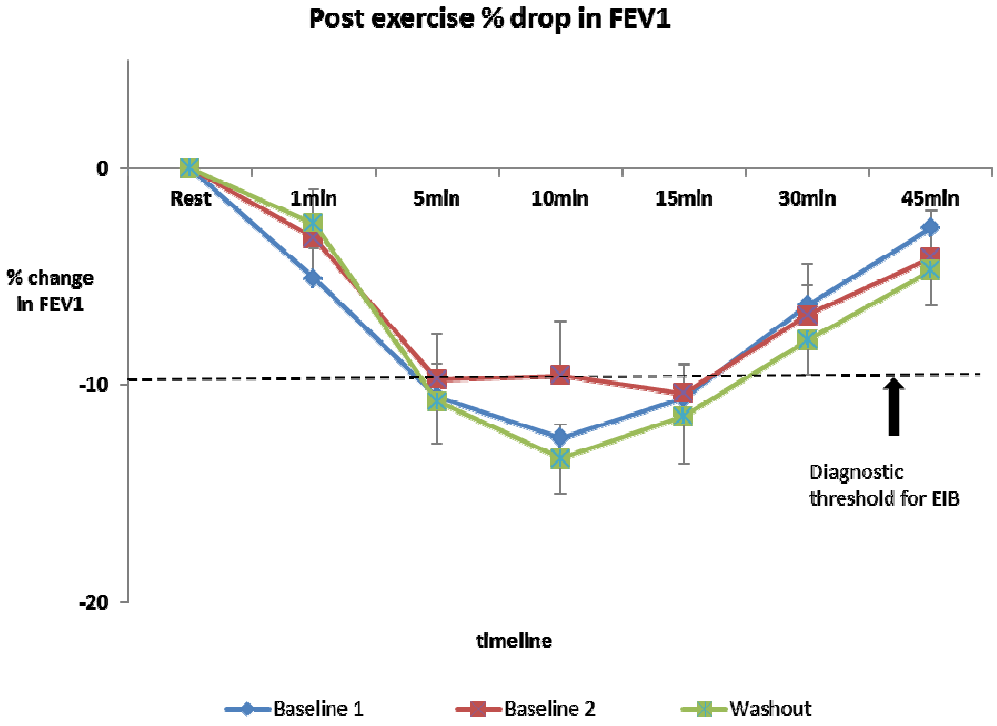

Figure 5.3 No carry over effects: There were no significant differences observed for % change in FEV_1 after exercise at baseline1, baseline 2, and end of washout phase for all participants using RM ANOVA. Reduction in FEV_1>10% shows a positive diagnosis for EIB.

Improvement in post exercise FEV_1 with Rv supplementation

Mean percentage change from pre exercise to post exercise FEV_1 was calculated. At the two baselines and after the placebo phase all participants demonstrated EIB symptoms with a >10% drop in post exercise FEV_1 at two time points (10 and 15 minutes). The mean values are displayed in figure 5.4. The reduction in FEV_1 at baseline and after the placebo phase was 13.1 % and 12.9 % respectively .The percentage drop in FEV_1 post Rv supplementation was only 9.4 % which is below the diagnostic threshold for EIB and indicates an attenuated EIB response. This value was significantly lower than the values recorded at baseline and after the placebo phase ($p=0.02$) and suggests a 30% improvement in post exercise FEV_1 with Rv supplementation. There was no difference observed between the two baselines, placebo and washout phase ($p>0.05$). Overall, the mean values of the group show an improvement in post exercise FEV_1 after supplementation and this is the first study in females with EIB demonstrating this effect.

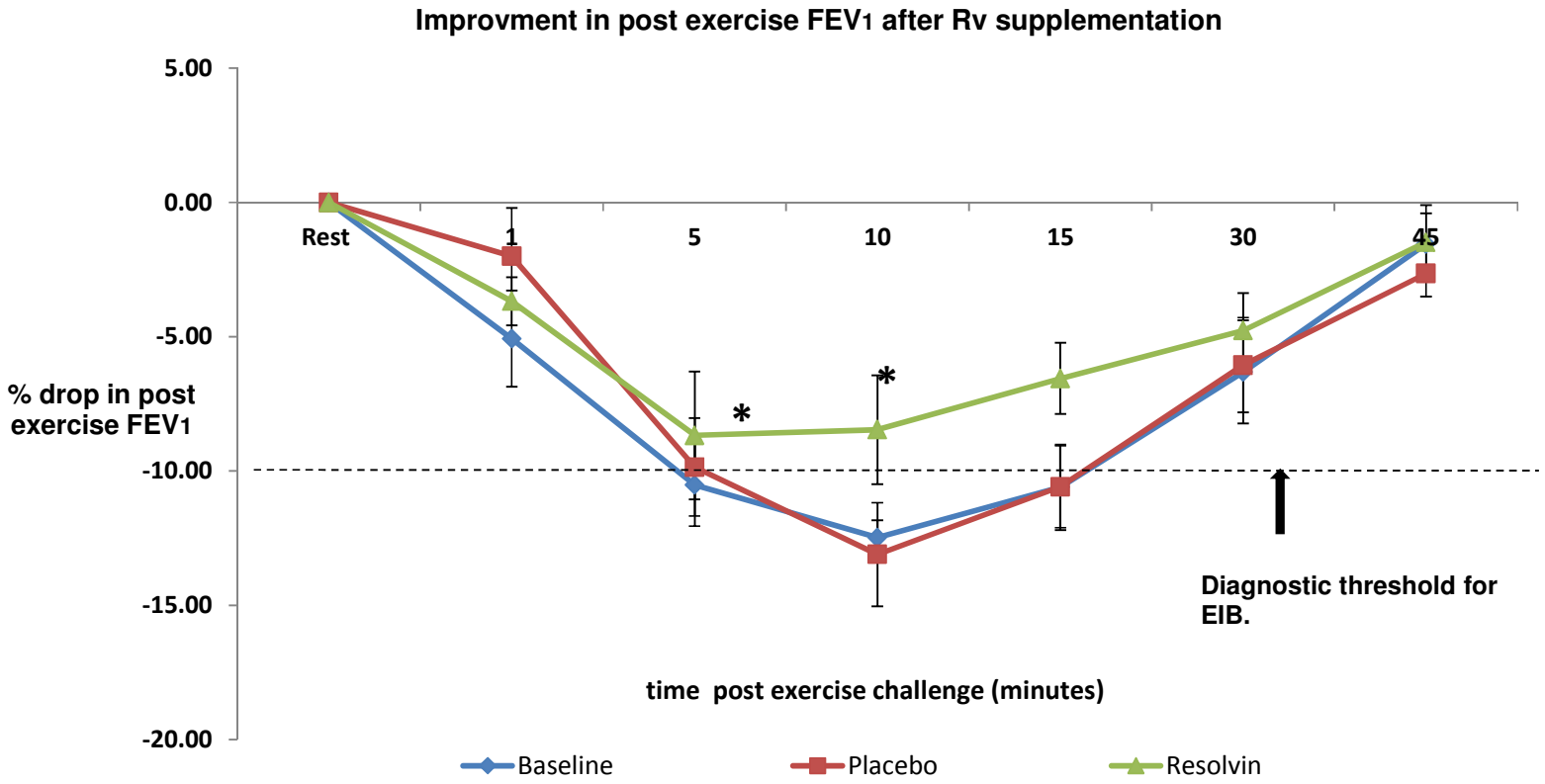

Figure 5.4 The percentage change in FEV_1 from before and after exercise at baseline, placebo and resolvin phase for all participants. Reduction in FEV_1 >10% shows a positive diagnosis for EIB
* Statistically significant difference in post exercise FEV_1 compared to baseline ($p<0.05$)

Pulmonary function tests

No changes in pre exercise FEV_1/FVC ratio with supplementation

FEV_1 and PEF are measures to assess the full potential of the lungs and there was no change in mean FEV_1 and PEF across all time points ($p>0.05$). FVC represents the vital capability of the lungs and as expected there was no change observed in the FVC value at any time point ($p>0.05$). FEV_1/FVC ratio is an important parameter for asthma diagnosis and no changes were observed in the FEV_1/FVC ratio across all time-points as shown in figure 5.5 ($p>0.05$). Overall; there was no effect of Rv supplementation on the pre exercise pulmonary function outcomes, however there was substantial improvement in post exercise FEV_1 as presented in the last section (figure 5.3).

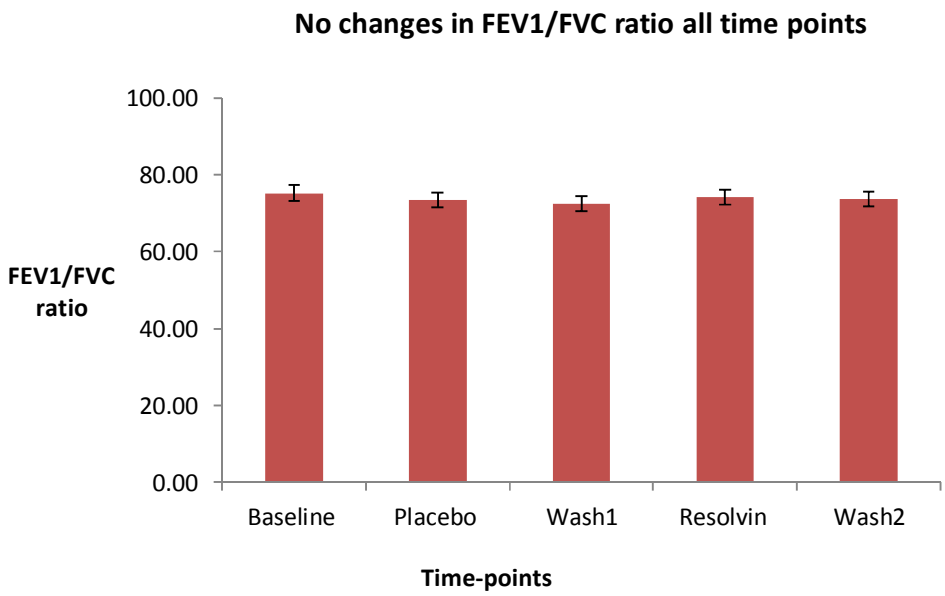

Figure 5.5 Changes in FEV_1/FVC ratio at all-time points; no change was observed after Rv supplementation or at any other time-point.

Bronchodilator usage changed within the first week of supplementation

The participants reported the number of puffs of bronchodilator used per week and each usage represents two puffs. There was no overall change in the mean bronchodilator usage across all time points (p=0.15, figure 5.6). There was no data collected for baseline 1 measurement.There was a reduced use of bronchodilators reported in week 1 of Rv supplementation (3 times or 6 puffs, figure 5.5). However, the pattern did not continue across the entire supplementation phase. The total bronchodilator usage was 11 times (22 puffs) during the placebo phase while it was 13 times (26 puffs) during the Rv supplementation phase. Bronchodilator usage was reported 17 times (34 puffs) during the washout after placebo phase and 15 times (30 puffs) during washout following the Rv supplementation phase.

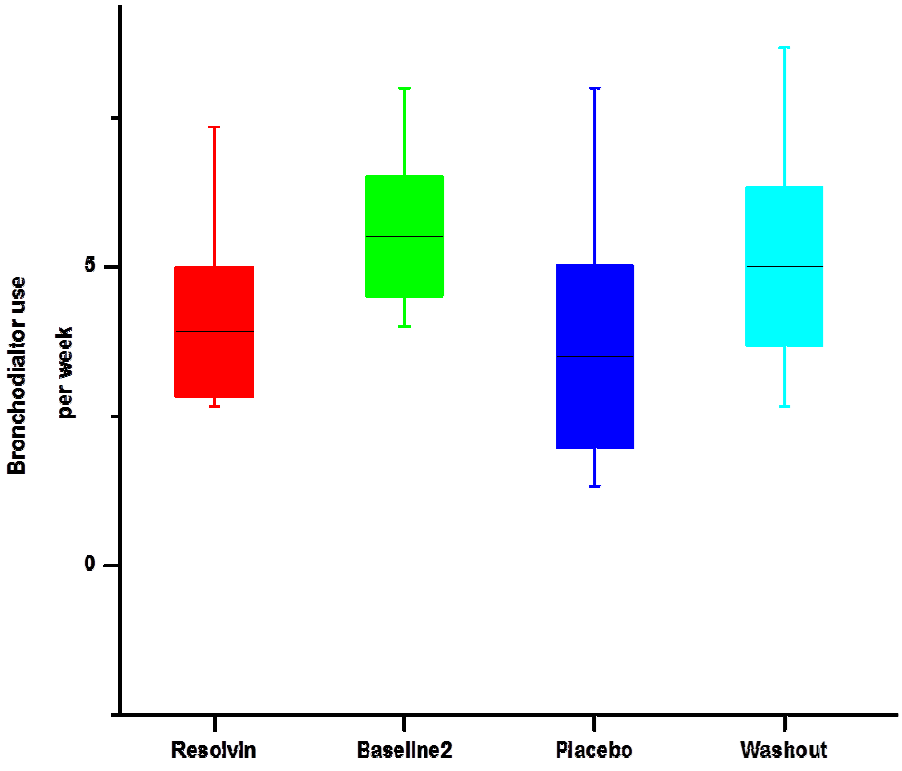

Figure 5.6. Changes in bronchodilator usage across all time-points

5.4.3 Secondary outcomes

No change in FeNO with Rv supplementation

All participants had elevated levels of FeNO compared to upper threshold of healthy individuals (25ppb) before the start of supplementation. Measurements for FeNO were collected at rest and post exercise challenge test at all-time points.

At rest, the values measured for baseline (46.5 ± 2.75ppb) were comparable to values measured after Rv supplementation (49.2 ± 11.58ppb) or placebo (43.75 ± 5.69ppb). Overall, there was no change in the FeNO levels at rest at any time points as displayed in figure 5.7, (p=0.59). Similarly, there was no change in the post exercise FeNO levels measured at each time point (p=0.29). Thus, there was no overall reduction in FeNO levels with 3-week Rv supplementation.

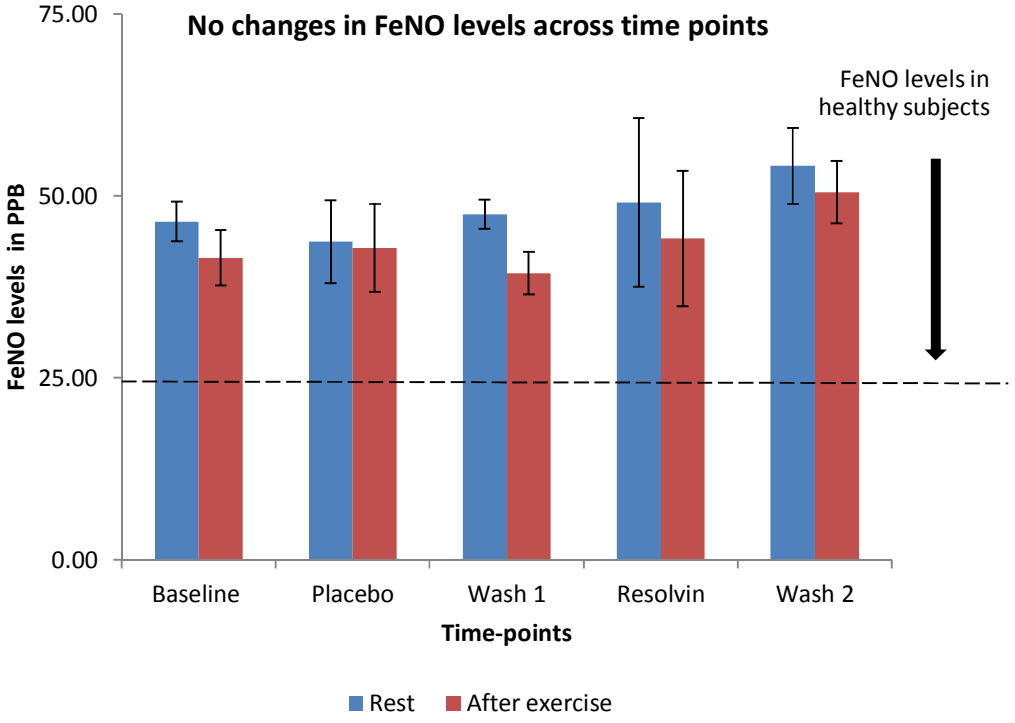

Figure 5.7 Changes in FeNO levels across all time points, normal levels for FeNO in healthy individuals is marked at 25ppb

5.4.4 A reduction in serum IL-10 and no change in serum IL-6 and TNF-α levels with Rv supplementation

There were no changes in the mean levels of serum TNF-α, IL-6 and IL-10 across the two baselines, placebo and the washout phases ($p>0.05$), subsequently all comparisons are reported with reference to baseline.

Though there was a reduction in TNF-α levels with Rv supplementation (4.99 ± 0.64pg/mL), this was not statistically different to baseline (7.26 ±1.02 pg/mL) ($p=0.09$). There was no significant change in IL-6 levels with Rv supplementation (4.43 ± 1.04 pg/mL) compared to baseline (2.67 ± 0.28 pg/mL). There was, however, a statistically significant reduction in IL-10 levels with Rv supplementation (4.63 ± 0.32 pg/mL) compared to baseline (6.22 ± 0.58) ($p=0.014$) as shown in figure 5.7.

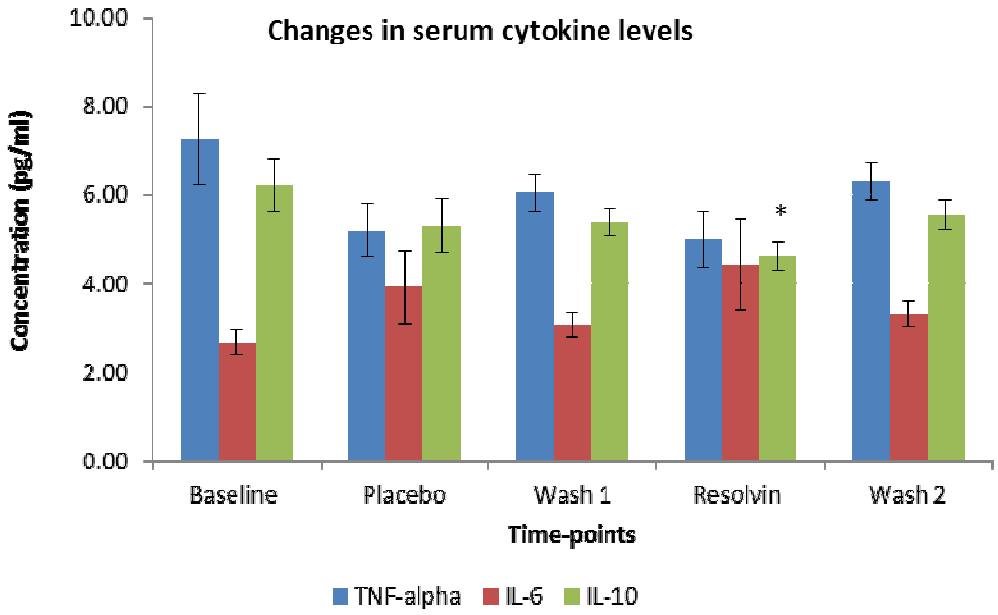

Figure 5.8 Serum cytokine levels across all time points;
* statistically significant difference between baseline and resolvin phase for IL-10 ($p<0.05$)

5.4.5 Relationship of oral contraceptive (pill) usage to study outcomes

Eight eumenorrheic participants (experiencing normal menstruation) completed the study and, of these, there were 5 participants currently taking an oral contraceptive (pill). There was no information available for the nature of the pill i.e. oestrogen only, progesterone only or a combination. The participants were grouped based upon whether they used a contraceptive pill or not, and the data was compared for the different study outcomes as reported below. It is important to note here that this study was not powered to detect the impact of oral contraceptive pill on pulmonary function. Thus, due to the small sample size recruited, it is difficult to make suitable inferences and caution is required while interpreting these findings.

Pill and pulmonary function

The pre exercise pulmonary function parameters including PEF, FEV_1 and FEV_1/FVC ratios were compared between the participants taking a contraceptive pill and those who were not. At baseline, there was no difference in mean PEF between the participants taking oral contraceptives (438 ± 26.81 l/min) and those who were not (398 ± 3.38 l/min) ($p=0.30$) suggesting no effect of oral contraceptives. Also, no differences were observed in FEV_1 values at baseline for the two groups (pill = 2.84 ±0.12 litres, no pill= 2.82 ± 0.05 litres; $p=0.90$). The FEV_1/FVC ratio of the two groups also showed no difference at baseline (pill =74.6 ± 1.5%; no pill =73.6 ± 1.6%, $p>0.05$). No difference in FEV_1/FVC ratio, PEF and FEV_1 were observed between the washout phases and placebo compared to baseline for those on or without an oral contraceptive pill ($p>0.05$).

The maximum percentage drop in post exercise FEV_1 at baseline for the group on pill was recorded as -13.96 ± 0.09% (group mean) which was similar to the drop demonstrated by the group without any pill [-12.25 ± 0.80%(group mean)]. Although there was an overall improvement in the post exercise FEV_1 for the entire study population following resolvin supplementation compared to baseline ($p<0.05$), there were no differences observed in the magnitude of the improvement between the two groups (pill = -8.82 ± 2.98%, no pill = -10.27 ± 2.12%). There were no differences in the washout or the placebo phase compared to the two baseline as displayed in figure 5.9 ($p>0.05$).

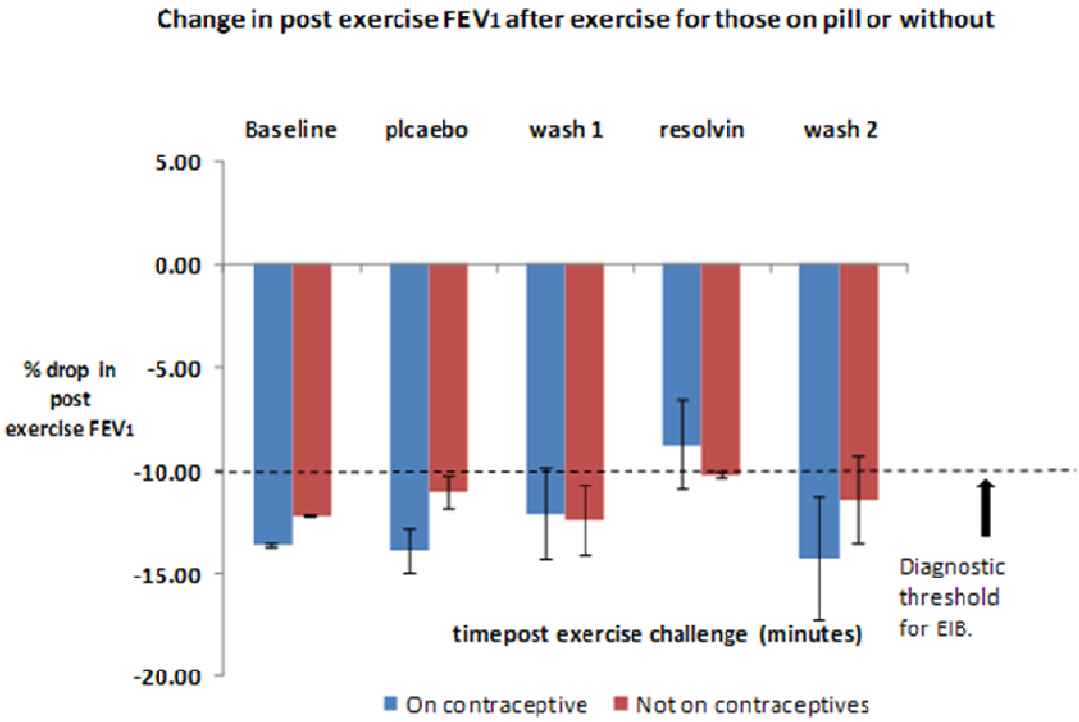

Figure 5.9 Post exercise change in FEV_1 across all time points for those on the pill and those without

Pill and FeNO

The group taking an oral contraceptive pill showed a mean baseline (pre exercise) FeNO level of 49 ± 4 ppb which was no different to the mean value measured for the group who were not taking the pill (42 ± 1 ppb) ($p=0.27$). These values remained unchanged across all time-points for both the groups ($p>0.05$). There was also no difference in the change in FeNO levels with resolvin supplementation between the two groups (pill=56 ± 18 ppb; no pill = 36 ± 7ppb).There was also no difference between the post exercise FeNO values of the two groups across any time point of the study ($p>0.05$). Overall, these data show that there was no relationship between FeNO levels and oral contraceptive intake.

Pill and cytokines

There were no differences in serum TNF-α levels for those taking an oral contraceptive pill or not at baseline (pill= 8.11 ± 1.51 pg/mL, no pill= 5.83 ± 0.78 pg/mL). Similarly, there were no differences measured in the IL-6 levels for those on an pill (2.68 ± 0.47 pg/mL) or those not (2.67 ± 0.13 pg/mL). In addition, the levels of IL-10 showed no differences between the two groups at baseline (pill= 5.19 ± 0.42 pg/mL, no pill = 7.60 ± 0.80). There were no differences in the levels of these cytokines after resolvin supplementation ($p>0.05$). On the whole, there was no relationship between oral contraceptive use and cytokine levels. Although there was a significant reduction in IL-10 levels for the two groups combined together as shown in figure 5.8 there were no differences between the two groups in terms of their response to supplementation ($p>0.05$).

5.5 Discussion

The aim of this chapter was to investigate the effect of EPA-derived bioactive mediators (E series Resolvins) on post exercise FEV_1 in EIB-prone females. This study was designed to test the hypothesis that there would be a statistically significant improvement in post-exercise FEV_1 after three-weeks of resolvin supplementation To investigate the hypothesis, a randomised, placebo controlled, double blind, cross over study was conducted comparing physiological and biochemical variables before and after 3-week resolvin/placebo supplementation phases. Additionally, the possible relationship between oral contraceptive pill use with pulmonary function changes and other outcomes of the study were evaluated. This is the first study to demonstrate a beneficial effect of 3-week of supplementation with E series resolvins in EIB prone females. The beneficial effect was marked by a significant improvement in post exercise FEV_1 (30% improvement) following supplementation which meant that the mean FEV_1 value for the group was below the diagnostic threshold for EIB. Although there was a significant improvement in post exercise FEV_1, the other outcomes (bronchodilator usage and FeNO levels) did not show any changes following supplementation. However, there was also a reduction in the levels of cytokine IL-10 but there were no changes in the levels of inflammatory cytokines (TNF-α and IL-6).

There are currently no prior studies, which have assessed the effect of resolvin supplementation in EIB prone females, thus it is difficult to compare the results to external, validated findings. Mickleborough and co-workers (2006) have demonstrated a 36% improvement in post exercise FEV_1 following three week supplementation with n-3 PUFA (3.2 grams EPA and 2.2 gram DHA) in EIB males and females. These changes were accompanied by a reduction in sputum concentrations of CystLTs (LTC_4-LTE_4), PGD_2 and circulating levels of IL-1β, and TNF-α before and after exercise following n-3 supplementation (Mickleborough et al., 2006). These findings are a confirmation of similar findings in elite athletes (males and females) with EPA/DHA supplementation using the same dose (Mickleborugh et al., 2003). In the study presented in chapter 4, it is reported that an approximately 45% improvement in post exercise FEV_1 is observed with three weeks of EPA/DHA supplementation in a dose similar to that given by Mickleborough et al ., (2006). Asthma is characterised as an airway disorder with airway and systemic inflammation (Macsali et al., 2012). The two prior studies discussed here, as well as this work, have provided some indication of an improvement in post exercise pulmonary function following n-3 supplementation. This beneficial effect as displayed by reduction in pro inflammatory eicosanoid production is signifying the hypothesis that n-3 PUFA have the ability to alter the eicosanoid synthesis by changing cellular composition (Bagga et al., 2003; Chapkin et al., 1992; Lee et al., 1985; Robinson& Stone, 2006; Sperling et al., 1993).

Rvs belong to a class of lipid mediators generated from EPA which are able to reduce and resolve inflammation and are found to be active in small concentrations (Bannenberg et al., 2005; Hisada et al., 2009, Serhan, 2011b). Resolvins along with protectins act during an inflammatory process in small quantities to facilitate the production of pro-inflammatory mediators and regulate the trafficking of inflammatory cells and mediators to sites of inflammation (Serhan et al., *2*008, 2012; Levy et al., 2007). Airway responses to exercise (EIB), injury, noxious stimuli, or microbes lead to leukocyte recruitment for host defence and as leukocytes respond, they may react with resident cells of the lungs and can produce a cascade of specific mediators such as Rvs. The Rvs may be enzymatically generated from EPA/DHA which could subsequently account for the anti-inflammatory effects of resolvins (Ariel & Serhan, 2007, Aoki et al., 2010). These bioactive, lipid-derived small molecules serve as agonists at specific receptors and are involved in resolution of localised inflammation (Kohli &Levy 2009, Serhan, 2011b). There is little information on

role of Rvs in lung diseases such as asthma; however Rvs are shown to exert anti-inflammatory effects on models of operitonitis, renal ischaemia–perfusion injury and dry eye (Bannenberg et al., 2005; Carlo & Levy, 2010; Resolvyx, 2012: ClinicalTrials.gov identifier: NCT00799552; Serhan, 2008). Based on these demonstrated anti-inflammatory effects, it is likely that resolvins would also be protective in the lungs and promote the resolution of airway injury and inflammation.

Our study shows that 3-week resolvin supplementation reduced the severity of EIB in female participants. In this study, resolvin supplementation improved lung function to below the diagnostic threshold of EIB which is a 10% fall in post exercise at FEV_1 at two time points after exercise. Consequently, there appears to be a suggestion that there is an effect of supplementation with n-3 or their bioactive metabolites on lung function.

Fractional exhaled breath nitric oxide (FeNO) is a non- invasive, clinically validated marker of airway inflammation. In our study no changes were observed in FeNO levels across any time point. Recent studies have used FeNO levels as a surrogate marker of eosinophilic airway inflammation and airflow obstruction in asthma based on the ATS, (2005) guidelines. The endogenously produced NO, as measured by FeNO, may reflect overall gas transfer across the alveoli in women with asthma (Borland & Higenbottam, 1989, Farha et al., 2009). However it has been shown that menstrual cycle has an impact over lung function which may also be affecting FeNO levels (Farha et al. 2009; Mickleborough et al. 2006). Stanford and co-workers (2006) have shown that pulmonary function in females is affected by menstrual cycle. The maximal percentage decline in post-exercise FEV_1 and forced expiratory flow from 25 to 75% of forced vital capacity (FEF (25-75%) was found to be significantly greater ($P<0.05$) on day 21 (mid-luteal phase), when salivary progesterone concentration was highest compared to day 5 (mid-follicular phase). This deterioration in the severity of EIB during the mid-luteal phase was mirrored by worsening asthma symptoms and increased bronchodilator use. Hence, a record of the menstrual cycle phase or control of the timing of experiments with regard to the menstrual cycle may be important when testing female participants for EIB and other respiratory ailments. Additionally, the frequency and severity of asthma symptoms is reduced when serum levels of oestrogen (oestradiol) are high, as observed after exogenous eostradiol administration, oral contraceptive use and during ovulation (de Oliveira et al., 2010; Matsuo et al., 1999).

Mandhane and co-workers (2009) monitored asthmatic females throughout a complete menstrual cycle and observed that increasing oestrogen levels were associated with decreasing FeNO values, whereas increasing progesterone levels were associated with increases in FeNO and atopy (measured by skin-prick tests). Additionally, it was found that participants using oral contraceptives showed no significant progesterone-associated increase in FeNO throughout their menstrual cycle (Mandhane et al., 2009). Asthmatics in our study were grouped based on their pill usage and there was no difference in the pulmonary function outcomes, cytokines as well as FeNO levels at baseline. Following resolvin supplementation for three weeks, no changes were observed in the overall FeNO levels as well as between the groups. A limitation of the present study was that a record of menstrual cycle was not maintained which could limit the understanding of changes in study outcomes during different phases of the menstrual cycle, although, again, this was not the primary focus of this supplementation study. Contraceptive pill usage was, however, recorded in the study but no relationships were observed between the pill usage and any of the study outcomes. Thus, whether the participants were taking oral contraceptive pills or not it made no difference to any of the outcomes of the study. This could be attributed to the small sample size of the study.

The FeNO measurements were conducted using hand held potable device that are widely used in fieldwork and clinical settings. However there are some concerns about limitations of this method arising due to their inability to control flow rates and it has been suggested that interpretations should be cautiously done for the results generated by hand held devices compared to stationary devices. This limitation of the procedure is discussed in chapter 4 (pages 164-165), however throughout the study the same hand held device was used so the likelihood of having variations is minimal and caution is required if these results are compared to results from stationary devices. It is suggested that the portable devices show relatively lower values than stationary devices (Kim et al., 2012). Considering this argument, the asthmatics in this thesis showed values >40 ppb for FeNO which suggests a high inflammation in the study population compared to healthy individuals (25 ppb). Subsequently, the duration or the dose of resolvin supplementation could be one of the reasons for observing no improvement in FeNO. Dose response and time course studies are required to evaluate the dose and duration of resolvins required to reduce FeNO levels significantly.

Levels of inflammatory cytokines including TNF-alpha, IL-6 and IL-10 were monitored before and after resolvin supplementation and only a reduction in serum IL-10 levels ($p<0.05$) was documented. No changes to IL-6 and TNF-alpha levels were observed. Thus, there is inconclusive evidence suggestive of further work is required with larger sample size and improved methods. The values recorded for these cytokines in our study represents the normal range for asthmatics found within the literature as discussed in chapter 4.Though the participants in the study were eumenorrheic with five out of eight on the pill, no adjustment was made for menstrual cycle phase. Owing to the cross-over nature of the study, it was considered important to start the resolvin supplementation on the individuals at the same time. Thus, the participants could possibly have been at different points of their menstrual cycle during the 3-week supplementation that could possibly account for poor improvement in cytokine and FeNO levels. Future studies should emphasise on recording and monitoring hormonal changes to appropriately evaluate menstrual factors in their analysis.

Conclusions

This study has shown for the first time that with three week Rv supplementation there is an improvement in post exercise pulmonary function in females with EIB. This was supported by a reduction in bronchodilator usage during the first week of Rv supplementation; however this trend was not maintained throughout that phase. No changes were observed in FeNO and inflammatory cytokine levels. There was a significant reduction in the levels of IL-10. There was no relationship of contraceptive pill usage to any of the study outcomes. Further studies with a larger sample size and control of timing during the menstrual cycle are required to assess dose response and duration of supplementation necessary to observe differences in other physiological and biochemical markers related to pulmonary function in EIB prone females

Chapter 6

General discussion

6 General discussion

6.1 Summary Background

Asthma is a large and increasing worldwide health problem to such an extent that despite medication being very effective in treating symptoms, there is still a major burden with regard to morbidity and mortality that is putting substantial pressure on public health services (Barnes et al., 2010; Masoli et al., 2004). As in many other common diseases, diagnosis presents with a range of issues. The characterisation of asthma in a majority of clinics is carried out via simple symptom scores and pulmonary function tests (PFTs). Asthma is primarily characterised by a fixed criteria (80% ratio for FEV_1/FVC) and due to the non-specificity of this ratio for asthma severity, a large proportion of young and old adults may be under or over characterised respectively (Cerveri et al., 2009). In addition, 'sub groups' including mild – moderate, atopic, allergic and exercise induced 'asthma' have the likelihood of under classification due to the variant nature of the symptoms (Pellegrino et al., 2005). The issues relating to the fixed criteria may be overcome by the introduction of a derived 'lower limit of normality' (LLN) criterion calculated from a matched control population (Miller et al., 2011; Pellegrino et al., 2005). Despite the American Thoracic Society and the European Respiratory Society (ATS/ERS) having issued position statements (Miller et al., 2009) encouraging the application of LLN for the diagnosis of respiratory diseases there are no specific guidelines for the use of LNN.

To address this issue, a study was conducted to characterise mild-moderate asthmatics based on a standard fixed criteria (80% ratio) and then separately characterise the same individuals using the LLN derived from a reference control population. Once the asthmatic population was identified using the 80% ratio and LLN, differences in pulmonary function, dyspnoea and cytokine levels were compared between asthmatics and controls. Young male participants (18-30 years) were recruited for this study, including mild – moderate asthmatics (based upon a previous clinical diagnosis and a history of asthma) and healthy controls. Using the 80% ratio based on ATS/ERS guidelines, 16 mild-moderate asthmatics (FEV_1/FVC ratio 60-80%) and 21 healthy controls (FEV_1/FVC ratio 80% and above) were characterised. The control population was used to calculate the LNN and a ratio of 82% was indicated meaning that all 16 individuals previously characterised as mild-moderate asthmatics were confirmed as mild-moderate asthmatics using this approach (below 82%).

The asthmatics, as identified by both the 80% ratio and LLN, displayed worse dyspnoea scores and elevated levels of serum cytokines (IL- 6 and TNF- α) than controls.

A number of recent studies have investigated the discrepancy in classification using fixed criteria and LLN; these studies document a range of false negatives (7-24%) in younger populations using different fixed criteria (Cerveri et al., 2009; Hansen et al., 2007; Miller et al., 2011; Roberts et al., 2006; Swanney et al., 2008). There is evidence that current airflow obstruction characterisation criteria are not adequate and alternative characterisation criteria should be investigated for defining airway obstruction in diseases such as asthma and COPD. LLN is one such alternative that may be used alongside the fixed criteria as demonstrated in this thesis (chapter 3). The lung matures by age 20–25 years, after which there is a progressive decline in the lung function due to various anatomical, physiological and immunological changes (Sharma & Goodwin, 2006). An age specific LLN may therefore be important for making a correct diagnosis. In our study, all of the 18-30 year old males previously characterised as mild-moderate asthmatics using an 80% ratio were confirmed as mild-moderate asthmatics using the LLN. This suggests that the two methods of characterisation are comparable for the classification of young, mild-moderate asthmatics; therefore, the LLN when used alongside 80% ratio may provide a reliable characterisation of mild-moderate asthmatics.

The discrepancy between our data and the studies described above (Cerveri et al., 2009; Hansen et al., 2007; Miller et al., 2011; Roberts et al., 2006; Swanney et al., 2008) may relate to a number of issues. The majority of the studies cited use ‘predicted values’ as opposed to ‘measured values’ for the calculation of LLN. Our study used a separate population of controls for calculation of LLN (using ‘measured values’), and applied it to the previously characterised mild-moderate asthmatics (by 80% ratio). This methodological difference may partly explain the discrepancies. Additionally, the current study used an age group of 18-30 years while larger studies have used an upper age range (up to 39 years) denoting younger population. This relates to the issue of lung age; although it could be argued that our samples include some young participants (18 years), however the narrow age band centred at the lower end of the range gives rise to a homogeneous sample in our study.

Clinically, the use of a predicted value is common; however, in young and or athletic populations these values are inappropriate due to the high proportion of individuals gaining values very close to and above 100% of predicted (Mickleborough et al., 2003; Stanford et al., 2006). It is not uncommon to observe values higher than 100% of predicted in young, fit, non-smoking population and this pattern is labelled as a 'normal physiologic variant' (Pellegrino et al., 2005). It is suggested that this is possibly due to dysanaptic growth or unequal growth of airways and lung parenchyma (Hyatt et al., 1986). In our study (chapter 3) the asthmatics displayed a mean predicted value of 86% for FEV_1/FVC ratio while the controls showed mean values of 106% for FEV_1/FVC ratio. Had the predicted values been used, a number of mild-moderate asthmatics would have been under characterised (false negatives) if an 80% ratio was used. This clearly demonstrates the limitation of using reference equations and may explain the comparable results for 80% ratio and LLN observed in our study due to the use of 'measured values'.

It could be argued that the comparable results observed for the two criteria for classification in our study (lack of difference between groups) could be attributed to a small sample size. However, despite the small sample size, there were significant differences in the FEV_1/FVC ratio of the asthmatics and controls (asthmatics = 71.23. ± 1.17%; controls = 88.74 ± 0.95%). In addition, there were worse symptom scores and elevated levels of inflammatory cytokines within the mild-moderate asthmatic group compared to healthy controls. Therefore, irrespective of the small sample size, the comparable findings in our results for the 80% ratio and LLN provides evidence that if matched reference population is used then an accurate characterisation of mild-moderate asthma may be achieved.

Markers of airway inflammation

Though pulmonary function tests are recognised worldwide as a diagnostic tool for the measurement of asthma it is suggested that other markers of asthma may help in a clearer definition of individual asthma subtype (such as EIB) and its severity/control (symptoms and medications) (GINA, 2011; Cerveri et al., 2009). In this thesis, asthma symptoms (dyspnoea) and levels of inflammatory cytokines were compared and asthmatics showed significantly elevated levels of inflammatory cytokines and poorer symptom scores. The asthmatics showed a significantly higher level of serum IL-6 (mean = 1.66 ± 0.08 pg/mL) than controls (mean = 1.38 ± 0.06 pg/mL) ($p<0.05$). Similarly, asthmatics had elevated

levels of TNF-α compared to controls (asthmatics, mean = 4.28 ± 0.25 pg/mL, controls, mean = 3.35 ± 0.26 pg/mL) (<0.05). The elevated levels of IL-6 and TNF-α reported in this study (chapter 3) are within the ranges found in previous literature for asthmatics and controls. For asthmatics, previous studies have reported a range of values for IL-6 (between 0.8 pg/mL to 15.49 pg/mL) and TNF-α values within the range of 3.6 – 8.0 pg/mL (Canoz et al., 2008; Halasz et al., 2002; Pukelsheim et al., 2010; Samy et al., 2010; Subrrahmanyam et al., 2011). The values obtained for controls (in chapter 3) also fall within the observed range from the literature (0.8 – 3 pg/mL for IL-6 and 3 – 8 pg/mL for TNF-α) (Kim et al., 2011; Pukelsheim et al., 2010). These results demonstrate that the 'mild-moderate' asthmatic group have elevated levels of inflammation; consequently, monitoring of inflammation may be important for assessing improvement with therapy. In clinical settings, it is not always possible to conduct measurement of venous blood based inflammatory mediators as a part of a routine assessment of asthma. Recently, indirect estimation of inflammation in the lung via exhaled breath nitric oxide (FeNO) has been suggested as a useful marker for assessing the efficacy of anti-inflammatory treatments (Dweik et al., 2011; Kharitonov et al., 1996; Zitt, 2005). FeNO levels were assessed in the supplementation studies reported in chapters 4 and 5 to evaluate the suitability of FeNO as a marker of inflammation.

Asthma and EPA/DHA supplementation

The currently available therapies for asthma and EIB are effective, however long-term use of these is associated with issues of tachyphylaxis and numerous health side effects (Barnes, 2010; GINA, 2011). There is evidence from supplementation studies to suggest a beneficial effect of fish oil [comprising of omega-3 (n-3) polyunsaturated fatty acids (PUFAs)] in asthma and other inflammatory diseases (Arm et al., 1989; Hodge et al., 1998; Mickleborough et al., 2003, 2006; Surette et al., 2008), therefore, there is an impetus towards using fish oil as a 'complimentary therapy' for asthma. The anti-inflammatory effects of fish oil are hypothesised to be linked with the alteration of cell membrane composition (primarily EPA/DHA) which can modify lipid mediator generation by producing less inflammatory eicosanoids. A newly identified group of lipid mediators derived from EPA/DHA (include resolvins, protectins and maresins) are proposed to be important in the resolution of inflammation (Serhan, 2011b). The reduced inflammatory and pro resolution effects of n-3 PUFA may attenuate the severity of asthma including symptoms (dyspnoea)

and exert a bronchodilatory effect (Hodge et al., 1998; Mickleborough et al., 2006). There is a lack of studies investigating the effects of fish oil supplementation in mild/moderate asthmatics with EIB; and most studies conducted have focussed on athletes. To address these issues related to the impact of EPA/ DHA supplementation on post exercise pulmonary function in EIB prone mild-moderate asthmatics, two supplementation studies were carried out in this thesis; (a) EPA/DHA supplementation in EIB males and (b) Resolvin supplementation in EIB females.

The LLN derived from the controls in chapter 3 was used alongside the 80% ratio for the characterisation of individuals for the n-3 supplementation study (chapter 4) to assess the effect of EPA/DHA supplementation on post exercise FEV_1 in mild – moderate EIB prone males. The study population comprised of EIB-prone individuals (n=9, 18-30 years) who were recruited following a positive test for EIB (a drop of >10% in FEV_1 following an exercise challenge) as well as elevated resting levels of FeNO in expired air (>40 ppb). These participants were recruited and tested within a very short time frame (12 weeks) in order to control for the possible seasonal variation in outcome measures. A sample size estimate to detect a minimum detectable difference of 0.3 litres in FEV_1 after exercise following the supplementation with EPA/DHA was selected based on previous studies showing the effect of n-3 PUFAs on pulmonary function in EIB subjects (Mickleborough et al., 2003, 2006). The required number of participants to show such a difference in post exercise pulmonary function was eight; therefore, the power requirements were met. All the participants in the supplementation study had abnormal pulmonary function and fell below the 82% LLN calculated in chapter 3 (mean baseline FEV_1/FVC ratio of asthmatics = 77 ± 1.4%). In addition, the participants showed elevated levels of upper airway inflammation confirmed by elevated FeNO (mean FeNO at baseline = 46 ± 2.7 ppb) and increased levels of circulating inflammatory cytokines (TNF-α at baseline = 7.2 ± 1.02 pg/mL, IL-6 = 2.67 ±0.28 pg/mL). The levels of inflammatory cytokines in the asthmatics of chapter 4 were higher than the values obtained for asthmatics in chapter 3, however these values were still comparable to the values previously observed in the asthma literature (Canoz et al., 2008; Halasz et al., 2002; Pukelsheim et al., 2010; Samy et al., 2010; Subrrahmanyam et al., 2011).

The differences observed in the level of cytokines measured within the mild-moderate asthmatics in chapters 3 and chapter 4 could be due to differences between the samples

used in the two studies and the assay methodology used, making direct comparison difficult. The differences in the performance of commercially available ELISAs from different manufacturers may be attributed to different specificities of antibodies and sensitivity of detection. The high sensitivity R&D kit used in chapter 3 for IL-6 had a minimum detectable limit of 0.039 pg/mL compared to 0.8pg/mL for the Tepnel (Diaclone) kit used in chapter 4 and 5. These differences in the performance of the assays could account for differences in the results. Considering these limitations, the study design for the intervention (chapter 4 and 5) was such that assay kits from the same manufacturer were used and samples from all the time points of the study were analysed on the same plate to minimise between plate variations.

Three weeks of dietary n-3 supplementation (3.2 gram EPA and 2.2gram DHA/per day) showed a beneficial impact on pulmonary function in EIB participants. The supplementation with EPA/DHA improved pulmonary function to below the diagnostic threshold value for EIB, i.e. 10% fall in post exercise FEV_1. There was approximately 50% improvement in post exercise FEV_1 at 10 and 15 minutes after exercise and these findings confirm previous reports by Mickleborough et al., (2003, 2006) using a similar dose of EPA/DHA where an improvement of approximately 60% and 25% were observed in the respective studies. These two studies by Mickleborough et al., conducted in athletes/recreationally active participants, have also shown a reduction in asthma medication use by end of supplementation or reduced medication use by 3rd week of supplementation which is mirrored by the findings of chapter 4 (>50% improvement by the third week of supplementation). These results clearly show that EPA/DHA supplementation can improve post exercise pulmonary function in EIB-prone males and significantly reduce reliance on medication. These observed differences in FEV_1 improvements maybe due to the mixed nature of the participants (males and females) in the studies conducted by Mickleborough et al., (2003, 2006). Our study was designed considering these issues and recruited only male participants and this may to some extent explain the 50% improvement in post exercise pulmonary function compared to the inconsistent improvements reported in the previous studies. For future studies, using a larger sample size to further elucidate our findings, it is suggested separate groups of males and females are included for intervention studies with a control for menstrual cycle in the female group.

In previous n-3 supplementation studies, bronchodilator use has been considered as a study outcome alongside pulmonary function. There have been no effects on asthma medication use in a number of studies using different doses of EPA/ DHA (Arm et al., 1989; Kirsch et al., 1998; McDonald et al., 1990; Schubert et al., 2009; Stenius-Aarniala et al., 1989; Thien et al., 1993). Using self-reported asthma status and medication use questionnaires, Surette et al., (2008) did report a reduction in medication at a dose (1.13 gram GLA+ 0.75 gram EPA) in mild-moderate asthmatics by the end of 3 weeks of supplementation. This was based on self-reported symptoms; therefore, in the absence of any PFT measurements the results from this study require careful interpretation. Studies by Emelyanov et al., (2002); Hodge et al., (1998) have shown an improvement in mean daytime wheeze/morning PEF in adults and children using <1.5 gram EPA and DHA. Thus, there is an encouraging trend to suggest a beneficial effect of EPA/DHA on pulmonary function and reduction in bronchodilator usage; however the inconsistencies in these studies are likely to be due to factors such as dose, duration and asthma type (sub group). In our study the symptom scores were already very low at the start of the supplementation trial and therefore the likelihood of seeing improvements in self-reported symptoms was minimal. However, by the third week of EPA/DHA supplementation, there was a 50% reduction in bronchodilator usage compared to placebo and this reduction was statistically significant. These results provide evidence for the bronchodilatory effects of EPA/DHA supplementation in mild-moderate EIB individuals.

EPA/DHA dose and duration response in asthma

Asthma supplementation studies have shown inconsistencies in pulmonary function outcomes however there is substantial information on the anti-inflammatory effect of n-3 PUFA in asthma. Arm et al., (1989) demonstrated a reduction in LTB_4 generation by stimulated neutrophils with supplementation; in addition, Schubert et al., (2009) have shown a reduction in CystLTs by activated leukocytes. Similar findings were reported by Mickleborough et al., (2006) with a reduction in LTB_4 and an increase in LTB_5 in stimulated polymophonuclear cells. In addition, they showed that there was a reduction in sputum levels of PGD_2, IL-1β and TNF-α, while in children, Hodge et al., (1998) demonstrated a drop in TNF-α production in cultured PBMC. Thus, n-3 supplementation has been shown to reduce inflammatory effects although heterogeneity between the studies (i.e. differences in dose and duration of supplementation) may be responsible for the different magnitudes

of effect seen. Studies with 3 weeks of supplementation, including the present study, have shown significant improvement in post exercise pulmonary function, reduction in inflammatory cytokines and a decrease in pro inflammatory lipid mediators with 3.2 gram EPA+2.2 gram DHA supplementation (Mickleborough et al., 2003, 2006). In atopic and allergic asthma 4 -10 weeks of supplementation with a dose between 0.5 mg EPA+DHA/day and 3.2 gram+2.2 gram DHA per day has shown beneficial effects on pulmonary function, medication usage and *in vitro* lipid mediator generation (Arm et al., 1989; Emelyanov et al., 2002; Hodge et al., 1998; Surette et al., 2008; Schubert et al., 2009). Thus there is no definite dose response relationship between the effects of n-3 PUFA supplementation in asthma as a range of doses and duration have shown beneficial effects.

In a dose response study by Rees et al., (2006) in healthy participants, a dose of 2.7 gram/day of EPA was shown to reduce ex vivo PGE_2 production by stimulated mononuclear cells compared to a lower dose of 1.35 grams of EPA/day. A number of studies, have shown a threshold for an anti-inflammatory effect to be exerted in the range of 1.3 -3.5 grams EPA per day (Calder., 2006, 2012; Dignass et al., 2004; Kelley et al., 1998; Kew et al., 2004; Kolahi et al., 2010; Grimble et al., 2003). In this thesis, significant improvement in post exercise pulmonary function has been observed with a supplementation of 3.2 grams EPA and 2.2 grams DHA which provides confirmation of the beneficial effects of n-3 PUFA in mild-moderate asthma; however lower doses require further investigation. Although there is no consensus about the optimal dose for improving lung inflammation, the beneficial results shown by n-3 PUFA in asthma suggest a possible anti-inflammatory effect of EPA /DHA and highlight the requirement for future dose response and time course trials in asthma to evaluate the impact of supplementation in asthma symptoms and inflammation.

The proposed mechanisms for the action of n-3 PUFA are associated with the modification of cell membrane composition with n-3 supplementation (Calder, 2012; Calder & Yaqoob, 2009). Modification of cell membrane phospholipid content can influence the membrane-linked enzyme systems and cell signalling pathways that affect the physiological responses of cells (Mickleborough & Rundell, 2005; Surette, 2008). Dietary supplementation with fish oil can alter cell membrane fatty acid profiles and there is an increase in overall EPA and DHA in different cell types including PBMC following

supplementation (Damsgaard et al., 2008; Yaqoob et al., 2000). Data from time course studies have shown that incorporation of EPA and DHA into human inflammatory cells starts within days of supplementation and reaches near maximum within four weeks; however, incorporation occurs in a dose response manner (Kew et al., 2004; Rees., et al. 2006; Thies et al., 2001; Yaqoob et al., 2000). Asthma supplementation studies using a dose of 3.2 gram EPA and 2.2 gram DHA per day for 3 weeks have previously shown significant increases in neutrophil, plasma and RBC phospholipid EPA content usually at the expense of AA (Arm et al., 1989; Schubert et al., 2009; Surette et al., 2003, 2006; Mickeborough et al., 2006; Hodge et al., 1998).

In this thesis, there was a trend toward an increase of EPA (3 fold) and DHA (2 fold) with a reduction in AA (2 fold) in PBMC, but this change was not statistically significant. The study was powered to detect a change in post-exercise pulmonary function, and, when post hoc power analysis was carried out for total PBMC lipid analysis it reflected a low power of 0.4 to detect statistically significant changes in lipid composition. There are studies which have previously shown incorporation of EPA/DHA into PBMC total lipids following supplementation with a dose of 1-2 grams EPA/DHA per day (Thies et al., 2001; Yaqoob et al., 2000). The difference between these studies and our study may be due to the duration of supplementation; as it was expected to see incorporation after 3 weeks of supplementation at a relatively high dose used in our study. In addition, there were limitations in the analytical method (GC-FID) used here which may have resulted in the lack of observed statistical significant incorporation.

FADS and n-3 supplementation

The lack of incorporation of EPA and DHA in this study may be attributed to factors other than dose/duration of supplementation and limitations of the analytical methods. The delta 5-and delta 6-desaturase enzymes encoded by FADS1 and FADS2 genes respectively have been shown to be critical in the metabolic pathway of n-3 PUFA and have an impact on chronic inflammatory diseases particularly in relation to a long chain PUFA (EPA and DHA) deficient diet (Aslibekyan et al., 2012; Lattka et al., 2010). Genome wide association studies have shown that the genetic variations in the FADS gene cluster are linked to PUFA content in serum phospholipid and RBC membranes in several populations including Caucasians, East Asians and Afro Americans (Lemaitre et al., 2011; Martinelli et

al., 2008). These variations have an impact on lipid metabolism, glucose metabolism and other quantitative characters and may be responsible for the pathogenesis of chronic diseases including coronary heart disease (Pizzolo et al., 2008), asthma and atopy (Schaeffer et al., 2006; Simopolous., 2009). Polymorphisms in FADS1 and FADS2 have shown positive associations with the level of AA and some association with levels of LA and n-3 fatty acids including ALA, EPA and DPA (Schaeffer et al., 2006). This particular study also reported that carriers of the rare alleles of several FADS1 and FADS2 polymorphisms and their respective haplotypes have a lower incidence of atopic eczema however there was no association with IgE. Thus, there may be a relationship of FADS polymorphisms with inflammation. There is, however, limited information about the associations of FADS polymorphism in different asthma 'sub groups' including mild-moderate asthma and EIB and further candidate and genome wide association studies are required to investigate the role of FADS in inflammation.

Supplementation of n-3 PUFA has been reported to affect cell signalling pathways either by altering the expression and activity of membrane receptors or by modification of the expression of genes by the activation of transcription factors such as NFκB and PPAR-α (Michaud & Renier, 2001; Rudkowska et al., 2009; Tai et al., 2005). To determine the long term effect of EPA/DHA on the cellular transcriptome, Bouwen et al., (2009) investigated changes in whole genome transcriptional profiles in PBMC and adipose tissue after 26 weeks of supplementation with 0.4 g EPA/DHA (n=36) and 1.8 g EPA/DHA (n=37) or a control oil (high–oleic acid sunflower oil, n=38) in an elderly population (66–80 years). Daily supplementation with the higher dose resulted in changes in the expression of approximately 1000 genes and there was significant down regulation of genes involved in inflammatory pathways, such as eicosanoid synthesis, interleukin signalling, and MAP kinase signalling. Additionally, the low dose of supplementation showed gene expression changes that were similar to those seen with a high dose which suggest that lower levels of supplementation for longer period may have similar effects on PBMC gene expression (Bouwen et al., 2009). However, the major limitation of this study was that it comprised of only elderly individuals and consequently the results cannot be generalised for all age groups. These findings are in line with a small study conducted by Gorjao et al., (2006) which showed a reduction in the expression of cytokine-related genes following n-3 supplementation. Therefore, evidence suggests that long-term n-3 supplementation has the potential to reduce the expression of leukocyte inflammatory genes and decrease

cytokine production following leukocyte stimulation. These anti-inflammatory effects of n-3 PUFA have a beneficial role in the management and control of airway inflammation in asthma.

In this thesis, levels of inflammatory cytokines (IL-6 and TNF-α) were measured before and after EPA/DHA supplementation and there was a significant reduction of 37% in the level of serum IL-6. The asthmatic population in this study had elevated levels of inflammatory cytokines at baseline and the reduction in the levels of these cytokines observed here maybe indicative of the anti-inflammatory effect of n-3 PUFA. In addition, in this thesis, there was no change in the level of TNF-α and IL-10 with supplementation. The serum IL-10 levels of asthmatics are generally lower than that of healthy controls, which indicates that asthmatics may have lower ability to release this cytokine and are unable to inhibit the formation of inflammatory factors (Guo et al., 2008). This could be attributed to the shorter duration of supplementation however to completely understand the relationship between IL-10 and n-3 supplementation dose response and time course studies will be required. Furthermore, studies are required to understand the regulation of genes involved in the asthma pathophysiology with n-3 supplementation.

The n-6/n-3 ratio vs. absolute amounts

A number of studies have described the n-6/n-3 fatty acid ratio by accumulating all n-6 and n-3 PUFAs and this approach assumes that all n-6 and n-3 PUFAs are biologically equivalent to one another (Calder & Deckelbaum, 2011; Harris, 2006). Since there is a competition between LA and ALA for metabolism by delta 6-desaturase, this ratio may be considered important as it is suggestive of the relative rates of metabolism of these two fatty acids *in vivo.* In those individuals whose dietary oily fish consumption and fish oil supplementation is absent, the n-6: n-3 ratio will approximate to the LA: ALA ratio as LA constitutes 95% of dietary n-6 while ALA makes up 90% of n-3 intake (Stanley et al., 2007). Furthermore there are a number of ways of changing the dietary n-6: n-3 ratio, either by changing LA intake alone and keeping ALA intake constant or vice versa. However, there is limited conversion of ALA to EPA and DHA (Brenna et al., 2009; Burdge & Calder, 2005; Goyens et al., 2006). Subsequently, changing the dietary n-6: n-3 ratio by modifying ALA can increase plasma, cell and tissue EPA to some extent, but not DHA content (Arterburn et al., 2006; Kelley et al., 1993). Thus, a ratio of n-6 to n-3 PUFA can be

misleading in relation to diet and biological status and it is suggested that the focus should be based on specific fatty acids and the absolute amounts/content rather than ratios. In addition, omega 3 index (EPA + DHA as a percentage of fatty acids in RBCs) or a ratio of AA: EPA+DHA or in plasma, cells and tissues could be used as a functional marker (Hibbeln et al., 2006; Rees et al., 2006; Stanley et al., 2007). Considering these arguments, the changes in the relative content of EPA, DHA and AA in PBMC total lipid reported in chapter 4 to assess the effect of supplementation can be considered a strength of the study.

Beneficial effects of resolvins on asthma

The biological effects of n-3 PUFA may be exerted via a range of mechanisms and to evaluate the role of newly identified lipid mediators a supplementation study was conducted to investigate the effect of EPA-derived bioactive mediators (E series Resolvins) on post exercise FEV_1 in EIB-prone females. The results showed a significant improvement in post exercise FEV_1 in EIB-prone females with three weeks of resolvin supplementation. There was a significant reduction in serum IL-10 levels but no overall reduction in serum TNF- α, IL-6 or FeNO levels. Additionally, there was no relationship between contraceptive pill use and any of the study outcomes.

This is the first study to demonstrate a beneficial effect of 3 weeks of supplementation with E series resolvins on pulmonary function in EIB prone females. The beneficial effect was supported by a markedly significant improvement in post exercise FEV_1 which put participants below the diagnostic threshold for EIB. Furthermore, there was a reduction in the levels of serum IL-10 levels although there was no change in the levels of inflammatory cytokines (TNF-α and IL-6). The findings from this study are encouraging as it suggests a beneficial role of a short duration supplementation with E series resolvin (100 mg Resolvinol per day). This dosage of resolvins is much lower than the required amount of EPA/DHA (range 1-3 grams/day) needed to exert anti-inflammatory effect. It is possible, based on these preliminary findings, that the newly identified resolvins can exert pro resolution effects in relatively small amounts. A short duration of supplementation was chosen for participant retention as longer phases have a greater likelihood of participant drop out (Max et al., 1991). The fact that there are few prior studies in this area and certainly none in asthma, meant that this particular part of the thesis study was

experimental in nature. As well as its strength in identifying the positive effect of resolvin supplementation on pulmonary function, this study has paved the way for future studies by determining an effective dose to promote a response in pulmonary function. However the lack of a systematic change in inflammatory mediators measured denotes that the mechanisms underlying the change in pulmonary function are not clear.

Fluctuations in pulmonary function are reported during the menstrual cycle and the symptoms are more severe during mid-luteal to mid-follicular stages, often with a dip near the time of ovulation. The mechanisms underlying the menstrual cycle and asthma and AHR are not clear and there appears to be reduced symptoms for those taking oral contraceptives (Lim & Kobzik, 2008; Stanford et al., 2006; Tan et al., 1997; Vrieze et al., 1997). There was no relationship of the pill use with any study outcome but this could be attributed to the small sample size.

Dietary Intake

In the supplementation studies (chapter 4 and 5) participants were advised to maintain their normal diet throughout their participation in the study. We are, however, unable to comment on possible variation in dietary intake during these phases which may have confounded these findings. Retrospective dietary recalls including 24-hour diet diaries and food frequency questionnaires (FFQs) were originally used for estimation of the dietary intake of participants in this study. Data were analysed using McCance & Widdowson's 'The Composition of Foods' published by the Department of Fisheries, Agriculture and Food (1998). One of the original aims of the thesis was to monitor the dietary behaviour to confirm the level of n-3 intake. The results of the planned dietary intake studies were found to be inconsistent and did not represent a normal daily intake as shown in the literature with regard to total energy and fat intake. It is likely that these inconsistencies could be due to errors in measurement, data collection and/or participant response. Quantitative estimates of dietary intake have been found to be error prone, for example, the co-efficient of variation (CV) of differences incurred from asking subjects to recall food portions rather than weighing them has been found to be in the 50% range for foods and 20% for nutrients (Hodson et al., 2008). It has been suggested that precision can be improved be improved by increasing the number of observations in individuals and by maintaining weighed food diaries (Bingham, 1991; Bingham et al., 1994; Buzzard, 1998; Todd et al., 1983). Although

attempts were made to collect reliable dietary information the inconsistencies in the analysis showed that data from these recalls carried possible measurement errors, lacked accuracy and may not represent normal dietary behaviour for participants. Consequently, on the advice of the examiners, the analysis was excluded from the thesis. The exclusion of these dietary data does not affect the primary aims of the thesis, however, due to this limitation of the work it is not possible to comment upon variation in dietary intake during supplementation (chapter 4 and 5) which may have confounded our findings relating to total PBMC lipid composition. For future studies, larger sample size, an improved analytical/dietary method and longer duration of study is required.

Relationship of plasma fatty acid content to atopy

Early life exposure to n-3 and n-6 PUFA is hypothesised to have an effect on asthma and atopy (Calder et al., 2010; Kremmyda et al., 2011). Evidence from observational studies has led to the hypothesis that an increased intake of n-6 PUFA accompanied by a low intake of fish oil [omega 3 (n-3) PUFA] may play a causal role in the increased incidence of asthma (Black & Sharpe., 1997; Hodge et al., 1994; Peroni et al., 2012). Epidemiological studies evaluating the effects of fish oil during infancy and childhood on asthma and atopy have been inconsistent, with a number of large-scale studies showing a beneficial effect of fish oil in asthma and atopy but some studies showing no effect (Mihrshahi et al., 2003; Nagakura et al., 2000; Peat et al., 1994, 2004; Vaisman et al., 2005). The fatty acid composition of plasma is affected by diet and various metabolic processes including those which regulate the fatty acid composition of the components (Sala-Vila et al., 2008). Thus, comparing the differences in the fatty acid composition of plasma between atopic and non-atopic individuals (asthmatics or healthy) is considered an important method for assessing the exposure of body components to fatty acids and may indirectly reflect diet and metabolism. There are a number of studies which have reported fatty acid composition in plasma and serum lipids in atopic children and adults (Laerum et al., 2007; Leichsenring et al., 1995; Wood et al., 2004). The pattern of results is inconsistent; a few studies show higher LA and lower AA, EPA and DHA levels in plasma in atopic individuals compared to matched controls (Griese et al., 1990; Haidopoulou et al., 2009; Leichsenring et al., 1995). In this thesis the asthmatics were not tested for atopy and future studies may incorporate tests for atopy within the different ‘sub groups’ of asthma and could compare the plasma/inflammatory cell lipid composition of these individuals with healthy controls.

6.2 Future directions

Since asthma is a growing health issue, public health interventions are becoming increasingly important to reduce its severity and prevalence. Diet and nutrition are potential factors that could affect the pathophysiology of asthma, decrease reliance on pharmaceutical drugs, and promote health and independence in later life. This study has confirmed the beneficial effects of EPA/DHA supplementation in decreasing EIB severity thus, there is a potential for using n-3 as a complimentary therapy for improving pulmonary function. The supplementation trials in asthma have been limited by sample size however there is a positive relationship of n-3 PUFA supplementation on various inflammatory outcomes in asthma.

Based on our results which confirm the previous findings of various studies in asthma including Arm et al., (1989); Emelyanov et al., (2002); Hodge et al., (1998); Schubert et al., (2009); there is a likelihood that if asthmatics with EIB compliment their daily diets with n-3 either in the form of oily fish or supplements, it may ameliorate their EIB symptoms. A number of asthmatics do not engage in physical activity as it triggers their asthma. For this population a dietary n-3 intervention may potentially change their lifestyle and activity levels with wide ranging health benefits. A large scale RCT in EIB patients with a daily n-3 intervention may be instrumental to understand the response of dietary n-3 at a population level, giving the ability to determine effects in different age groups, different body mass classes, with gender and ethnicity. To reduce under classification in younger adults or those with mild/moderate/EIB symptoms and over classification in older adults due to fixed criteria for asthma diagnosis a LLN criteria maybe used together with the 80% ratio to get a more reliable diagnosis of the study population. This requires gathering data on appropriate reference populations and translating these data into usable clinical guidelines. Correct and timely diagnosis will provide an early opportunity for intervention to improve the quality of life of those affected.

The research findings from this thesis along with the previous literature showing the beneficial effect of EPA /DHA in asthma were taken forward and a proposal was submitted to the ESRC/MRC (Interdisciplinary ESRC / MRC Fellowship) with an aim of understanding the interactions between dietary behaviour (omega 3 intake) and the severity and prevalence of asthma at the population level. It was emphasised that the

Department of Health's (DH) initiative to promote the campaign 'small change, big difference' encourages people to make minor changes to their lifestyles to give them a better chance of living longer and healthier lives. The proposed method included an integrative approach of behavioural sciences, psychology and biological sciences to ascertain the level of community preparedness for a dietary change (n-3) by using the Community Readiness Model for behavioural change (Edwards et al., 2000; Plested et al., 2006). Though this grant was not successful, there were positive comments from the review committee which supported the concept of the proposed community based intervention to meet the DH's initiatives.

Currently there are no guidelines for the recommended dosage of resolvin required to exert anti-inflammatory and pro resolution effects in asthma. The beneficial effects shown in this study on female participants are promising for future studies that should investigate the effects of dose and time response with resolvins on inflammation. Clinical trials are currently underway (Brooks, 2009; Resolvyx, 2012; ClinicalTrials.gov identifier: NCT00799552) and the results will hopefully add important data to the growing literature base.

6.3 Final Conclusions

The LLN criteria for asthma characterisation is comparable with standard fixed criteria for young males with mild-moderate asthma. Therefore, LLN can be used alongside fixed criteria for reliable characterisation of asthma in this population. Asthmatics have greater inflammation compared to controls, confirmed by their pulmonary function tests, symptoms, and circulating IL-6 and TNF-α level. FeNO is an attractive non-invasive measure for airway inflammation; further work is required to evaluate the dose and duration of EPA/DHA supplementation to reduce FeNO levels and how these changes relate to pulmonary function. There was a beneficial effect of 3-weeks of supplementation (3.2 gram EPA+ 2.2 grams DHA/day) in the post exercise pulmonary function of mild-moderate EIB prone asthmatic males which was supported by reduced bronchodilator usage and a reduction in circulating IL-6 levels. Three-weeks of supplementation with the recently identified E series resolvins (100 mg/day) improved post exercise pulmonary function in mild-moderate EIB prone asthmatic females; however, controls for menstrual

cycle and pill usage were confounding variables. Studies with a large sample size and carefully controlled study methods are required to confirm these findings at a population level.

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Appendix 1

Appendix 1 contains details of the materials ad reagents required for the biochemical procedures used during the study.

Table 1: Balanced salt solution for PBMC isolation

Stock solution A		**Conc. (g/l)**
Anhydrous D-glucose	0.1%	1.0
CaCl2 × 2H2O	5.0 × 10-5 M	0.0074
MgCl2 × 6H2O	9.8 × 10-4 M	0.1992
KCl	54 × 10-3 M	0.4026
Tris	0.145 M	17.565
Conc. HCl	10 N	to pH 7.6
Distilled water*		to 1 l
Stock solution B		**Conc. (g/l)**
NaCl	0.14 M	8.19
*Dissolve in approximately 950 ml distilled water and add 10 N HCl until pH is 7.6 before adjusting the volume to 1 l. To prepare the balanced salt solution, 1 volume Solution A was mixed with nine volumes Solution B and sterilized.		

Table 2: Materials required for TNF-α measurement from blood

Wash Buffer	To avoid crystal formed in the concentrate, the buffer was warmed at room temperature and mix gently until the crystals have completely dissolved. 100 ml of Wash Buffer Concentrate was missed into deionized water to prepare 1000 mol of Wash Buffer.
Substrate Solution	Lyophilized Substrate was reconstituted with 6.0 ml of Substrate Diluent at least 10 minutes before use. The vials were re-stopped, capped, and mix thoroughly.
Amplifier Solution	Lyophilised Amplifier was reconstituted with 6.0 ml of Amplifier Diluent at least 10 minutes before use. The vials were re-stopped, capped, and mix thoroughly.
TNF-α Standard	The TNF- α Standard was reconstituted with the volume of Calibrator Diluent RD6-13 printed on the Standard vial label to produce a stock solution of 32 pg/ml. This reconstitution produced a 1X stock solution. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. **To prepare dilutions:** 500uL of Calibrator Diluent RD6-13 was aliquoted into each tube. The stock solution was used to create dilutions of 16, 8, 4, 2, 1, and 0.5pg/ml. The undiluted standard served as the high standard (32 pg/mL). Calibrator Diluent RD6-13 served as the zero standard (0 pg/mL).

Table 3: Materials required for IL-6 measurement from blood

Wash Buffer	To avoid crystal formed in the concentrate, the buffer was warmed at room temperature and mix gently until the crystals have completely dissolved. 100 ml of Wash Buffer Concentrate was mixed into deionized water to prepare 1000 mol of Wash Buffer.
Substrate Solution	Lyophilized Substrate was reconstituted with 6.0 ml of Substrate Diluent at least 10 minutes before use. The vials were re-stopped, capped, and mix thoroughly.
Amplifier Solution	Lyophilised Amplifier was reconstituted with 6.0 ml of Amplifier Diluent at least 10 minutes before use. The vials were re-stopped, capped, and mix thoroughly.
IL-6 Standard	The IL-6 Standard was reconstituted with 5.0 mL of the calibrator Diluent RD6-11 Concentrate. This reconstitution produces a stock solution of 10pg/ml. The standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. 500 ul of the Calibrator Diluent RD6-11 Concentrate was added into each tube. The stock solution to was used to produce a dilution series To produce a dilution series of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 pg/ml. The appropriate reconstituted standard serves as the high standard (10 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).

Appendix 2
Appendix 2 illustrates the recruitment flyers, participant information sheet and related documents used for recruitment during the study.

ASTHMATICS NEEDED

Can Fish Oil Intake Reduce Severity of Asthma?

? Help us answer this question?

Who can take part?
* Males
* Asthmatics
* Age 18-65 years

Why?
Studies suggest that fish oil intake can reduce severity of asthma. This project aims to determine relationship between dietary intake of fish oil (Omega-3) and asthma/lung inflammation at cellular level.

What will the study involve?
The experiment will involve:
One visit to the lab (lasting up to an hour)
Short questionnaires
Lung function test and a small blood sample

Where and How?
If interested please contact:
Ash
a.varma@lboro.ac.uk
01509 22 81 59
Department of Human Sciences
Wavy Top building
Map reference: 49

Omega 3 and Asthma Study

Q. Can this help treat Exercise Induced Asthma?

For Information Contact:
Miss A Varma (PhD student), Email: A.Varma@lboro.ac.uk (01509 228159) or
Dr M R Lindley, Email: M.R.Lindley@lboro.ac.uk
School of Sport, Exercise and Health Sciences, Loughborough University

What is the purpose of the study?

- The study is designed to look at the effects of taking fish oil supplementation (Omega-3) on lung health (Pulmonary Function test). In order to do this we need participants to take three different supplements, each for a period of 3 weeks and with 3 weeks washout in between the phases. Participants will be asked to come into the lab for testing before and after each supplementation period.

- This means a total duration of 15 weeks however, this is only six visits to the lab for testing and only 9 weeks of actually taking the supplements.

- The Christmas period will remain free from testing and supplementation so that you can carry on your normal holiday activities.

The omega-3 capsules are made of gelatine, which are not suitable for vegetarians.

Who can take part in the study?

- Males and Females
- Mild-moderate Asthmatics and healthy individuals
- Age 18-65 years
- No self-reported respiratory infection within the past 8 weeks
- Not taken steroids, of any kind, Hay-fever or other allergy medication (past 7 days)

What will I have to do?

- All testing will take place in the laboratories within the School of Sport Exercise and Health. Short questionnaires (including University health screening) lasting up to 20 minutes will be completed.
- Participants will receive a dose of three different supplements, each for a period of 3 weeks.
- For each lab visit (lasting up to a maximum of 2 hours); spirometry measurements and exercise challenge testing will be completed.
- In addition, three blood samples (9ml each) will be collected for associated biochemical analysis.

The total supplementation period is 6 weeks. There will be six lab visits required lasting up to a maximum of 2 hours.

Participant Information Sheet
Isolation and Analysis of Peripheral Blood Mononuclear Cell.

What is the purpose of the study?
Studies suggest that fish oil intake can reduce severity of asthma. This project aims to determine relationships and effects of fish oil (Omega-3) on asthma and lung inflammation at cellular level. Blood cells (Peripheral blood mononuclear cells) will be isolated and Omega 3 constitution of cell membranes will be analysed. These results will be compared against pulmonary function test results. This will allow us to investigate any possible relationships between fish oil intake and asthma.

Who can take part in the study?
- Males
- Both groups - Asthmatics and non-asthmatics
- Age 18-65 years
- No respiratory infection within the past 8 weeks
- Not taken steroids, of any kind, Hay-fever or other allergy medication (past 7 days)

What will I have to do?
- One visit to the lab(lasting up to an hour)
- Short questionnaires (including University health screening)
- Lung function test and a small blood sample

What will I gain from participating in this study?
You will receive the results from your lung function test as well as the standard ranges.

Are there any risks?
- The risk involved are minimal
- Lung Function test will involve a number of maximal breaths.
- All invasive procedures will follow University Health and Safety guidelines.

What will happen to my test results and information?
All of your information will be kept confidential and anonymous. The blood sample will be used to determine the cell membrane composition and inflammatory markers. Remaining samples will be destroyed after completion of the study.

What if I change my mind?
You are free to withdraw at any time during the study.

Whom do I contact if I have any more questions?

Aishwarya Varma	**Dr Martin Lindley**	**Dr S S Mastana**
PhD Student	Exercise Physiologist	Snr. Lecturer, human Biology
a.varma@lboro.ac.uk	m.r.lindley@lboro.ac.uk	s.s.mastana@lboro.ac.uk
Tel: 01509 22 8159	Tel: 01509 22 3017	Tel: 01509 22 3041

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Participant Information Sheet
Exercise challenge test screening

What is the purpose of the study?
Exercise related symptoms are very common in young people with even well controlled asthma and because they limit participation in physical activity can have quite marked effects on the general health, quality of life, mental health and social interactions of young people. Exercise challenge test is an important method to assess exercise-induced asthma. Exercise related symptoms occur in up to 50% of patients with asthma; they are often the most incapacitating aspect of the disease in younger patients. This study aims to assess exercise induced asthma symptoms in adults with stable asthma.

Who can take part in the study?
- Males and females
- Both groups – Mild-moderate Asthmatics and non-Asthmatics
- Age 18-65 years
- No respiratory infection within the past 8 weeks
- Not taken steroids, of any kind, Hay-fever or other allergy medication (past 7 days)

What will I have to do?
- One visit to the lab(lasting up to an hour)
- Short questionnaires (including University health screening)
- Lung function test using standard spirometry.

What will I gain from participating in this study?
You will receive the results from your lung function test as well as the standard ranges.

Are there any risks?
- The risk involved are minimal
- Exercise challenge will involve maximum exhaustion.
- Lung Function test will involve a number of maximal breaths.
- All invasive procedures will follow University Health and Safety guidelines.

What will happen to my test results and information?
All of your information will be kept confidential and anonymous.

What if I change my mind?
You are free to withdraw at any time during the study.

Whom do I contact if I have any more questions?

Aishwarya Varma	**Dr Martin Lindley**	**Dr S S Mastana**
PhD Student	Exercise Physiologist	Snr. Lecturer, human Biology
a.varma@lboro.ac.uk	m.r.lindley@lboro.ac.uk	s.s.mastana@lboro.ac.uk
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Loughborough University

Name/Number .....…….

Health Screen Questionnaire for Study Volunteers

Note to Investigators: This HSQ can be used in its entirety but you can also remove some of the questions if you know they are not relevant to your study.

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research ***[only include for projects involving invasive procedures].***

Please complete this brief questionnaire to confirm your fitness to participate:

1. At present, do you have any health problem for which you are:

(a)	on medication, prescribed or otherwise	Yes		No	
	If Yes , please mention:				
(b)	attending your general practitioner	Yes		No	

2. In the past two years, have you had any illness which required 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 ..... Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? ... Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....
.....

5. **Allergy Information**

(a) are you allergic to any food products? Yes ☐ No ☐

(b) are you allergic to any medicines? Yes ☐ No ☐

(c) are you allergic to plasters? Yes ☐ No ☐

If YES to any of the above, please provide additional information on the allergy

……
……..

5. Have you ever taken any Omega 3 dietary supplements? Yes ☐ No ☐

If YES, Please mention which supplements, duration in which taken etc.

……

6 Additional questions for female participants

(a) are your periods normal/regular? ..... Yes ☐ No ☐

(b) are you on "the pill"? ..... Yes ☐ No ☐

(c) could you be pregnant? ..... Yes ☐ No ☐

(d) are you taking hormone replacement therapy (HRT)? Yes ☐ No ☐

6. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name:
……
.

Telephone Number:
……
.

Work ☐ Home ☐ Mobile ☐

Relationship to
Participant:……
..

7. Are you currently involved in any other research studies at the University?

Yes ☐ No ☐

If yes, please provide details of the study

Loughborough University

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.

I agree to participate in this study.

Your name _____

Your signature _____

Signature of investigator _____

Date _____

Participant Information Sheet
Omega 3 Dietary Supplementation

What is the purpose of the study?
Several biochemical studies suggest beneficial effects of Omega 3 supplementation on asthmatics. Participants will be required to take Omega 3 supplements consisting of 7 gelatine capsules containing approximately containing 2.6 grams of Eicosapentaenoic acid (EPA) and 1.4 grams of Docosahexaenoic acid (DHA) per day. EPA and DHA are Omega 3 PUFAs that have shown to have positive impacts on inflammation with respect to asthma, cystic fibrosis, cardiovascular diseases and rheumatic diseases. EPA has possible anti-inflammatory properties. The supplementation will be carried for a maximum of 6-week period.

Who can take part in the study?
- Males and Females
- Mild-moderate Asthmatics and healthy individuals
- Age 18-65 years
- No respiratory infection within the past 8 weeks
- Not taken steroids, of any kind, Hay-fever or other allergy medication (past 7 days)

Who cannot take part in the study?
- Children under 18 years of age
- People over 65 years of age
- People with mental illness
- Prisoners/other detained persons
- Pregnant women

What will I have to do?
- Short questionnaires (including University health screening)
- Participants will receive a dose of Omega 3 PUFAs in the form of seven gelatine capsules (comprising approximately 2.6 grams of EPA and 1.4 grams of DHA) for a maximum of 6 weeks.
- Participants will return to the lab at the beginning of each week for a maximum of 6 weeks to collect supplements and undergo additional physiological assessments (lasting for a maximum of one and half hour).

What will I gain from participating in this study?
You will receive your dietary analysis information.

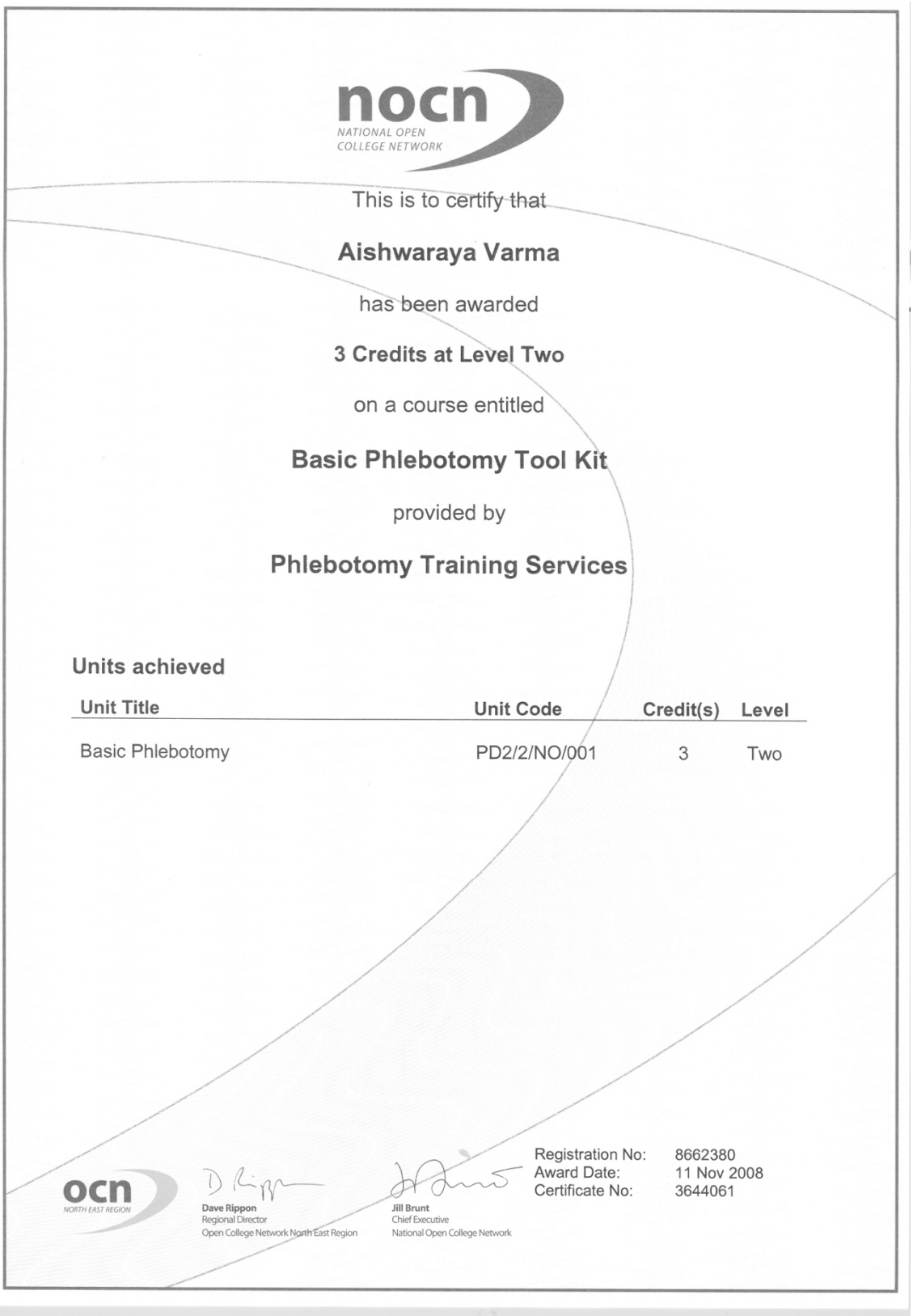
Are there any risks?
- The risk involved are minimal
- There is no major adverse health side effect linked with Omega 3 supplementation.

What will happen to my test results and information?
All of your information will be kept confidential and anonymous.

What if I change my mind?
You are free to withdraw at any time during the study.

Who do I contact if I have any more questions?

Dr Martin Lindley — Lecturer — m.r.lindley@lboro.ac.uk — Tel: 01509 22 3017
Aishwarya Varma — PhD Student — a.varma@lboro.ac.uk — Tel: 01509 22 8159
Dr S S Mastana — Snr. Lecturer, human Biology — s.s.mastana@lboro.ac.uk — Tel: 01509 22 3041
Department of Sports, Exercise and Health Sciences, Wavy Top building, Loughborough University, LE11 3TU



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NATIONAL OPEN COLLEGE NETWORK

This is to certify that

Aishwaraya Varma

has been awarded

3 Credits at Level Two

on a course entitled

Basic Phlebotomy Tool Kit

provided by

Phlebotomy Training Services

Units achieved

Unit Title	Unit Code	Credit(s)	Level
Basic Phlebotomy	PD2/2/NO/001	3	Two

ocn NORTH EAST REGION

Dave Rippon
Regional Director
Open College Network North East Region

Jill Brunt
Chief Executive
National Open College Network

Registration No: 8662380
Award Date: 11 Nov 2008
Certificate No: 3644061

Appendix3

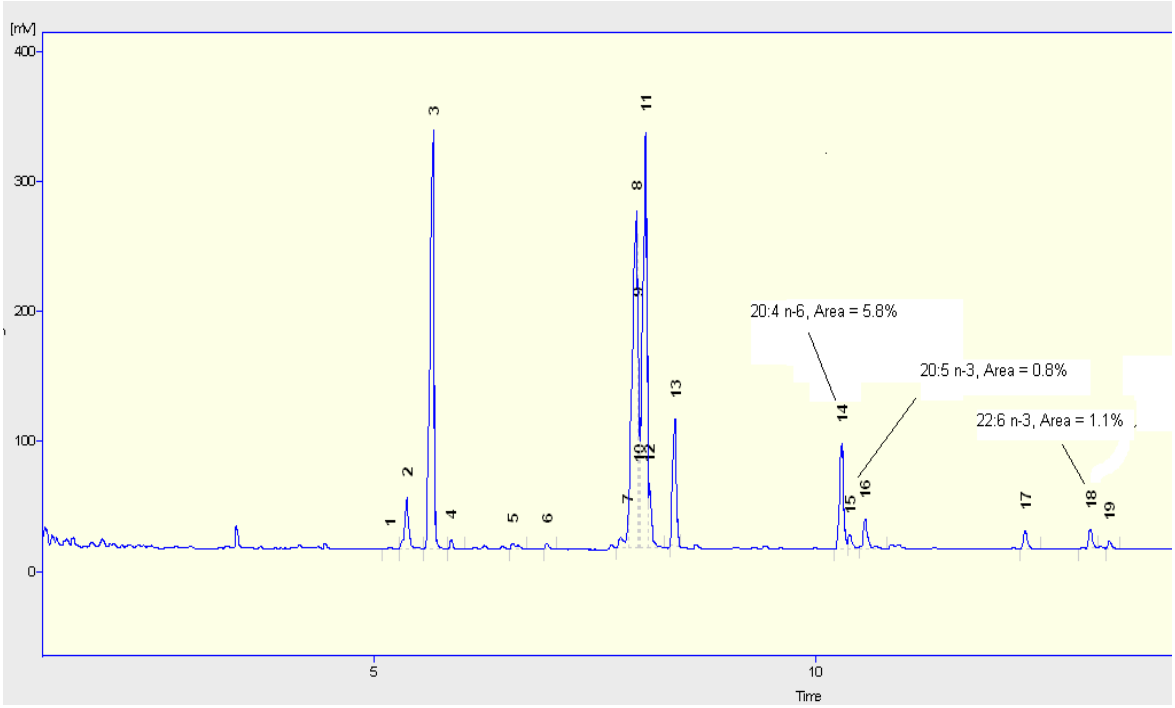
This appendix includes supplementary tables and figures from chapter 4

Table 4.1: Percentage change in post exercise FEV_1 at all time-points for all participants, all values are negative showing percentage (%) drop.

Baseline						
Participant ID	**1min**	**5min**	**10min**	**15min**	**30min**	**45min**
1	10.09	12.33	10.09	9.19	5.16	0
2	10.71	22.06	19.33	11.55	3.15	0
3	+ 4.69	1.23	13.58	14.32	+ 2.22	+ 4.94
4	5.26	11.37	10.32	11.58	6.11	5.05
5	+ 1.34	5.15	10.96	12.53	8.28	5.37
6	2.3	12.64	10.11	11.72	5.52	0.69
7	7.48	14.49	7.01	4.21	6.07	4.21
8	20.78	22.37	31.28	27.17	4.57	0.46
9	9.81	12.15	10.87	3.62	5.33	0.85
Mean ± SD	**6.71 ± 7.52**	**12.64 ± 6.85**	**13.73 ± 7.40**	**11.77 ± 6.84**	**4.66 ± 2.92**	**1.30 ± 3.21**
Placebo						
	1min	**5min**	**10min**	**15min**	**30min**	**45min**
1	5.16	9.19	14.57	17.49	6.73	4.71
2	2.1	7.98	12.39	14.29	2.94	3.78
3	+ 5.19	+ 1.23	2.47	10.37	8.4	+ 7.41
4	+ 2.95	17.68	12.63	6.74	2.32	1.89
5	+ 2.46	10.51	10.96	10.51	5.82	+ 1.34
6	12.64	10.11	11.72	6.21	7.59	3.45
7	14.49	7.01	4.21	6.54	8.88	6.54
8	1.83	7.31	10.96	11.87	9.13	1.83
9	12.15	10.87	3.62	2.77	4.05	+ 0.21
Mean ± SD	**4.20 ± 7.37**	**8.83 ± 4.94**	**9.28 ± 4.54**	**9.64 ± 4.56**	**6.20 ± 2.58**	**1.47 ± 4.11**
Omega 3						
IDs	**1min**	**5min**	**10min**	**15min**	**30min**	**45min**
1	10.54	18.39	11.88	9.19	10.31	4.93
2	0.42	7.35	8.61	4.2	4.41	4.2
3	+7.65	+ 3.96	-3.46	12.84	8.4	+ 2.47
4	+ 3.79	1.05	+1.89	0.84	+ 0.63	2.95
5	2.68	1.57	5.59	3.36	+ 0.67	0.45
6	2.3	0.92	2.3	4.6	0.23	0.23
7	9.35	7.71	3.97	4.21	6.07	+ 0.93
8	+ 0.68	0.68	+ 0.22	+ 5.71	+ 7.31	+ 6.16
9	4.69	9.59	2.56	0.64	0.85	+ 0.21
Mean ± SD	**1.98 ± 5.82**	**4.81 ± 6.67**	**4.03 ± 4.24**	**3.80 ± 5.26**	**2.41 ± 5.43**	**0.33 ± 3.45**

Figure 4.1: A sample GC-FID trace showing changes in relative percentages of AA, EPA and DHA levels in PBMC total lipid profile before and after EPA/DHA supplementation.

Before supplementation



After EPA/DHA supplementation

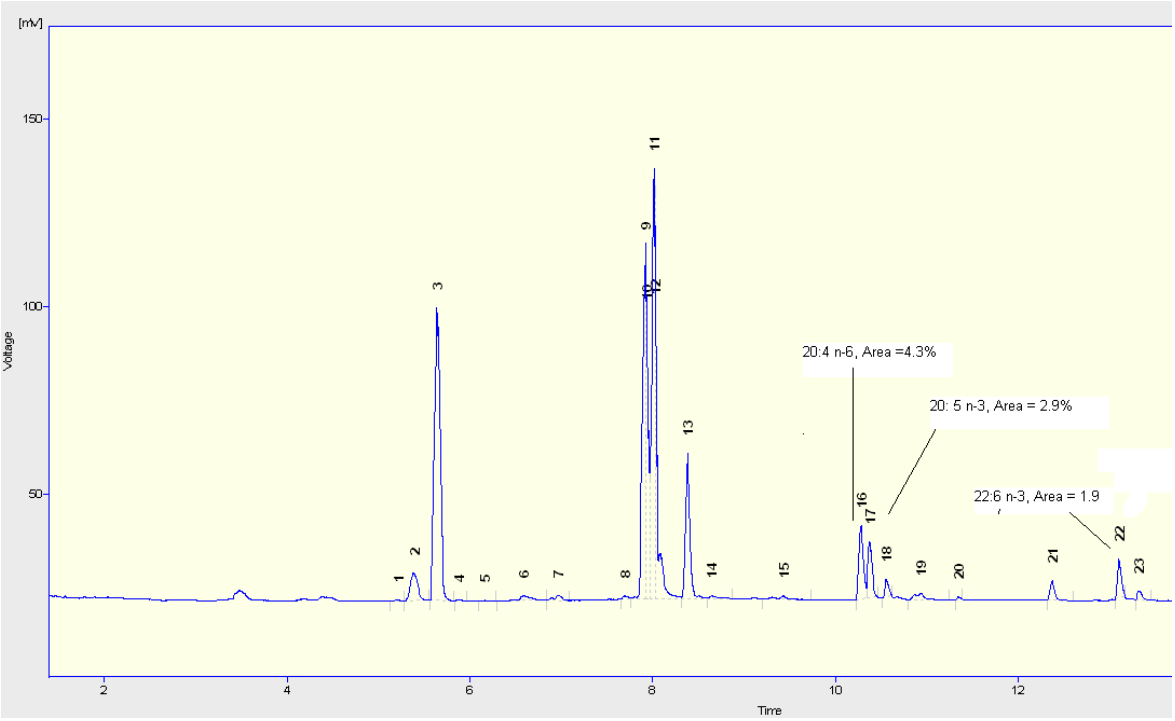


Table 4.3: Mean PBMC total lipid composition of the mild-moderate asthmatics all time points of the intervention study with EPA/DHA.

	Time-points					RM ANOVA				
Fatty Acids	**Baseline**	**Omega 3**	**Placebo**	**wash 1**	**wash 2**	**F value**	**error**	**P value**	**Post hoc significance**	**Observed power**
SFA										
Capric acid										
Lauric acid	5.81 ± 1.98	3.80 ± 2.15	4.29 ± 2.43	5.81 ± 2.88	4.00 ± 1.33	0.26	28.00	0.89	No	0.10
Myristic acid	6.84 ± 2.68	8.36 ± 4.28	4.11 ± 1.98	4.90 ± 2.56	3.55 ± 1.67	0.81	28.00	0.53	No	0.23
Pentadecanoic	1.38 ± 0.47	2.89 ± 1.93	2.58 ± 2.31	2.53 ± 0.95	1.10± 0.68	0.22	28.00	0.93	No	0.09
Palmitic acid	18.28 ± 3.24	15.31 ± 3.63	14.90 ± 2.72	14.07 ± 4.06	18.48 ± 2.38	0.34	28.00	0.85	No	0.16
Heptadecanoic acid	0.39 ± 0.23	0.81 ± 0.26	0.97 ± 0.44	0.54 ± 0.12	1.17 ±0.62	0.79	12.65	0.57	No	0.21
Stearic acid	9.74 ± 1.69	4.52 ± 1.18	4.58 ± 0.94	6.42 ± 0.47	6.49 ± 0.63	4.05	28.00	0.01	No	0.86
MUFA										
Palmitoleic acid	9.39 ± 3.28	3.76 ± 1.19	3.26 ± 1.13	6.70 ± 1.65	4.17 ± 1.80	1.75	28.00	0.21	No	0.30
Oleic acid	10.13 ± 1.54	9.01 ± 2.91	10.39 ± 2.31	7.81 ± 2.95	17.18 ± 1.52	2.77	28.00	0.05	Yes, between baseline and wash 1	0.64
PUFA										
Linoleic Acid (LA)	8.83 ± 2.51	9.40 ± 2.70	10.29 ± 11.36	8.18 ± 2.88	5.73 ± 0.90	0.56	28.00	0.69	No	0.17
Alpha - linoleic Acid (ALA)	0.70 ± 0.09	0.73 ± 0.16	0.77 ± 0.12	1.85 ± 0.54	1.13 ± 0.18	3.36	9.85	0.09	No	0.43
Arachidonic acid (AA)	4.93 ± 0.37	2.28 ± 0.62	6.32 ± 1.64	4.57 ± 0.75	5.61 ± 1.08	2.52	14.32	0.12	No	0.43
Eicosapentaenoic Acid (EPA)	1.33 ± 0.34	3.06 ± 0.73	1.20 ± 0.26	1.36 ± 0.47	1.36 ± 0.22	2.77	12.35	0.11	No	0.28
Docosahexaenoic Acid (DHA	0.60 ± 0.06	1.08 ± 0.23	1.54 ± 0.46	0.82 ± 0.14	0.86 ± 0.19	1.53	28.00	0.22	No	0.41

Appendix 4:

Table 5.1: Percentage change in post exercise FEV_1 at all time-points for all participants, all values are negative showing percentage (%) drop.

Baseline						
Participant ID	**1min**	**5min**	**10min**	**15min**	**30min**	**45min**
1	4.51	15.79	15.04	17.67	14.66	4.89
2	15.65	11.9	15.25	6.12	6,46	1.36
3	3.77	13.36	10.96	4.11	1.37	2.05
4	(+) 2.46	8.42	12.28	10.53	5.26	1.75
5	6.57	14.19	11.42	13.49	12.8	5.88
6	5.71	11.11	10.79	7.62	0.63	(+) 0.95
7	3.85	6.73	11.86	14.1	8.65	3.85
8	2.96	2.63	11.84	11.18	0.66	2.96
Mean ± SD	**5.07 ± 5.06**	**10.52 ± 4.35**	**12.43 ± 1.74**	**10.60 ± 4.50**	**6.31 ± 5.44**	**2.72 ± 2.16**
Placebo						
	1min	**5min**	**10min**	**15min**	**30min**	**45min**
1	2	3.01	20.86	24.23	5.01	(+) 1.50
2	1.36	11.22	18.03	18.37	4.76	1.36
3	7.53	12.44	13.41	14.49	7.53	5.82
4	(+) 0.70	8.07	7.37	4.91	5.26	2.46
5	2.55	5.11	14.6	10.22	10.22	2.55
6	(+) 0.23	10.47	12.09	4.19	3.49	1.63
7	3.85	8,65	6.73	10.9	10.26	3.85
8	3.67	10	15	11.33	8.33	3
Mean ± SD	**3.09 ± 0.52**	**8.62 ± 3.18**	**13.51 ± 4.83**	**12.33 ± 6.67**	**6.86 ± 2.60**	**2.40 ± 2.11**
Omega 3						
IDs	**1min**	**5min**	**10min**	**15min**	**30min**	**45min**
1	1.52	20.83	15.91	11.36	12.5	(+) 3.03
2	6.02	12.37	11.71	6.02	2.68	1.34
3	3.22	5.14	9.97	5.14	1.93	3.54
4	0.7	14.34	14.69	10.49	6.64	5.24
5	8.03	8,63	7.55	7.69	6.23	7,27
6	4.76	1.9	1.27	2.86	0.95	0
7	3.85	4.17	0.32	(+) 0.96	0.96	(+) 4.17
8	1.32	1.97	6.25	9.54	6.25	1.64
Mean ± SD	**2.49 ± 3.85**	**8.67 ± 5.74**	**8.46 ± 5.74**	**6.52 ± 4.16**	**4.53 ± 4.26**	**1.48 ± 3.90**

Appendix 5:

SSHB Conference
25th May 2011, Loughborough University (Oral Presentation)

Impact of a Three Week Dietary Supplementation with Omega 3 on Exercise Induced Asthma in Recreationally Active Males.

Aishwarya Varma, Sarabjit S Mastana, Martin R Lindley , Human Cell and Molecular Biology Research Lab, School of Sports, Exercise and Health Sciences, Loughborough University.

Abstract
Omega 3 (n3) and Omega 6 (n6) are Essential Fatty Acids playing integral role in cell membrane composition and signalling pathways. To evaluate the possibility of using n3/n6 ratio as a biomarker for asthma, a double blinded Randomised Crossover Trial was conducted. Mild asthmatics (Males, n=9, mean age 21.77 ± 2.71years) with a history of Exercise Induced Bronchoconstriction (EIB, >10% drop in post exercise FEV1, >40ppb Exhaled breath Nitric Oxide (FeNO)) completed the trial. Institution's ethics committee approved the study.

Participants entered the study on normal diet; they were supplemented with dietary n3 (comprising 3.2 grams of EPA and 2.2 grams of DHA or placebo) for three weeks before they crossed over to alternate diet (2 week wash). Venous blood samples were used to isolate Peripheral blood Mononuclear Cells (PBMC), genotyping and measurement of inflammatory markers. Standard FFQ and dietary recall was completed to ascertain n3/n6 intake and Exercise challenge test (ATS/ERS guidelines) were used to assess % change in FEV1 after exercise in pre and post supplementation phases.

Significant increase in post exercise FEV1 (p<0.05, Figure 1) and reduction in serum IL-6 levels were observed post n3 supplementation, with no reduction in serum TNF-α and IL-10 levels. The n3/n6 ratio in PBMC membrane was not modified significantly, requiring further investigation. Data suggests that higher n3/n6 can improve lung function and potentially serve as a biomarker for asthma.

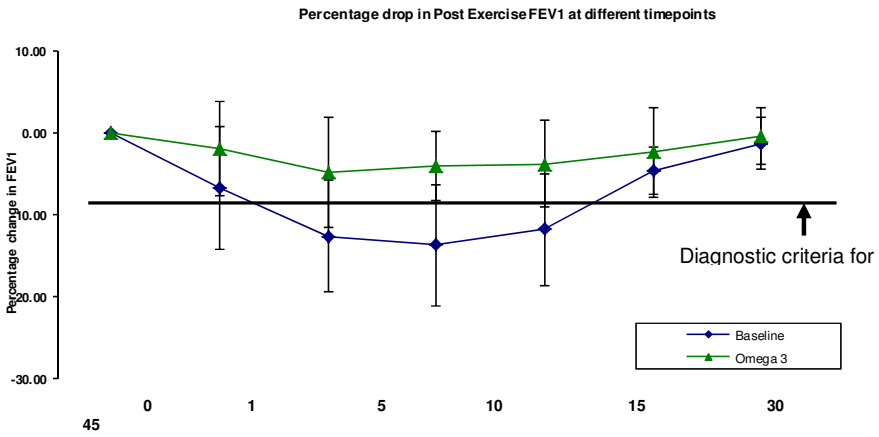


Figure 1

Fatty acids: medicine and menace YLS 2010 (Oral Presentation)
Fatty acid conference, Biochemical Society, Charles Darwin House, London.
27th May 2010

Omega 3/Omega 6 Ratio: A Biomarker for Asthma?
Aishwarya Varma, M R Lindley, Sarabjit S Mastana, Human Cell and Molecular Biology Research Lab, School of Sports, Exercise and Health Sciences, Loughborough University

Biochemical studies suggest beneficial effects of Omega 3 fatty acids on inflammatory diseases such as cardiovascular diseases, diabetes, obesity, asthma and related chronic diseases. Therefore, there is a need to investigate the mechanisms guiding the proposed effect. This study aims to investigate the relationship between Omega 3(n3) and Omega 6(n6) ratio in immune function cell [Peripheral Blood Mononuclear Cell (PBMC)] membranes and lung function.

Mild-moderate asthmatics (N=17, mean age 26.5±5.1, BMI 24.3±1.8) and healthy individuals (N=24, 24.1±7.6, BMI 24±2.8) were recruited from the East Midlands, U.K. The study was approved by Loughborough Universities' ethics committee . Spirometry (BTS/ERS) guidelines measured lung function while venous blood was used to isolate PBMC and measure inflammatory mediators. Dietary recall was completed to ascertain fatty acid intake. A modified method for fatty acid esterification with gas chromatography was adapted for PBMC analysis.

The data demonstrates that the total amount of n3 (sum of ALA, EPA and DHA) and n6 (sum of LA and AA) was significantly different within the two groups ($P<0.05$). The amount of AA was significantly higher in the asthmatic population when compared to the controls; however there was no significant difference between the EPA levels of the two groups. This suggests that asthmatics have a different cell membrane composition with respect to n3/n6; despite the similar dietary n3/n6 intake, as confirmed by the dietary recall analysis.

These findings imply a relationship between n3/n6 in PBMC cell membranes and inflammation, indicating that n3/n6 ratio may be used as a possible biomarker for asthma.

European Respiratory Society Annual Congress (Thematic Poster)
Vienna, Austria
September 2009

Omega 3/Omega 6 ratio in peripheral blood mononuclear cells - a biomarker for asthma?

Aishwarya Varma, M R Lindley, Sarabjit S Mastana. Human Cell and Molecular Biology Research Lab, School of Sports, Exercise and Health Sciences, Loughborough University, Loughborough, Leicestershire, United Kingdom, LE113TU

Biochemical studies suggest beneficial effects of omega 3 fatty acids on asthma and related lung inflammatory diseases (Mickleborough et al Clin Nutr. 2009;28(1):71-7). Therefore, there is a need to investigate the mechanisms guiding the proposed effect. This study aims to investigate the relationship between Omega 3(n3) and Omega 6(n6) ratio in peripheral blood mononuclear cell (PBMC) membranes and lung function. Asthmatics (mild-moderate, n=21; mean age 26.05±12.19) and healthy individuals (n=22; 27±9.75) were recruited from Leicestershire, U.K. Institution's ethics committee approved the study.

Spirometry based on BTS/ERS guidelines measured lung function and venous blood was used to isolate PBMC and measure inflammatory mediators, while dietary recall was completed to ascertain fatty acid intake. A modified method for fatty acid esterification with gas chromatography (Masood et al J Lipid Res.2005;46(10):2299-305) was adapted for PBMC analysis. Preliminary findings suggest asthmatics and controls can be distinguished based on their n3/n6 ratio. These findings imply a relationship between n3/n6 in PBMC cell membranes and inflammation, indicating that n3/n6 ratio may be used as a possible biomarker for asthma.

Table1:Preliminary ratio data for Gas chromatography

	Omega 3	Omega 6	Ratio
Asthmatics(FEV1/FVC <70%)	1	1.8	0.54
n=2	1	7.2	0.13
Controls	1	1.2	0.83
n=2	1	0.98	1.02