

**Exercise-induced bronchoconstriction in athletes:  
influence of airway dehydration on bronchial hyper-  
responsiveness, epithelial injury and mast cell activation**

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By

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# ABSTRACT

Exercise-induced bronchoconstriction (EIB) is the most common chronic medical condition affecting elite athletes; our understanding of the condition remains however incomplete. The over-arching aim of this thesis was therefore to investigate the underlying mechanisms of EIB in athletes. More specifically, *via* induced and inhibited bronchoconstriction, the influence of airway dehydration on bronchial hyper-responsiveness, epithelial injury and inflammatory mediator release was investigated.

The results of our first experiment suggest that mild, whole-body dehydration does not affect the severity of EIB in athletes; however, signs of small airway dysfunction were noticed post-dehydration. The clinical and functional relevance of these findings are yet to be evaluated.

Our next two experiments showed that administration of a single, therapeutic dose of the inhaled  $\beta_2$ -agonist terbutaline before bronchial provocation challenge with dry air i) reduced the severity of bronchoconstriction by 54% in athletes, ii) attenuated the rise in urinary CC16 (a marker of airway epithelial injury), and iii) inhibited the release of the mast-cell derived broncho-constrictive mediator prostaglandin (PG) $D_2$ . These results suggest that local airway dehydration and mast cell activation have a key role in hyperpnoea-induced epithelial injury and bronchoconstriction in athletes.

In our final study, using a newly developed mass spectrometry platform, we identified for the first time that exercise provocation is not solely associated with

the release of potent broncho-constrictive mediators, such as PGD<sub>2</sub> and thromboxane, but also with the release of the broncho-protective mediators PGE<sub>2</sub> and PGI<sub>2</sub>. These results of naturally occurring broncho-protective agents in response to exercise open exciting new opportunities for drug development for EIB.

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- Table 6.1 Participant characteristics.

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## CHAPTER 7: Study 4

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Table 7.1 Participant characteristics.

Table 7.2 Lung function results.



# LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
AChE	Acetylcholinesterase
AMP	Adenosine 5' monophosphate
AHR	Airway hyper-responsiveness
AUC	Area under curve
ASL	Airway surface liquid
ASM	Airway smooth muscle
ATS	American Thoracic Society
Ca <sup>++</sup>	Calcium
cAMP	Cyclic adenosine mono-phosphate
Cl <sup>-</sup>	Chloride
CO	Carbon monoxide
COX	Cyclooxygenase
CC16	Club cell protein
Cyst-LT	Cysteinyl leukotriene
DLCO	Carbon monoxide diffusion capacity
EIA	Exercise-induced asthma
EIB	Exercise-induced bronchoconstriction
ERS	European respiratory society
EVH	Eucapnic voluntary hyperpnoea
FEF <sub>25-75</sub>	Mean expiratory flow rate between 25 and 75% of FVC
FEF <sub>25</sub>	Expiratory flow rate at 25% of FVC
FEF <sub>50</sub>	Expiratory flow rate at 50% of FVC
FEF <sub>75</sub>	Expiratory flow rate at 75% of FVC
FEV <sub>1</sub>	Forced expiratory volume in 1 second
FIS	Federation Internationale de Ski
FOT	Forced oscillometry technique
FRC	Functional residual capacity
FVC	Forced vital capacity
IC	Inspiratory capacity
ICS	Inhaled corticosteroid
IGE	Immunoglobulin E
IRV	Inspiratory reserve volume
IVC	Inspiratory vital capacity
GINA	Global Initiative for Asthma
Hb	Haemoglobin
He	Helium
H <sub>2</sub> O	Water
IOC-MC	International Olympic Committee-Medical Commission
K <sup>+</sup>	Potassium
KCO	Transfer coefficient (i.e. DLCO/VA)
LT	Leukotriene
MBW	Multiple breath washout
MCC	Mucociliary clearance

MVV	Maximal voluntary ventilation
N <sub>2</sub>	Nitrogen
Na <sup>+</sup>	Sodium
NO	Nitric oxide
PEF	Peak expiratory flow
PD	Provoking dose
PG	Prostaglandin
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
Raw	Airway resistance
REC	Research ethics committee
RH	Relative humidity
RHE	Respiratory heat exchange
RV	Residual volume
SPT	Skin prick testing
sRaw	Specific airway resistance
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TMB	Tetramethylbenzidine
TLC	Total lung capacity
VA	Alveolar volume
VC	Vital capacity
VE	Expiratory volume
VEGF	Vascular endothelial growth factor
V <sub>T</sub>	Tidal volume

# PUBLICATIONS ARISING FROM THIS THESIS

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## Full papers

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Simpson, A. J., Bood, J., Anderson, S. D., Romer, L. M., Dahlén, B, Dahlén, S-E., & Kippelen P. A standard, single, dose of inhaled terbutaline attenuates hyperpnoea-induced bronchoconstriction and mast cell activation in athletes. *Journal of Applied Physiology*. Under review.

Simpson, A. J., Romer, L. M., & Kippelen, P. (2015). Self-reported symptoms after induced and inhibited bronchoconstriction in athletes. *Medicine and science in sport and exercise*. 47(10) 2005-2013.

Simpson, A. J., Tufvesson, E., Anderson, S. D., Romer, L. M., Bjermer, L., & Kippelen, P. (2013). Effect of terbutaline on hyperpnoea-induced bronchoconstriction and urinary club cell protein release in athletes. *Journal of Applied Physiology* 115, 1450-1456.

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## Abstracts

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Simpson, A. J., Kippelen. P., Bood, J., Dahlén S-E., & Anderson, S. D. (2015). Inhaled terbutaline attenuates hyperpnoea-induced bronchoconstriction and mast cell activation in athletes. ANZSRS Oral Presentations. *Respirology*, 20: 1–6.

Simpson, A. J., Romer, L. M., & Kippelen P (2015). Exercise-induced whole-body dehydration does not affect airway responsiveness in athletes but may impair small airway function. ANZSRS Oral Presentations. *Respirology*, 20: 1–6.

Simpson, A. J., Romer, L. M., & Kippelen, P. (2014). Dissociation in the effect of terbutaline on hyperpnoea-induced respiratory symptoms and bronchoconstriction in athletes. *Proceedings of the 19th Annual Congress for the European College of Sport Science*, p143.

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Kippelen, P., Simpson, A. J., Greenwood, A. G., Romer, L. M., & Anderson, S. D. (2012). Acute protection against hyperpnoea-induced bronchoconstriction by terbutaline in athletes. *Medicine and Science in Sports and Exercise*. 44, 566.

# **CHAPTER 1**

## **Introduction**

## 1-1 Introduction

Exercise-induced bronchoconstriction (EIB) is a transient narrowing of the airways that occurs during or shortly after vigorous exercise (usually within 15 min). The term exercise-induced asthma (EIA) was originally used to describe the same phenomenon. However, due to the recognition that EIB can occur in individuals without clinical asthma (König & Godfrey, 1973) the term EIB is now preferred, and hence, will be used throughout this thesis.

One of the earliest descriptions relating to EIB comes from Arataeus the Capadocian and is dated back to around the end of the 1<sup>st</sup> century AD, as relayed by Brewis (1990):

“If from running, gymnastic exercises, or any other work, the breathing become difficult, it is called Asthma (asqma)”.

The first investigation of EIB was documented by Herxheimer (1946), who conducted spirometry on six patients before and after exercise and observed a reduction in lung function. Herxheimer (1946) made the following observations: that the severity of the airway response could be influenced by the duration of exercise, that the change in lung function was usually associated with wheeze and, that often, the changes in lung function occurred a few minutes after the completion of exercise. This study and these observations laid the foundations for the following 60+ years of research in the area. As highlighted by a quick search for

“exercise-induced bronchoconstriction” OR “exercise-induced asthma” in PubMed, which returned over 2800 referenced articles (PubMed.gov, accessed 26/08/14), EIB has been the subject of much interest. It is not surprising then, that our understanding and management of the condition has improved a lot over the past half-century.

Whilst historically EIB was a condition whereby sufferers would refrain from exercise and would therefore result in reduced levels of aerobic fitness (Clark & Cochrane, 1988), the advancements in our understanding of the condition and the development of new treatments means that individuals with EIB can currently enjoy a physically active lifestyle that is comparable to their peers without the condition (Fitch, 2010). A testament to the advancements in the management of EIB is that, at recent Olympic Games, athletes with asthma/EIB have out-performed their peers who did not suffer from respiratory ailments (Fitch, 2012).

However, of much interest, athletes are at increased risk for EIB (Carlsen et al., 2008) and it is believed that the various effects (osmotic, thermal and mechanical) of high ventilatory demands during strenuous exercise may lead to the development of the condition (Anderson & Kippelen, 2005). For the majority of athletes with EIB, an acute episode of bronchoconstriction will resolve spontaneously within 30-60 minutes. Of much importance however, in extreme cases EIB can be fatal. Indeed, between 1993 and 2000, 263 sport-related deaths

were recorded in American athletes of which asthma / EIB accounted for 61 (23%) (Becker, Rogers, Rossini, Mirchandani, & D'Alonzo, 2004).

Although the treatment of EIB has improved greatly over the past 50 years, our understanding of the pathophysiology and aetiology of the condition, especially in athletes, remains incomplete. A better understanding of the underlying mechanisms of EIB could further improve the treatment of the condition and may lead to the development of prevention strategies. The aims of this thesis were therefore to i) investigate factors that could influence the severity of EIB, ii) further our understanding of the pathophysiology of the condition and iii) identify treatment strategies that could improve the management and/or prevent the development of EIB in athletes.



## **CHAPTER 2**

### **Literature review**

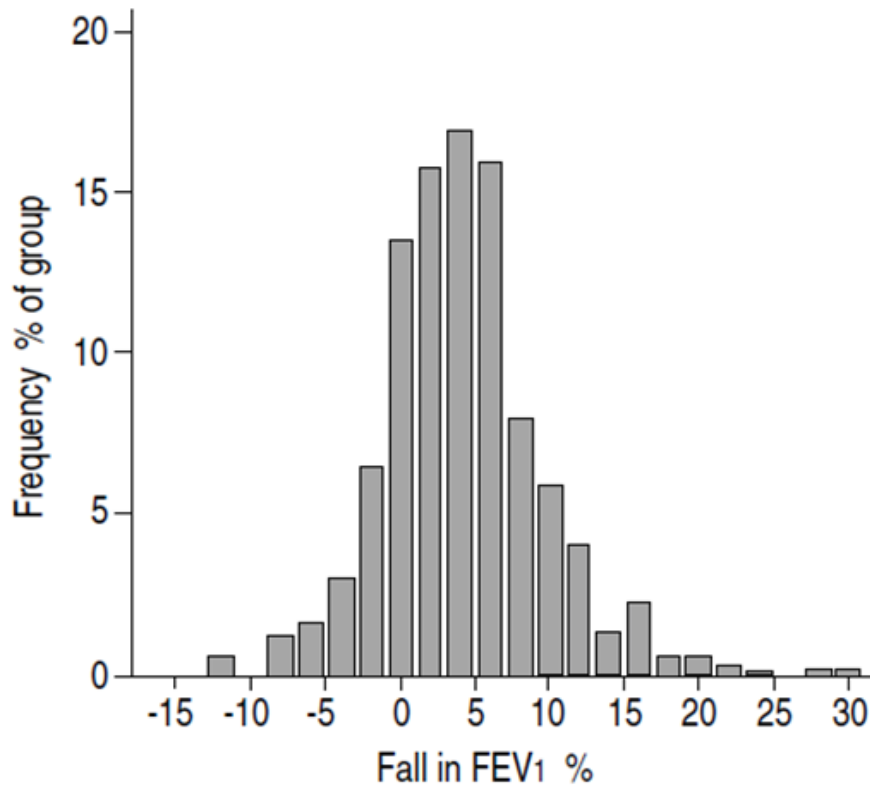
## **2-1 Prevalence of EIB**

### **2-1.1 Prevalence in individuals with asthma**

The airway response to exercise in the general population, which is usually measured by a fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) post-exercise, follows a normal distribution curve [Figure 2.1. (Haby, Peat, Mellis, Anderson, & Woolcock, 1995)]. Reductions in FEV<sub>1</sub> of more than 1.96 standard deviations (SD) (corresponding to 95% confidence interval) away from the mean are often considered to be abnormal airway responses to exercise and are used for the diagnosis of EIB. Using this criterion, reductions in FEV<sub>1</sub> of 8.2% (Godfrey, Springer, Noviski, Maayan, & Avital, 1991), 10% (Kattan, Keens, Mellis, & Levison, 1978) and of 15.3% (Haby et al., 1995) following exercise have been utilised for the diagnosis for EIB. This variation reflects the different exercise provocation protocols used by the different research groups [i.e., a laboratory-based exercise provocation challenge on a treadmill, such as used in the studies by Godfrey and colleagues (1991) and by Kattan and colleagues (1978), or field-based challenges, such as described by Haby and colleagues (1995)]. According to the current guidelines from the American Thoracic Society (ATS) a  $\geq 10\%$  fall in FEV<sub>1</sub> after exercise is considered an abnormal response to exercise and, given that the majority of healthy individuals display an increase in FEV<sub>1</sub> following exercise, is proposed a “reasonable” criterion for the diagnosis of EIB (Crapo et al., 2000).

Asthma is a common respiratory disease characterised by chronic inflammation and reversible airflow obstruction. Individuals with asthma are at an increased risk for EIB. The Global Initiative for Asthma (GINA) describes asthma as a heterogeneous disease whereby patients experience variable symptoms of wheeze, shortness of breath, chest tightness and cough and by variable airflow limitation; the respiratory symptoms vary over time and intensity and are often triggered by factors such as exercise, allergen or irritant exposure (GINA, 2014). Worldwide, it is estimated that as many as 300 million people are currently suffering from asthma (Masoli, Fabian, Holt & Beasley, 2004). The UK has one of the highest rates of asthma in Europe, with the condition affecting 4.3 million adults and 1.1 million children [i.e., around 8% of the population, ("Asthma facts and FAQs," 2014)]. Prevalence rates of EIB in individuals with asthma vary from 36 to 90% (Benarab-Boucherit et al., 2011; Cabral, Conceição, Fonseca-Guedes, & Martins, 1999; Karjalainen, 1991). This large variation may reflect the varying criteria and broncho-provocation methods used for diagnosis. Nonetheless, it indicates that asthmatic individuals are at increased risk for EIB.

Airway hyper-responsiveness (AHR) describes an abnormal airway response to normally innocuous stimuli. AHR encompasses EIB, but also includes increased airway reactivity from exposure to allergens, industrial chemicals and laboratory tests, such as histamine, methacholine and hyperosmotic aerosols. Importantly, the high level of ventilation associated with exercise may increase the exposure of the airways to these inhaled irritants and, thus, exercise may indirectly cause AHR.



**Figure 2.1.** Relative frequency distribution of % fall in forced expiratory volume in 1 s ( $FEV_1$ ) in 435 “normal” children. Data demonstrates the normal distribution in the airway response to exercise. From Haby et al., (1995).

### 2-1.2 Prevalence in athletes

Similarly to the general population, wide variations in the prevalence rate of EIB have been reported in athletes over the past 25 years, with values ranging from 7% to >50% (Carlsen et al., 2008). In 2001, the International Olympic Committee-Medical Commission (IOC-MC) made a decision that all Olympic athletes must demonstrate current asthma and/or AHR to warrant the use of asthma medication at the Olympic Games. This led to the standardisation of diagnostic tests for asthma/AHR detection in the athletic population and to the accumulation of a

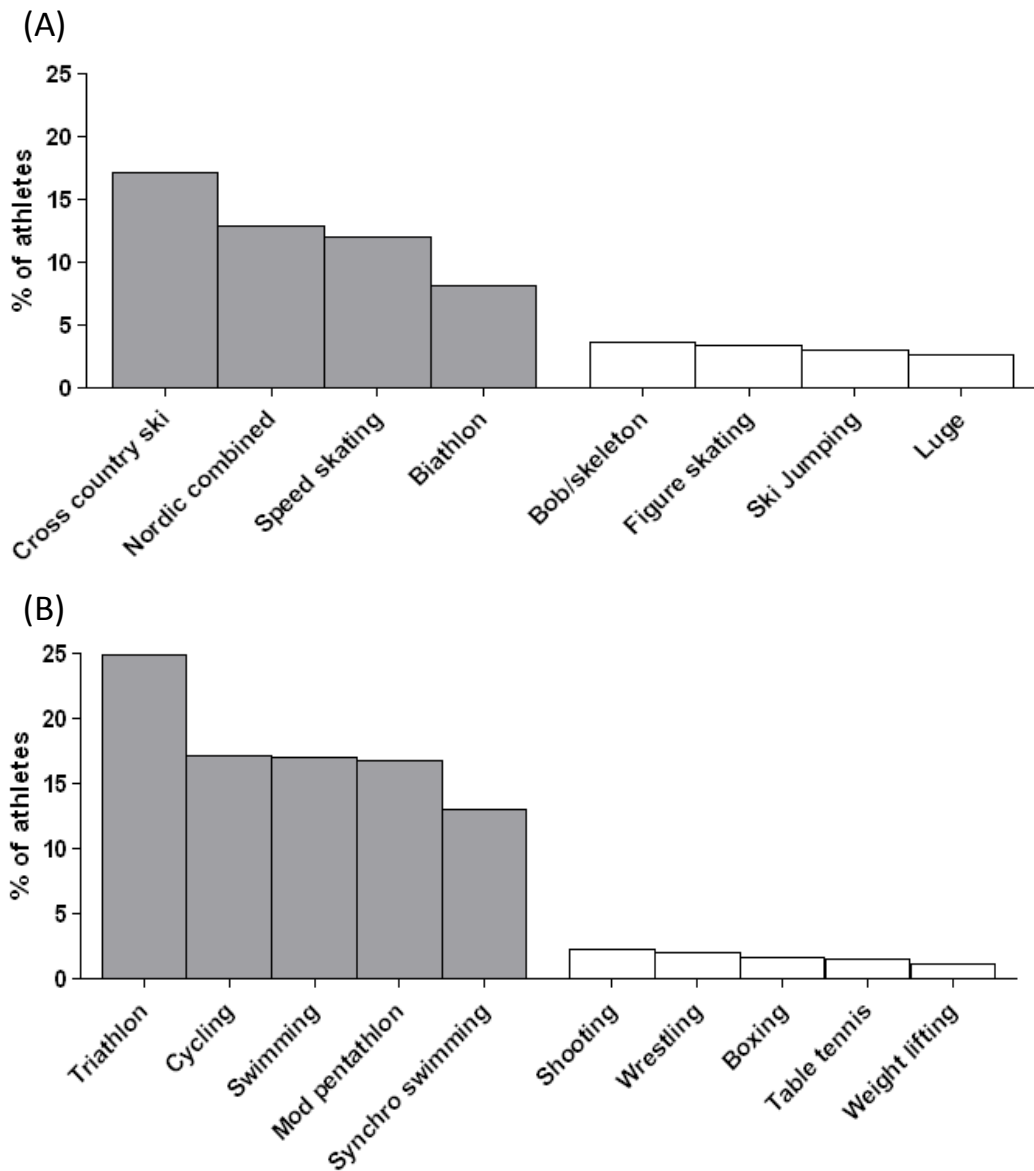
wealth of data on the prevalence of asthma and AHR in elite sport (Anderson et al., 2003; 2006b; Carlsen et al., 2008; Dickinson, 2005; Fitch, 2006; 2012). Data obtained by the IOC-MC for athletes requesting to use inhaled  $\beta_2$ -agonists at the Olympic Games between 2002 and 2010 suggests the prevalence of asthma/AHR to be around 8%. This makes asthma/AHR the most common chronic medical condition in Olympians (Fitch, 2012).

Further analysis of the data obtained by the IOC-MC revealed a discrepancy in the prevalence of asthma/AHR between endurance-trained athletes and athletes who train for sports that require short 'bursts' of effort (Carlsen et al., 2008; Fitch, 2012). For example, Fitch (2012) noted a prevalence rate of 12.9% in Nordic combined competitors (i.e., those who ski jump and complete a 15 km cross-country race), which was four times that of athletes who perform ski jumping alone (Figure 2.2). This finding is consistent with many other reports in endurance sports (Carlsen et al., 2008; Fitch, 2012). Figure 2.2 clearly illustrates the increased prevalence of asthma/AHR in endurance sports. These data suggest that athletes who regularly partake in sports in which high ventilation levels are sustained for long periods of time are at an increased risk for asthma/AHR.

Another interesting observation is the propensity for athletes to develop asthma/AHR relatively late in their careers. At the 2006 Winter Olympic Games, 48.7% of athletes positive for asthma or AHR reported the onset of respiratory symptoms after the age of 20 (Fitch, 2006). Similarly, at the 2008 Summer Olympics, 36.9% of the athletes positive for asthma or AHR reported the first

symptoms after the age of 25 yr (Fitch, 2012). This is contrary to asthmatic individuals who usually present first EIB symptoms during childhood, and then develop asthma later in life (Stern, Morgan, Halonen, Wright, & Martinez, 2008). This also suggests that, in athletes, EIB/AHR may develop gradually over years of intensive training.

The environment of practice may also contribute to the increased prevalence of asthma/AHR in some athletic populations. Cold weather athletes (Larsson et al., 1993; Rundell et al., 2000; Sue-Chu, Larsson, & Bjermer, 1996), swimmers (Dickinson, 2005; Langdeau et al., 2000; Zwick, Popp, Budik, Wanke, & Rauscher, 1990) and ice-rink athletes (Lumme et al., 2003; Mannix, Manfredi, & Farber, 1999) have all been shown to be at increased risk for asthma/AHR. This is likely to be due to their airways being repeatedly exposed to cold dry air or to airborne noxious agents (Rundell & Sue-Chu, 2013).



**Figure 2.2.** Percentage of athletes approved for inhaled  $\beta_2$ -adrenoceptor agonist use at (A) the Winter Olympics Games in 2002, 2006 and 2010 and (B) at the Summer Olympics in 2004 and 2008. Only sports with highest and lowest percentages are included. Data provides evidence of an increased prevalence of asthma/exercise-induced bronchoconstriction in athletes from endurance based disciplines. Reconstructed from Fitch (2012).

## **Summary**

Exercise-induced bronchoconstriction is highly prevalent in elite athletes.

Athletes partaking in endurance sports and/or chronically exposed to cold dry air or noxious inhaled agents may develop the condition over many years. That EIB develops over long periods of time in these athletes suggests that protecting against the harmful effects of the inhalation of large volumes of cold dry air or noxious agents may prevent the development of the condition.

## **2-2 Diagnosis of EIB**

### **2-2.1 Symptom-based diagnosis**

Respiratory symptoms are a common occurrence following exercise. In a large survey of 1085 members of the general public in the USA, 29% of the population reported experiencing respiratory-related symptoms (incl. shortness of breath, wheezing, coughing, difficulty taking a deep breath, noisy breathing, or chest tightness) during or immediately following exercise (Parsons et al., 2011). In the UK, results from a survey of 257 family practitioners suggest that one third encounter an individual with exercise-induced respiratory symptoms at least once *per* month (Hull, Hull, Parsons, Dickinson, & Ansley, 2009). Athletes also frequently report exercise-induced respiratory symptoms. Results from a questionnaire completed by nearly 700 athletes from 33 sports suggest that 16% experience periods of wheezing, marked breathlessness and chest tightness when exercising (Turcotte,



Langdeau, Thibault, & Boulet, 2003). These findings are supported by numerous observations of a high prevalence of respiratory symptoms in athletes across a variety of sports (Bougault, Turmel, & Boulet, 2010; Holzer, Anderson, & Douglass, 2002; Randolph, Dreyfus, Rundell, Bangladore, & Fraser, 2006; Rundell et al., 2001; Stadelmann, Stensrud, & Carlsen, 2011). Exercise-induced cough is particularly prevalent in athletes, with rates as high as 64% in cross-country skiing (Sue-Chu et al., 1996).

A study by Rundell and colleagues (2001) assessed the efficacy of using self-reported symptoms for the diagnosis of EIB. One hundred and fifty eight elite athletes completed a questionnaire to determine the prevalence of self-reported respiratory symptoms prior to the completion of a sport-specific exercise challenge to determine the presence of EIB. The percentage of athletes who reported experiencing at least one respiratory symptom was 51%; the prevalence was not significantly different between athletes who demonstrated EIB (61%), and those athletes who were either negative to the challenge (45%) or borderline (57%). In the same study, 39% of athletes who demonstrated EIB were asymptomatic. These results are consistent with the findings from Holzer and colleagues (2002), who noted 40% of athletes with a positive bronchial provocation challenge reported no respiratory symptoms. Furthermore, 45% (Rundell et al., 2001) and 68% (Holzer et al., 2002) of athletes with a negative bronchial provocation challenge reported respiratory symptoms. For respiratory symptoms to be of any diagnostic value, the sensitivity and specificity need to be around 85% and 92%, respectively; in previous

studies (Holzer et al., 2002; Rundell et al., 2001) however respiratory symptoms fell short of these thresholds (Table 2.1). Rundell and colleagues (2001) therefore suggested that “symptom-based diagnosis of EIB is not more accurate than a coin toss”.

**Table 2.1.** The sensitivity and specificity of self-reported respiratory symptoms to predict exercise-induced bronchoconstriction.

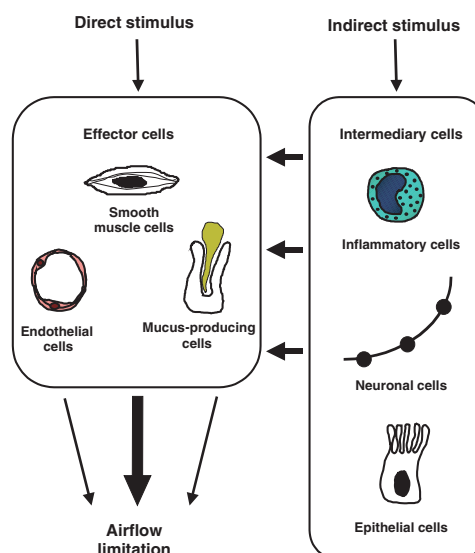
	<b>Sensitivity (%)</b>	<b>Specificity (%)</b>
Cough	61	69
Wheeze	17	82
Chest tightness	20	80
Excess mucus	22	85

Based on findings from Rundell et al., (2001). Data suggests that self-reported respiratory symptoms are neither sensitive nor specific for the diagnosis of exercise-induced bronchoconstriction in athletes.

In 2001, in light of the poor diagnostic value of respiratory symptoms for EIB diagnosis, and due to an alarming increase in notification of Olympic athletes using inhaled  $\beta_2$ -adrenoceptor agonist medication, a meeting was called by the IOC-MC. The result of this meeting was that all athletes wishing to use inhaled  $\beta_2$ -agonists at the upcoming Olympic games were required to provide objective evidence of airway obstruction, either at rest, or post-bronchial provocation (Anderson et al., 2003). The IOC-MC provided a list of direct and indirect bronchial provocation challenges from which objective evidence of EIB/AHR should be sought (Anderson et al., 2003).

## 2-2.2 Direct and indirect bronchial provocation challenges

Bronchial provocation challenges can be classified as direct or indirect and are categorised as such depending on their main site(s) of action. Direct challenges (e.g., methacholine and histamine) act on effector cells, such as airway smooth muscle, mucus producing cells and endothelial cells, causing airway narrowing (Figure 2.3). In contrast, indirect challenges [e.g., exercise, eucapnic voluntary hyperpnoea (EVH), hypertonic aerosols and adenosine 5' monophosphate (AMP)] act on intermediary cells, such as inflammatory cells, neuronal cells and epithelial cells that release mediators or neurotransmitters. Released mediators and neurotransmitters have a subsequent action on effector cells, leading to airway narrowing [Figure 2.3, (Van Schoor et al., 2005)].



**Figure 2.3.** The mechanisms by which direct and indirect bronchial stimuli induce airflow limitation. From Van Schoor et al., (2005). Examples of direct stimuli include methacholine and histamine. Indirect stimuli include exercise, eucapnic voluntary hyperpnoea, hypertonic aerosols and adenosine 5' monophosphate.

### **2-2.3 Exercise broncho-provocation challenges**

Exercise is a 'natural' provocation for bronchoconstriction in susceptible individuals and is therefore a logical choice for EIB diagnosis. A bronchial provocation challenge with exercise can be performed in a laboratory or in field-based settings, and the selection of the most appropriate setting depends on the anticipated trigger for EIB. If noxious agents in the environment of practice [such as chlorine derivatives for swimmers (Bernard et al., 2003) or particulate matter from fossil-fuelled ice resurfacing machines in ice-hockey players (Rundell, 2003), for example] are thought to be at least partly responsible for EIB, a field-based test might be particularly appropriate. Field-based testing has been shown to detect cases of EIB missed by laboratory-based exercise and by EVH (Mannix et al., 1999; Rundell et al., 2000; Rundell, Anderson, Spiering, & Judelson, 2004). However, the lack of standardisation, with no control of temperature and humidity [two main determinants in the severity of the airway response (Anderson et al., 1982)], reduces the reliability of field-based challenges, especially in the context of research investigations that require a repeated measures design.

A laboratory-based exercise provocation challenge involves a short bout of exercise (usually around 8 min) at a high intensity. According to the ATS guidelines, the intensity of the exercise should be increased rapidly over 4 minutes and then sustained at a high level for the remaining 4 minutes (Crapo et al., 2000). The intensity has to be high so that ventilation is significantly increased above baseline value. That ventilation reaches >50% of maximal voluntary ventilation (MVV) is key

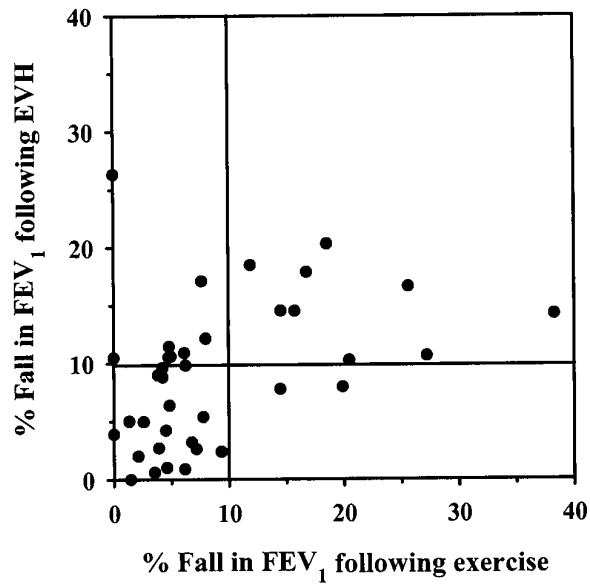
(Crapo et al., 2000), in that ventilation has been shown to directly affect the severity of the airway response post-exercise (Kivity, Souhrada, & Melzer, 1980). Because environmental conditions (i.e., temperature and humidity) are also regarded as key factors in the severity of EIB (Anderson et al., 1982), the ability to manipulate the inspired air conditions (i.e., modify the temperature and humidity of the air) makes bronchial provocation with exercise a particularly useful bronchial provocation challenge to investigate the mechanisms of EIB.

#### **2-2.4 Eucapnic voluntary hyperpnoea**

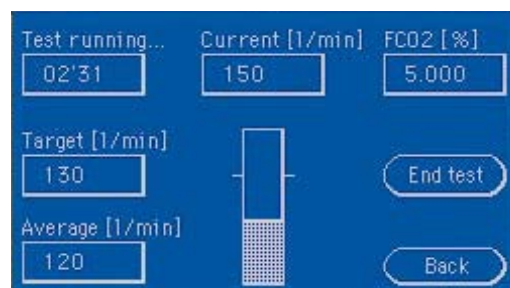
EVH is a bronchial provocation challenge that was originally designed by members of the US Army as a surrogate for exercise to identify EIB (Phillips, Jaeger, Laube, & Rosenthal, 1985). The principles of bronchial provocation with EVH mimics that of bronchial provocation with exercise, i.e., a high level of ventilation should be rapidly achieved and sustained for a short period of time (normally 6 min). During an EVH challenge participants inhale a dry gas mixture that is delivered *via* compressed gas cylinders. The target ventilation during EVH provocation is very high, usually set at 30 x FEV<sub>1</sub> (approx. 85% MVV) (Anderson, Argyros, Magnussen, & Holzer, 2001a). Most athletes should easily achieve 25 x FEV<sub>1</sub> (approx. 60% MVV) (Anderson, Argyros, Magnussen, & Holzer, 2001a), which is the lowest required threshold for results interpretation. The low levels of humidity and the high level of ventilation make provocation with EVH more potent, and therefore more sensitive, than provocation with exercise for the diagnosis of EIB (Eliasson, Phillips, Rajagopal, & Howard, 1992; Mannix et al., 1999; Rundell et al., 2004). This is demonstrated in

Figure 2.4. However, it should be noted that a recent systematic review highlights that in many of the investigations that compared the sensitivity of EVH to exercise provocation, the ATS guidelines for exercise testing (Crapo et al., 2000) were not adhered to. Specifically, the authors noted that rarely were the low levels of humidity suggested by the ATS guidelines met (Stickland, Rowe, Spooner, Vandermeer, & Dryden, 2011). This may have resulted in a reduction in the stimulus for EIB and reduced the sensitivity of exercise to detect EIB (Stickland et al., 2011). As a result the authors suggest that additional high quality studies comparing EVH to exercise should be conducted before any conclusions are made regarding the sensitivity of EVH (Stickland et al., 2011). Nevertheless, for now EVH is considered as 'gold standard' for the diagnosis EIB in athletes (Anderson, Argyros, Magnussen, & Holzer, 2001a).

Recently, the development of purpose-built EVH equipment [such as used in our experiments (Figure 2.5)] has contributed to a larger use of this technique, both for clinical and research purposes. Bronchial provocation with EVH offers participants visual feedback of their level of ventilation, allowing for the tight matching of ventilation between trials. That the level of ventilation can be tightly regulated between tests and that the humidity level of the inhaled air remains constant, makes bronchial provocation challenges with EVH an ideal tool for repeated-measures study designs.



**Figure 2.4.** The fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) following bronchial provocation with eucapnic voluntary hyperpnoea (EVH) and exercise. A fall in FEV<sub>1</sub> of  $\geq 10\%$  is consistent with a positive diagnosis of exercise-induced bronchoconstriction (EIB). Data suggests EVH is a more sensitive test for the diagnosis of EIB. From Rundell et al., (2004).



**Figure 2.5.** The Eucapsys system (SMTEC, Nyon, Switzerland), a purpose-built device for broncho-provocation with eucapnic voluntary hyperpnoea. On the right, a visual display that helps participants to reach their target ventilation.

## 2-2.5 Other bronchial provocation tests

### *Hypertonic challenges*

Hyperosmolar aerosols can be used as a surrogate for exercise bronchial provocation. Hypertonic challenges simulate the effects of respiratory water loss that occurs during exercise by increasing the osmolarity of the airway surface liquid (ASL) (Smith & Anderson, 1986). Bronchial provocation with hypertonic aerosols can be conducted using hypertonic saline solution or dry powder mannitol. The latter is a naturally occurring alcohol sugar that can be dried and encapsulated for inhalation (Anderson et al., 1997). The mannitol challenge was developed and standardised by Anderson and co-workers (1997). Both hypertonic saline and mannitol provocation challenges use a dose response protocol, whereby the dose of the inhaled agent is doubled and spirometry is taken following each dose. A test is deemed positive if the fall in FEV<sub>1</sub> is  $\geq 15\%$  (Anderson et al., 1997; Sterk et al., 1993). If a  $\geq 10\%$  decrease in FEV<sub>1</sub> is observed between any two consecutive doses, it is also regarded as a positive test for bronchial provocation with mannitol (Anderson et al., 1997) and the same dose would be repeated during a hypertonic saline bronchial provocation challenge (Sterk et al., 1993).

One of the limitations of using mannitol or hypertonic saline for bronchial challenge is that they induce cough in the majority of individuals (85% and 73%, respectively) (Brannan et al., 2005). Severe cough has been reported to interfere with the testing protocol in 13% and 8% of tests, respectively (Brannan et al., 2005). However, advantages of using hypertonic aerosols for the diagnosis of EIB/AHR are several:

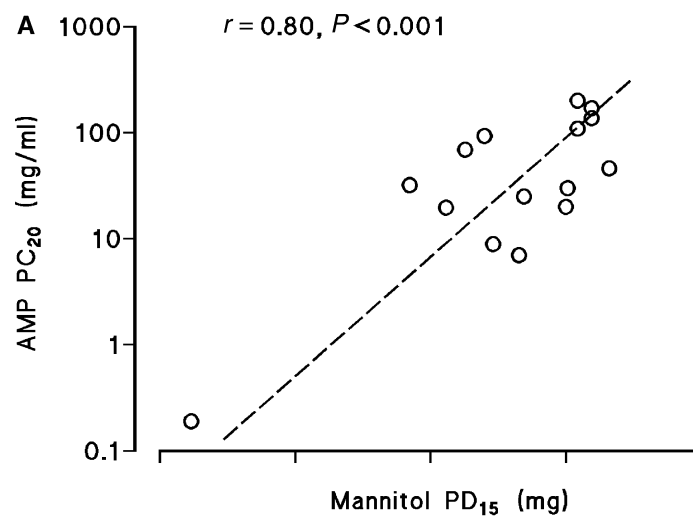


the dose-response protocol reduces the risk of a severe response in individuals with severe or uncontrolled asthma; the dose response protocol allows for identification of a change in sensitivity of the airways to a given stimulus (rather than just measuring the severity of the response, as with exercise or EVH challenges); the inhalation of hypertonic solutions removes any effects of respiratory heat loss, as occurs during the conditioning of inspired air with exercise and EVH. Removing respiratory heat loss as a confounding factor in EIB has offered important insights into the pathophysiology of the condition (see following section for further details).

### ***Adenosine 5' monophosphate (AMP)***

*In vitro* preparations show that AMP has a very weak constrictor effect on bronchial smooth muscle (Finney, Karlsson, & Persson, 1985). AMP is therefore considered an indirect bronchial provocation challenge. Like hypertonic aerosols, AMP is delivered *via* inhalation in doubling doses. The dose of AMP that provokes a 20% reduction in FEV<sub>1</sub> (PD<sub>20</sub>) correlates significantly with the dose that provokes a 15% fall in FEV<sub>1</sub> (PD<sub>15</sub>) following bronchial provocation with mannitol (Figure 2.6), suggesting that both challenges act primarily on inflammatory cells (Currie, Haggart, Brannan, et al., 2003a). The mode of action of AMP is likely mediated by AMP binding with the A<sub>2</sub>-purinoceptors receptors on the inflammatory cells and subsequent release of mediators (Lee, Gray, & Lipworth, 2003). Although AMP and hypertonic aerosols work *via* activation of the same inflammatory cells, AMP does not mimic any aspect of exercise. Hence, AMP is not a method of choice for the

diagnosis of EIB in athletes and was omitted from the IOC-MC list of accepted bronchial provocation challenges (Anderson et al., 2003).



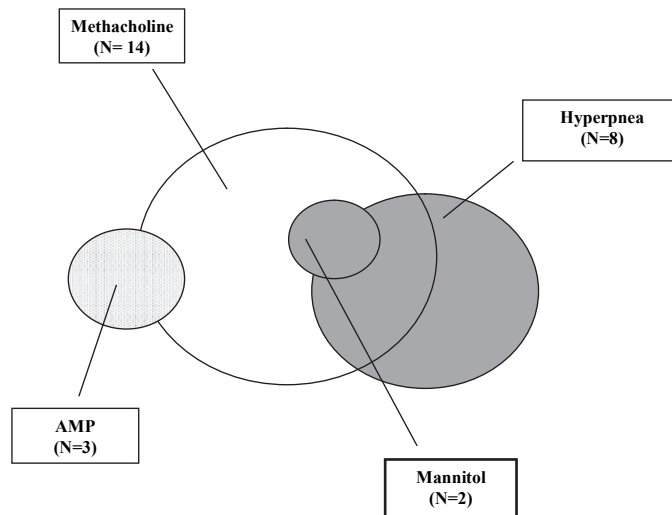
**Figure 2.6.** Correlation between provocation with Adenosine 5' monophosphate (AMP) and dry powder mannitol. The strong correlation is suggestive that both bronchial provocation challenges act on the same inflammatory cells. From Currie et al., (2003)

### ***Methacholine and histamine***

Bronchial provocation using methacholine and histamine challenges are widely used in clinical and research settings for the assessment and diagnosis of asthma and AHR. Methacholine chloride and histamine stimulate muscarinic receptors and histamine receptors, respectively, at the site of the airway smooth muscle

(Woenne, Kattan, Orange, & Levison, 1978). Due to their direct action on the airway smooth muscle they are considered direct bronchial provocation challenges. Like bronchial provocation challenges with hypertonic aerosols and AMP, methacholine and histamine use a dose response protocol. Standardised guidelines for methacholine challenges have been published by the ATS (Crapo et al., 2000) and guidelines for histamine have been devised by the European Respiratory Society (ERS) (Sterk et al., 1993).

A positive response to a methacholine challenge is consistent with the presence of current active asthma (Crapo et al., 2000). However, due to its modes of action, methacholine may not identify EIB in individuals without asthma. Indeed the sensitivity of methacholine to diagnose EIB is low; a positive response to a methacholine challenge was observed in only 36% of elite athletes with EIB (as confirmed by a positive EVH challenge) (Holzer et al., 2002). Moreover, in cold-weather athletes (such as cross-country skiers) a positive methacholine challenge has been suggested to be a marker of airway epithelial injury rather than asthma (Sue-Chu, Brannan, Anderson, Chew, & Bjermer, 2010). Airway epithelial injury (due to inhalation of large volumes of poorly conditioned air) is thought to enhance access of methacholine to the muscarinic receptors on the airway smooth muscle (Sue-Chu et al., 2010). This idea is in line with the fact that a large proportion of cross country skiers are positive to methacholine, yet negative to indirect challenges such as mannitol, AMP and EVH [Figure 2.7 (Sue-Chu et al., 2010)].



**Figure 2.7.** Interrelationship of airway hyperresponsiveness to methacholine, adenosine 5'-monophosphate (AMP), mannitol and eucapnic voluntary hyperpnoea (EVH) in 33 cross country skiers. Data demonstrates a disconnect of positive responses to methacholine and to indirect provocation challenges. From Sue-Chu et al., (2010).

### Summary

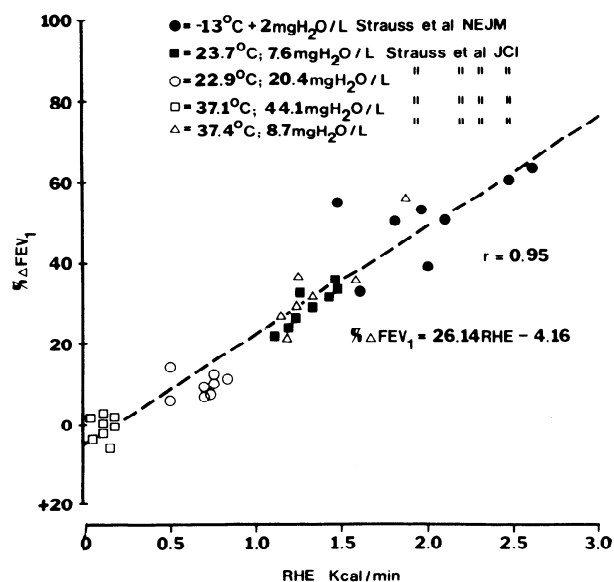
Various bronchial provocation challenges can be used for the diagnosis of EIB. Bronchial provocation with exercise allows for modifications of the temperature and humidity of the inspired air and may therefore be utilised to determine the respective roles of water and heat loss in EIB. In contrast, EVH offers tight control of the ventilation and humidity of the inspired air, rendering this test particularly suitable for investigations on the effects of preventive strategies (such as pharmacological treatments) on the severity of EIB.

## 2-3 Pathophysiology of EIB

During the late 1970's and early 1980's two main theories emerged to explain EIB; the osmotic theory and the thermal theory. Both of these theories acknowledge that it is not exercise *per se* that induces EIB, but rather the effects of increased ventilation associated with strenuous exercise. All inspired air is heated and humidified to body conditions before reaching the alveoli. At rest, when ventilation is low ( $\sim 6 \text{ L}\cdot\text{min}^{-1}$ ), air is primarily inhaled through the nose, where it is heated and humidified by the nasal mucosa. During exercise, when ventilation exceeds 35 - 45  $\text{L}\cdot\text{min}^{-1}$ , there is a shift from nasal breathing to mouth breathing (Niinimaa, Cole, Mintz, & Shephard, 1980; Saibene, Mognoni, LaFortuna, & Mostardi, 1978); an action which increases the demand for the airways to provide heat and moisture to the inspired air. The conditioning of large volumes of air during exercise therefore has the potential to affect the temperature of the airways and to alter the composition of the ASL. The thermal theory proposes that it is a change in temperature of the airways and of the surrounding bronchial microvasculature that is responsible for EIB (Deal, McFadden, Ingram, & Jaeger, 1979a; McFadden, Lenner, & Strohl, 1986). The osmotic theory proposes that evaporative water loss affects the volume and composition of the ASL, which, through a cascade of events, triggers EIB (Anderson et al., 1982).

### 2-3.1 Thermal Theory

The thermal theory is an extension to the airway cooling theory proposed by Deal and colleagues (1979b). The airway cooling theory was based on evidence that the severity of bronchoconstriction increases as the temperature of the inspired air decreases (Deal et al., 1979b). The authors concluded that the severity of bronchoconstriction was directly proportional to the thermal load placed on the airways and proposed respiratory heat loss as a determinant for the severity of EIB (Figure 2.8). Evidence of respiratory heat loss was provided by the same research team in a subsequent paper (McFadden et al., 1985). In that latter paper the authors demonstrated, by placing a probe containing multiple thermistors down the tracheobronchial tree of 6 healthy participants, that airway cooling progresses distally when ventilation increases.

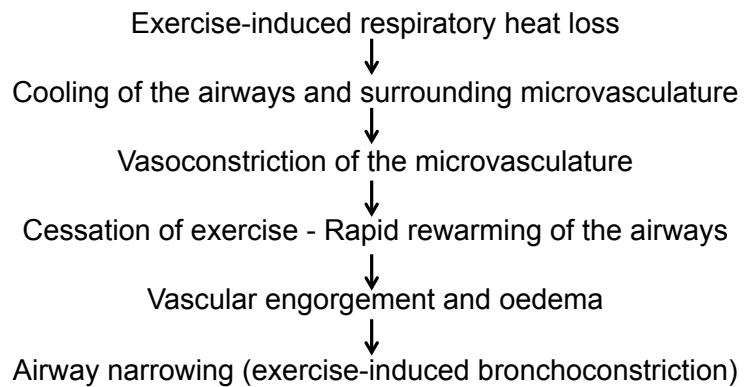


**Figure 2.8.** Relationship between respiratory heat exchange (RHE) and post-exercise change in forced expiratory volume in 1 s ( $FEV_1$ ). From Deal et al., (1979b).

The thermal theory added to the airway cooling theory that rapid rewarming of the airways immediately after exercise is essential for the development of EIB (McFadden et al., 1986). The thermal theory is supported by the fact that the severity of the bronchoconstriction can be reduced when participants breathe cold air immediately after exercise, therefore removing the thermal gradient at the end of exercise (McFadden et al., 1986).

The mechanism proposed to explain the thermal theory is that an increase in ventilation, particularly of cold air, during exercise cools the bronchial microvasculature causing vasoconstriction. When the cooling stimulus is removed (i.e., during recovery) reactive hyperaemia of the blood vessels occurs, causing luminal narrowing through vascular engorgement and oedema in the mucosa and submucosa [Figure 2.9, (McFadden et al., 1986)]. In support to this theory, an increase in vascular endothelial growth factor (VEGF) – a factor known to increase vascular permeability (Dvorak, Brown, Detmar, & Dvorak, 1995) and to contribute to the formation of oedema (Kanazawa, Hirata, & Yoshikawa, 2002) – has been observed in induced sputum in individuals with asthma (Kanazawa et al., 2002). The concentration of VEGF has also been shown to correlate with the severity of EIB in asthmatic individuals (Kanazawa et al., 2002), suggesting that an increased vascular permeability and the formation of oedema induced by VEGF may contribute to the severity of EIB. Finally, the number and percentage of blood vessels surrounding the airways is known to be increased in individuals with asthma (Hoshino,

Takahashi, Takai, Sim, & Aoike, 2001); this could therefore amplify the vascular effects and explain the high prevalence of EIB in individuals with asthma.

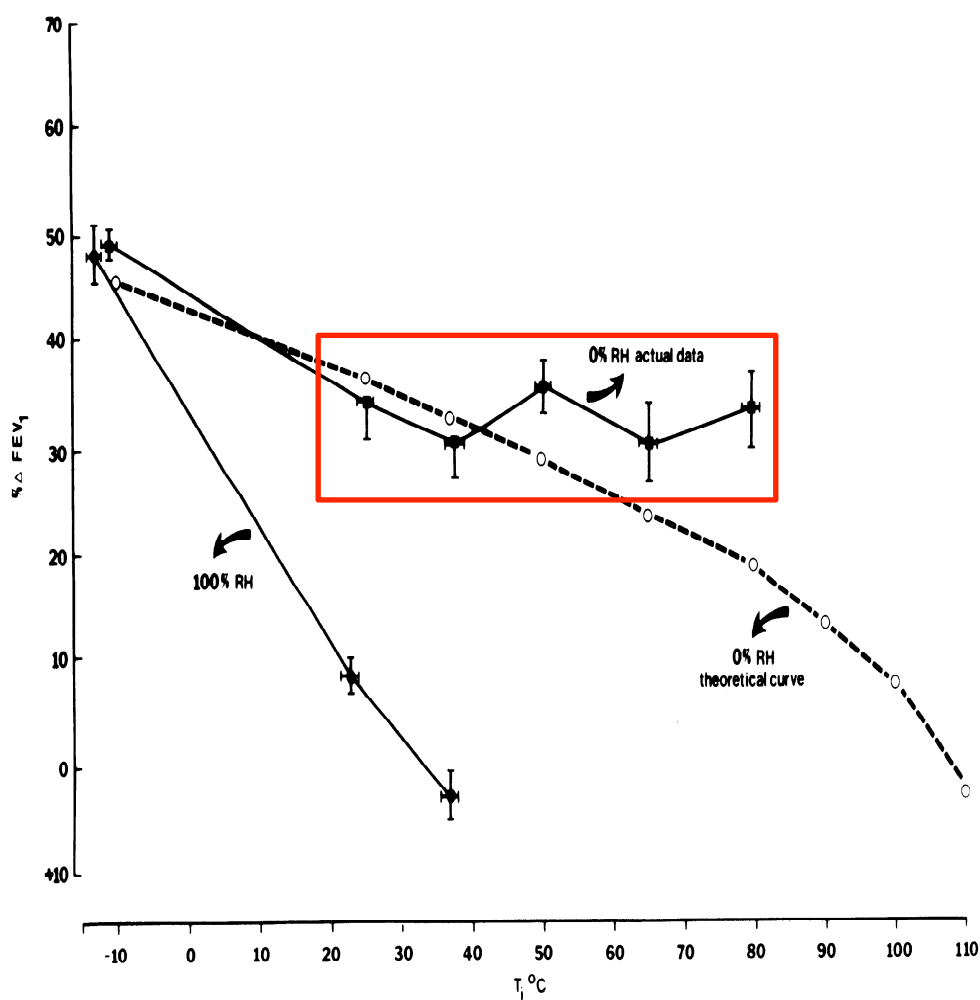


**Figure 2.9.** A flow chart of the thermal theory of exercise-induced bronchoconstriction as proposed by McFadden et al., (1986).

The thermal theory however fails to explain many observations of EIB. Probably the most irrefutable evidence that the thermal effects are not solely responsible for EIB is that inhalation of hot dry air during exercise can induce bronchoconstriction [Figure 2.10 (Anderson, Schoeffel, Black, & Daviskas, 1985; Deal et al., 1979b; Hahn, Anderson, Morton, Black, & Fitch, 1984)]. Whilst the inhalation of hot dry air will induce some degree of heat loss from the airways (due to the evaporation of moisture from the airway surface), this would be insufficient to significantly cool the airways. Indeed, Anderson and colleagues (1985) demonstrated that the inhalation of hot dry air only reduces the retro-tracheal temperature by 0.1°C, yet



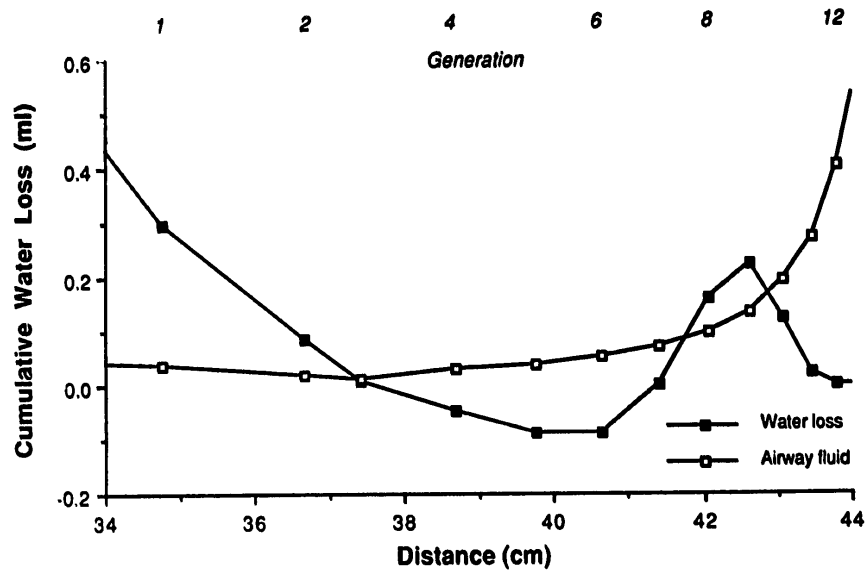
EIB can occur. The thermal theory of EIB also fails to explain bronchoconstriction caused by inhalation of hypertonic aerosols, such as described in many studies (Anderson et al., 1997; Brannan et al., 2005; Brannan, Gulliksson, Anderson, Chew, & Kumlin, 2003; Holzer, Anderson, Chan, & Douglass, 2003; Kersten, Driessen, van der Berg, & Thio, 2009; Larsson et al., 2011; Smith & Anderson, 1986).



**Figure 2.10.** Post-exercise fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) when humid air [100% relative humidity (RH)] and dry air (0% RH) of different temperature were inhaled during exercise. Red box highlights that the inhalation of hot dry air can cause bronchoconstriction. Adapted from Deal et al., (1979b).

### 2-3.2 Osmotic Theory

The osmotic theory suggests that evaporative water loss and the subsequent increase in osmolarity of the ASL during the conditioning of inspired air is the main stimulus for EIB (Anderson et al., 1982). According to the mathematical model developed by Daviskas and colleagues (1990), a significant amount of water loss can occur in generations 8-10 of the airways when ventilation levels  $>60 \text{ l}\cdot\text{min}^{-1}$  are reached in temperate conditions [ $26.7^{\circ}\text{C}$  and 36.5% (Figure 2.11)]. During strenuous exercise, ventilation can reach values of  $200 \text{ l}\cdot\text{min}^{-1}$  or above (particularly in endurance-trained athletes), thus the need to heat and humidify inspired air is greater, and respiratory water loss would extend distally. Evidence of reduced mucociliary clearance (MCC) following hyperpnoea with dry air (Daviskas et al., 1995) supports a loss of ASL during exercise. The consequence of reduced ASL would be an increase in its osmolarity. An increase in the osmolarity of the ASL has been documented following hyperventilation of dry air in animal models (Freed & Davis, 1999). Similarly, in human models, the osmolarity of nasal secretions has been shown to increase following nasal inhalation of cold dry air (Togias, Proud, Lichtenstein, Adams, Normal, et al., 1988). There is therefore good support for a reduction in ASL and increase in ASL osmolarity following periods of hyperpnoea of dry air.



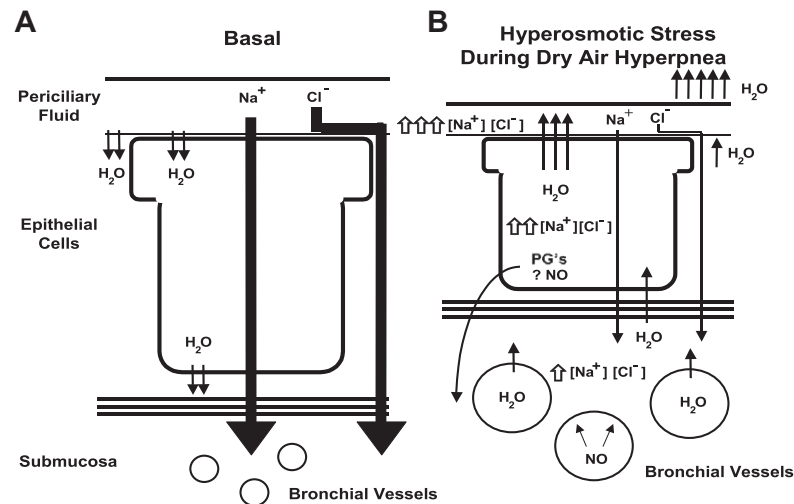
**Figure 2.11.** Cumulative loss of water past the trachea after 4 min of exercise, under inspiratory conditions of 26.7°C and 8.8 mg·l<sup>-1</sup> (approx. 36.5% relative humidity) at a ventilation rate of 60 l·min<sup>-1</sup>. From Daviskas et al., (1991).

According to the osmotic theory, evaporative water loss from the airway surface results in an increase in the concentration of Na<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>++</sup> and K<sup>+</sup> ions in the ASL, thus increasing its osmolarity. As demonstrated in Figure 2.12, fluid would then move from nearby cells across the osmotic gradient towards the airway surface, resulting in cell shrinkage and in release of intracellular mediators (Eveloff & Warnock, 1987). This fluid shift and concurrent release of mediators is believed to occur from a variety of cells located at, or around the airway surface, including epithelial cells and inflammatory cells, such as mast cells and eosinophils. Inflammatory cells have been the focus of much attention due to the potent constrictor effects of their mediators (O'Byrne, 1997). It is the action of these mediators on the airway smooth muscle that is thought to cause

bronchoconstriction in susceptible individuals. However, the exact mode of action of these mediators and their interplay remains unclear; hence one of the focal points of this thesis will be to decipher the role of inflammatory mediators in EIB.

Observations that the severity of EIB is associated with the water content of the inspired air (Strauss, McFadden, Ingram, Deal, & Jaeger, 1978), that EIB can be blocked in individuals with severe asthma with the inhalation of fully conditioned air (Anderson, Daviskas, Schoeffel, & Unger, 1979) and that hyperosmotic aerosols can mimic EIB (Smith & Anderson, 1986), all implicate local airway dehydration and subsequent increase in airway osmolarity as the main determinant for EIB (Anderson & Daviskas, 2000).

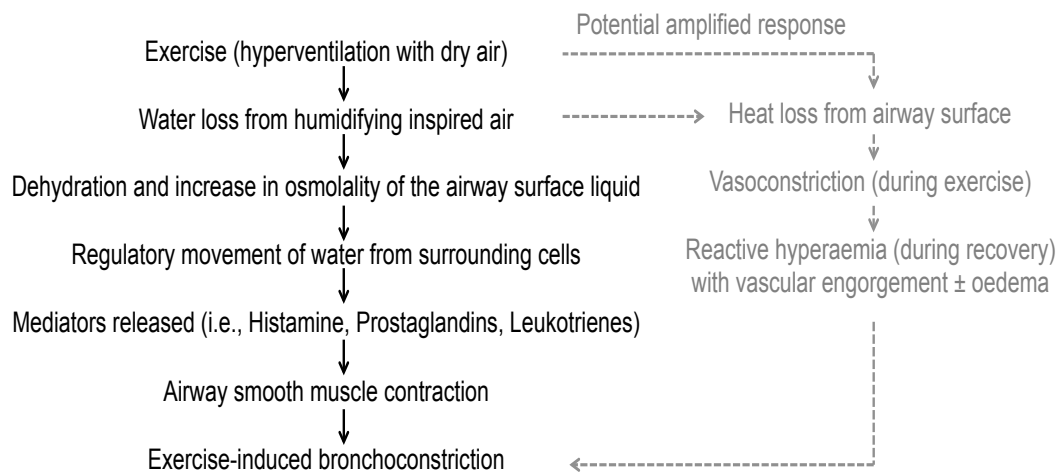
Whilst the effects of local airway dehydration in EIB have been investigated in depth, little attention has been given so far to the effects of whole-body dehydration. This is surprising given that whole-body dehydration can increase plasma osmolarity (Cheuvront & Kenefick, 2014) and may therefore interfere with the volume and composition of the ASL. Furthermore, athletes are at increased risk for both, whole-body dehydration following exercise (Rüst, Knechtle, Knechtle, Wirth, & Rosemann, 2012; Whiting, Maughan, & Miller, 1984) and EIB. Whether both phenomena (i.e., whole-body and local airway dehydration) are inter-related and occur concomitantly remains to be established.



**Figure 2.12.** Epithelial cells and ion transport under basal conditions (A) and hyperosmotic stress during dry air hyperpnoea (B). Under basal conditions,  $\text{Na}^+$  ions are absorbed via an apical sodium channel, and  $\text{Cl}^-$  ions move paracellularly. Under basal conditions, water moves into the epithelial cells and submucosa due to the osmotic gradient created by the movement of these ions. During hyperpnoea, evaporative water loss reduces the periciliary fluid layer and increases the ion concentration, which creates an osmotic stimulus for water to move out of the epithelial cells. As a result, the epithelial cells shrink, creating an osmotic stimulus for water to move from the submucosa. Hyperosmolarity of the epithelial cells and the submucosa is a possible stimulus for the release of nitric oxide (NO) and prostaglandins (PGs). From Anderson & Daviskas (1999)

### 2-3.3 Unifying theory

Currently, the most widely accepted theory for the pathophysiology of EIB is that presented by Anderson & Daviskas (2000). Due to a large number of observations that remain unexplained by the thermal theory, the unifying theory of EIB is heavily influenced by osmotic factors, but it does also acknowledge a possible thermal influence (Anderson & Daviskas, 2000). This theory suggests that it is the osmotic events (as described in the previous section) that are responsible for EIB and that the thermal events (i.e., reactive hyperaemia of the microvasculature) may amplify the airway response (Anderson & Daviskas, 2000). A flow chart of this unifying theory is presented in Figure 2.13.



**Figure 2.13.** The unifying theory of exercise induced-bronchoconstriction as proposed by Anderson and Daviskas (2000).

The only significant update to the unifying theory since its initial proposal in 2000 is the addition of a possible role of the sensory nerves. When activated, sensory nerves release tachykinins, which can cause smooth muscle contraction and mucus secretion. Animal models have demonstrated that the antagonism of neurokinin receptors can inhibit bronchoconstriction by 50% (Freed, McCulloch, Meyers, & Suzuki, 2003). In human models, an inhaled formula of the neurokinin antagonist FK-888 agent did not significantly alter the severity of EIB, but did enhance the speed of recovery from bronchoconstriction (Ichinose et al., 1996). The activation of sensory nerves in animal models of EIB is likely mediated by inflammatory mediator release (Freed et al., 2003; Lai & Lee, 1999). In humans the levels of the cysteinyl leukotrienes (cyst-LTs) and the neuropeptide neurokinin-A (a marker of sensory nerve activation) in induced sputum correlate following exercise in individuals with EIB (Hallstrand, Debley, Farin, & Henderson, 2007); this suggests that sensory nerves are activated by, or alongside inflammatory mediator release.

### **Summary**

That hyperpnoea-induced local airway dehydration, with subsequent increase in osmolarity of the ASL, is the main stimuli for EIB is widely accepted. What remains to be established is whether exercise-induced whole-body dehydration accelerates the process and may, therefore, amplify the airway narrowing.

Moreover, the effect of airway water loss on inflammatory mediator release in individuals with EIB remains to be clarified.

## **2.4 Inflammatory mediators in EIB**

The main inflammatory cells implicated in EIB are eosinophils and mast cells (Parsons et al., 2013). These inflammatory cells contain bioactive substances that are believed to instigate many of the features of EIB. In this section, evidence of an involvement of various inflammatory cells in EIB will be provided and prevention strategies for blocking inflammatory cell release will be proposed.

### **2-4.1 Inflammatory cells**

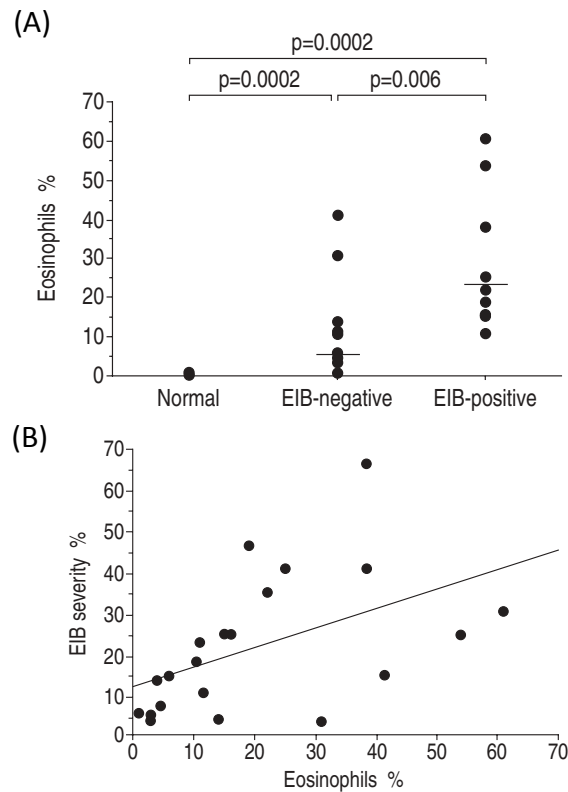
#### ***Eosinophils***

Eosinophils are pro-inflammatory white blood cells that are implicated in numerous inflammatory processes. Eosinophilic inflammation is a hallmark of asthma and it may also contribute to EIB. The association between eosinophils and asthma/EIB is illustrated in a study by Yashikawa and colleagues (1998). The authors demonstrated that individuals with asthma had a significantly higher percentage of eosinophils in induced sputum than healthy controls (Figure 2.14). Furthermore, it was noted that individuals with both asthma and EIB had a significantly greater percentage of eosinophils in induced sputum than individuals with asthma alone, and that the percentage of eosinophils in induced sputum correlated with the severity of bronchoconstriction following exercise (Figure 2.14). These initial observations have recently been confirmed by several other research teams (Duong, Subbarao, Adelroth, Obminski, Strinich, et al., 2008; Hallstrand, Moody,



Aitken, & Henderson, 2005a) and implicate eosinophilic inflammation in both asthma and EIB.

Interestingly, an increased number of eosinophils has also been noted in the airways of athletes. Indeed, Karjalainen and colleagues (2000) examined lung biopsies from cross country skiers and compared the results to biopsies from patients with mild asthma and to healthy controls. The authors noted that the number of eosinophils in the lung biopsies of cross country skiers was twice that of the healthy controls; this number remained however lower compared to individuals with asthma (Karjalainen et al., 2000). An increase in eosinophil and mast cell numbers has also been reported in lung biopsies of competitive swimmers (Bougault et al., 2012). These results are consistent with the presence of chronic inflammation in the airways of athletes.



**Figure 2.14.** (A) Comparison of eosinophils (%) in induced sputum of healthy controls and individuals who have asthma with or without exercise-induced bronchoconstriction (EIB). (B) A significant correlation was noticed between EIB and the sputum eosinophil percentage ( $r=0.59$ ,  $P=0.009$ ). From Yoshikawa et al., (1998).

### ***Mast Cells***

Mast cells are pro-inflammatory cells that play a key role in the inflammatory process. The cytoplasm of mast cells contains large amounts of granular material that have a multitude of physiological effects. An increase in mast cell density in the airways is usually noticed in individuals with asthma. Carroll and colleagues (2002) examined lung biopsies taken from autopsies of individuals with and without asthma, and observed an increase in mast cell density in the individuals who

suffered with the condition. In the same study, the authors noted that the density of mast cells in the airways increased peripherally, and that mast cells are more densely populated on smooth muscle cells and mucosal glands (Carroll et al., 2002). The increased density of mast cells in individuals with asthma supports a role of those cells in the pathophysiology of the disease, and their proximity to airway smooth muscle and mucosal glands is in keeping with their function as sensory cells (Dahlén & Kumlin, 2004).

Evidence for the role of mast cells in EIB originally came from pharmacological studies that demonstrated that mast cell stabilising agents reduce the severity of bronchoconstriction following exercise. A study by Patel and colleagues (1982) demonstrated a dose response improvement in the severity of bronchoconstriction following exercise with the administration of the mast cell stabilising agent sodium cromoglycate. Subsequent studies have confirmed the broncho-protective effects of mast cell stabilisers. A Cochrane review, published in 2002 that pooled 17 investigations, suggests the mast cell stabilising agent nedocromil sodium reduced the post-exercise fall in FEV<sub>1</sub> by 16% (Spooner, Saunders, & Rowe, 2002). These results offer indirect evidence for the role of mast cells in EIB. As presented in the following section, direct evidence is also available through published data on the measurement of bio-markers of airway inflammation during asthma attacks or EIB.

## 2-4.2 Inflammatory mediators

### *Histamine*

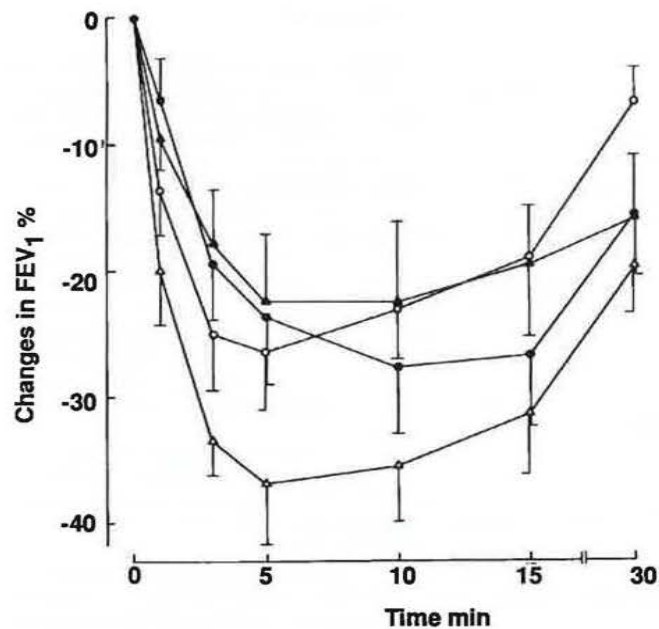
Histamine is a mediator released from a variety of inflammatory cells within the body, these include; mast cells, eosinophils and basophils. Histamine is a potent constrictor of airway smooth muscle and, as such, can be used for bronchial provocation challenge. Histamine was the first mediator to be directly measured during the occurrence of EIB. In the late 1970s and early 1980s many studies investigated the effect of exercise on plasma and serum histamine levels. The findings of ten early studies on the topic were reviewed by Ind and colleagues (1983). The authors revealed contradictory results regarding the impact of exercise on histamine levels and no consensus was reached as to whether individuals with asthma displayed a greater increase in histamine concentrations compared to individuals without asthma.

In the same review article, Ind and colleagues (1983) raised concerns about some of the methodological practises regarding histamine sampling from plasma and serum. The authors proposed that results of previous studies that had used histamine as a marker of inflammatory mediator release could be confounded by the disruption and subsequent release of histamine from basophils during the sampling process (Ind et al., 1983). Furthermore, the authors suggested that any differences in the histamine levels between individuals with and without asthma may simply reflect the differences in the leakage of histamine from basophils between the two groups [as demonstrated *in vitro* by Findlay & Lichtenstein (1980)]. In line with the idea

that exercise-induced basophilia may be responsible for post-exercise histamine release, Howarth and colleagues (1984) demonstrated that an increase in basophils occurs post-exercise in both, individuals with and without asthma. Therefore conclusions drawn from early investigations using histamine as a marker of inflammatory mediator release should be made with caution.

Due to the methodological problems arising with the direct measurement of histamine in bodily fluids, much of the evidence of a role of histamine in EIB has been provided by pharmacological studies. Many such studies have shown that treatment with histamine antagonists can reduce the severity of bronchoconstriction following exercise (Finnerty & Holgate, 1990; Patel, 1984), or reduce the sensitivity of the airways following bronchial provocation with mannitol or AMP (Brannan et al., 2001; Currie, Haggart, Lee, et al., 2003b). Finnerty and colleagues (1990) demonstrated that the histamine antagonist terfenadine was able to reduce the severity of bronchoconstriction following exercise by 34%. Interestingly, the authors observed that the protective effect of anti-histamines was most prominent at 5 min following the exercise challenge (Figure 2.15). This is in contrast to the prostaglandin (PG) inhibitor, flurbiprofen, that reduced the severity of EIB by 31% but had most of its effect at 30 min of recovery [Figure 2.15, (Finnerty & Holgate, 1990)]. Similarly, Currie and colleagues (2003b) noted that the administration of the anti-leukotriene medication, montelukast, in combination with the anti-histamine drug, desloratadine, reduced the sensitivity of the airways to mannitol compared to montelukast alone. However both montelukast alone and

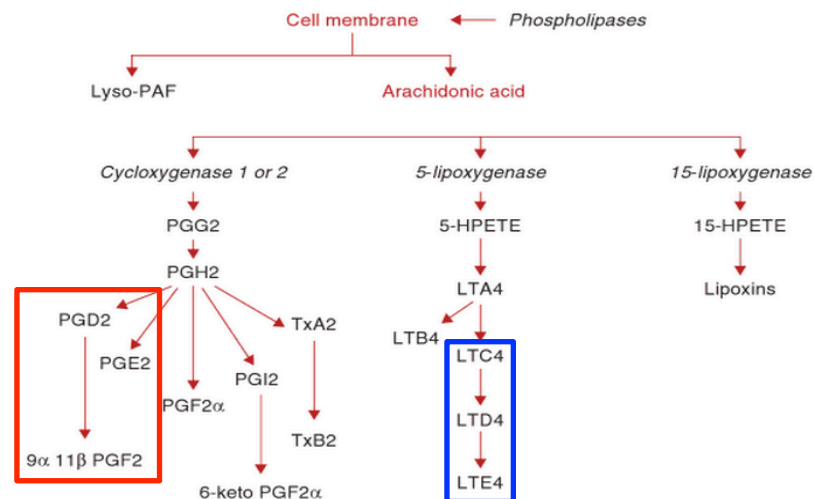
in combination with desloratadine increased the speed of recovery from bronchoconstriction compared to a placebo. These findings suggest that histamine is an important mediator in initiating the broncho-constrictor response and that other mediators are responsible for sustaining the bronchoconstriction.



**Figure 2.15.** Percentage fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) over 30 min following bronchial provocation with pre-treatment with: placebo (open triangles), terfenadine (closed triangles), fluribiprofen (open circles) and fluribiprofen plus terfenadine (closed circles). Data suggests that histamine is an important mediator in initiating the bronchoconstrictor response to exercise and that other mediators may be responsible for sustaining the bronchoconstriction. From Finnerty & Holgate, (1990).

## ***Leukotrienes (LTs)***

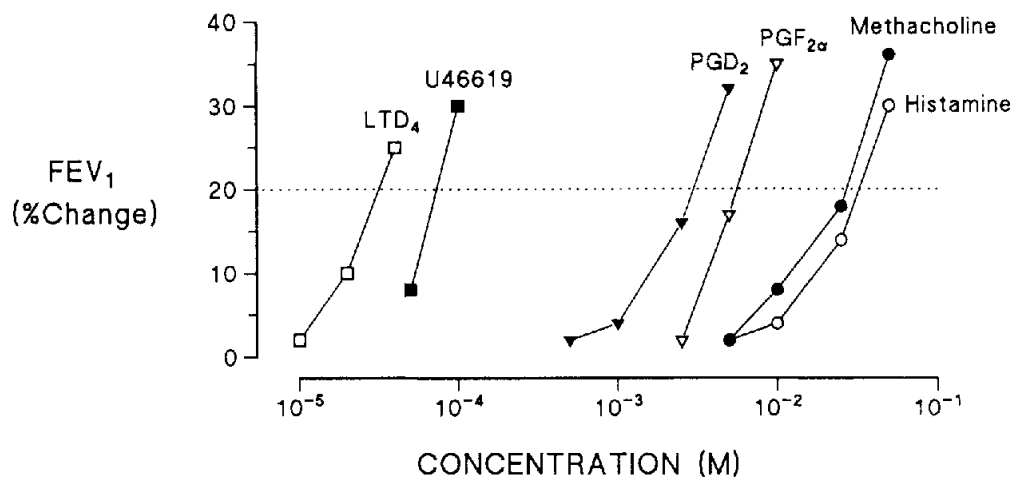
LTs are part of a group of compounds called eicosanoids. The main LTs measured in relation to asthma/EIB are the cysteinyl leukotrienes (cyst-LT) LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>. These mediators are formed from the phospholipid membranes of inflammatory cells, such as eosinophils, basophils and mast cells [Figure 2.16 (Rodger, Botting, & Dahlén, 1998)]. Following stimulation of these inflammatory cells, arachidonic acid is formed from phospholipid cell membranes by the action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes. The intermediate LTA<sub>4</sub> is formed from the action of 5-lipoxygenase (5-LO) on arachidonic acid. The cyst-LTs, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> are then formed from LTA<sub>4</sub> by LTC<sub>4</sub> synthase (Rodger et al., 1998). These cyst-LTs act on the cyst-LT<sub>1</sub> receptors within the airways and have been implicated in a multitude of physiological processes (Rodger et al., 1998).



**Figure 2.16.** Eicosanoid biosynthesis from arachidonic acid. The main eicosanoids studied in relation to exercise-induced bronchoconstriction are the cysteinyl leukotrienes, i.e., LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> (highlight in the blue box), and the prostaglandins, i.e., PGD<sub>2</sub>, PGE<sub>2</sub> and the PGD<sub>2</sub> metabolite 11β-PGF<sub>2</sub>α (highlighted in the red box). Their formation is described in the text. Adapted from O’Byrne (2009).

Cyst-LT have been implicated in smooth muscle contraction, mucus secretion, microvascular permeability, airway inflammation and proliferation and remodelling of airway smooth muscle (Hallstrand & Henderson, 2009). Cyst-LTs are potent constrictors of airway smooth muscle; indeed LTD<sub>4</sub> has been shown to be 1000 times more potent than histamine at causing smooth muscle contraction [Figure 2.17 (Adelroth, Morris, Hargreave, & O'Byrne, 1986)]. Furthermore the duration of action of LTC<sub>4</sub> and LTD<sub>4</sub> is approximately 2-3 times longer than that of histamine (Barnes, Piper, & Costello, 1984). Cyst-LTs may also contribute to airway obstruction by initiating mucus secretion and increasing microvascular permeability. *In vitro* preparations of the human airways displayed a dose response increase in mucous glycoprotein production following exposure to LTC<sub>4</sub> and LTD<sub>4</sub> (Marom, Shelhamer, Bach, Morton, & Kaliner, 1982). This is in line with *in vivo* observations that the number of cyst-LTs in induced sputum correlated with MUC5AC (a mucin protein) following exercise in individuals with EIB (Hallstrand et al., 2007). Finally cyst-LTs have been implicated in the infiltration of inflammatory cells into the airways, which may perpetuate EIB. Laitinen and colleagues (1993) noted an increase in the number of eosinophils and neutrophils in mucosal biopsies 4 h after the inhalation of LTE<sub>4</sub>, suggesting that LTE<sub>4</sub> may initiate airway inflammation. Taken together there is strong evidence for the role of cyst-LTs in a range of deleterious physiological processes associated with asthma/EIB.





**Figure 2.17.** The airway response to the inhalation of a variety of bronchoconstrictor mediators in one asthmatic participant. Data shows that the leukotriene mediator LTD<sub>4</sub> is approximately 1000 times more potent than histamine and that the prostaglandin mediator PGD<sub>2</sub> is approximately 10 times more potent than histamine. From O'Byrne (1994).

Urinary excretion of LTE<sub>4</sub> is often used as a marker of cyst-LT production. *In vitro* LTC<sub>4</sub> is rapidly metabolised almost exclusively to LTE<sub>4</sub> with no further conversion (Kumlin & Dahlén, 1990). Thus, LTE<sub>4</sub> appears to be the end product of cyst-LTs in the lung and, as such, is a useful marker of cyst-LT production. An early demonstration of an increase in urinary LTE<sub>4</sub> following exercise was documented by Kikawa and colleagues (1992). Urine samples from 13 children with asthma and 10 healthy children were collected before and after 6 min of high intensity exercise on a treadmill. Results revealed that urinary LTE<sub>4</sub> levels increased by 150% in the children with asthma, in contrast to no change in the healthy children (Kikawa et al., 1992). Since then, many investigations have demonstrated an increase in urinary LTE<sub>4</sub> following various provocation challenges, including: exercise

(Mickleborough, Murray, Ionescu, & Lindley, 2003; Reiss et al., 1997), EVH (Kippelen et al., 2010a; 2010b) and mannitol (Brannan et al., 2003; 2006; Larsson et al., 2011).

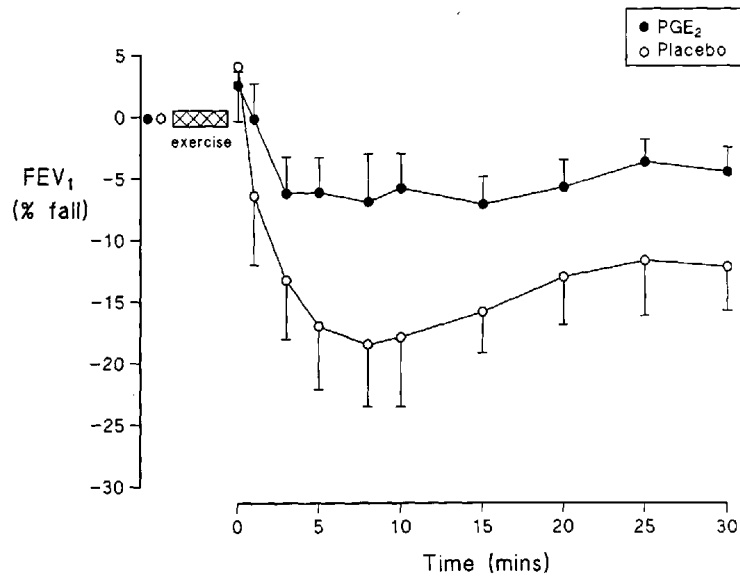
The first pharmacological trial that demonstrated efficacy of LT antagonists in preventing EIB was conducted by Israel and colleagues (1989). Following a fortnight of treatment with the LT antagonist LY17883, the stimulus required to cause a 20% fall in FEV<sub>1</sub> following isocapnic hyperpnoea was increased by 25% (Israel et al., 1989). Following this initial observation, further studies were conducted with the results demonstrating similar, but incomplete, broncho-protective effects (Finnerty, Wood-Baker, Thomson, & Holgate, 1992; Makker, Lau, Thomson, Binks, & Holgate, 1993; Robuschi et al., 1992). A pharmacological study that combined a cys-LT<sub>1</sub> receptor antagonist with a histamine antagonist demonstrated that the fall in FEV<sub>1</sub> following exercise was attenuated by only 54% in individuals with asthma and mild-to-moderate EIB (Hallstrand, Moody, Wurfel, Schwartz, Henderson, et al., 2005b); this incomplete inhibition of EIB indicates that other mediators are involved in EIB.

### ***Prostaglandins (PG)***

Prostaglandins, like LTs, are part of the eicosanoid family. Their formation differs from LTs in that arachidonic acid is broken down by cyclooxygenase enzymes (COX<sub>1</sub> and COX<sub>2</sub>) to form PGG<sub>2</sub> and PGH<sub>2</sub> (Figure 2.16). Prostaglandin isomerases then produce the PGD<sub>2</sub> and PGE<sub>2</sub> [Figure 2.16 (O'Byrne (2009))]; these mediators and their metabolites have widely been studied in relation to EIB.

PGs, much like cyst-LTs, can affect airway smooth muscle tone and have been implicated in the infiltration of inflammatory cells into the airways. PGD<sub>2</sub> and its metabolite 11β-PGF<sub>2α</sub> can cause airway smooth muscle contraction (Beasley, Varley, Robinson, & Holgate, 1987; Hardy, Robinson, Tattersfield, & Holgate, 1984). PGD<sub>2</sub> has been shown to be 30 times more potent than histamine at causing smooth muscle contraction [Figure 2.17 (Hardy et al., 1984)]. Further, similar to LTE<sub>4</sub>, PGD<sub>2</sub> may perpetuate airway inflammation by initiating migration of eosinophils to the airways (Emery, Djokic, Graf, & Nadel, 1989).

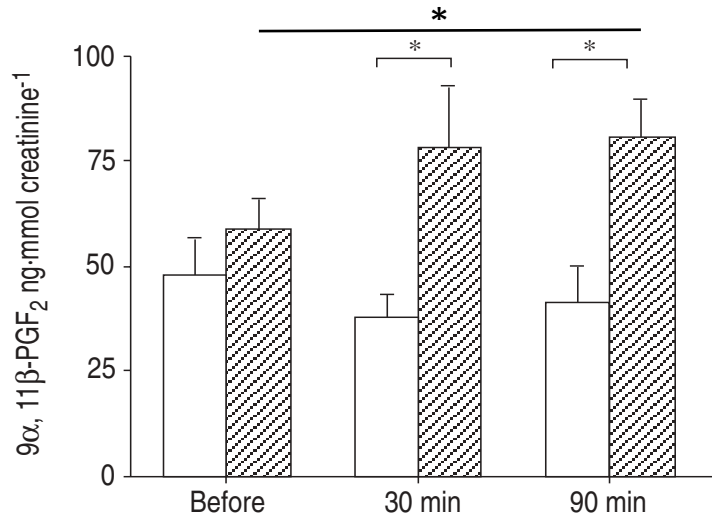
A protective prostaglandin, PGE<sub>2</sub>, has also been identified. The inhalation of PGE<sub>2</sub> 30 min prior to an exercise challenge has been shown to reduce the fall in FEV<sub>1</sub> from around 26% to 10%, and to significantly reduce the duration of bronchoconstriction [Figure 2.18 (Melillo, Woolley, Manning, Watson, & O'Byrne, 1994)].



**Figure 2.18.** The fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) after treatment with inhaled PGE<sub>2</sub> or a placebo in individuals with asthma and exercise-induced bronchoconstriction. Data demonstrates that PGE<sub>2</sub> significantly attenuated the magnitude of EIB and shortened the recovery time. From Melillo et al., (1994).

In humans, only mast cells produce PGD<sub>2</sub> in significant quantities (O'Sullivan, 1999). PGD<sub>2</sub> and its metabolite, 11β-PGF<sub>2α</sub>, can therefore be utilised as a specific marker of mast cell activation (O'Sullivan, 1999). This is in contrast to LTs which may also be produced from eosinophils (Moloney, Griffin, Burke, Poulter, & O'Sullivan, 2003), and PGE<sub>2</sub> that can be produced from airway epithelial cells (Stuart-Smith & Vanhoutte, 1988). The urinary measurements of 11β-PGF<sub>2α</sub> is currently considered the most sensitive marker of mast cell activation *in vivo* (Dahlén & Kumlin, 2004). It is therefore a logical choice for investigations assessing mast cell mediator release and for pharmacological investigations of mast cell stabilising treatments (such as done in this thesis).

The first investigation using  $11\beta$ -PGF<sub>2α</sub> as a marker of mast cell activation in EIB was conducted by O'Sullivan and colleagues (1998). The authors measured urinary  $11\beta$ -PGF<sub>2α</sub> before and after 5 min of heavy cycling in individuals with asthma. The authors noted that individuals with EIB, defined by a post-exercise reduction in FEV<sub>1</sub> of >15% FEV<sub>1</sub>, displayed a significantly higher post-exercise  $11\beta$ -PGF<sub>2α</sub> concentration than individuals without EIB (Figure 2.19). Similar observations of an increase in urinary  $11\beta$ -PGF<sub>2α</sub> following exercise have been documented by other teams (Mickleborough et al., 2003; Nagakura et al., 1998). Furthermore, increases in urinary  $11\beta$ -PGF<sub>2α</sub> have also been detected when EVH (Kippelen, Larsson, Anderson, Brannan, Dahlén, et al., 2010b; Kippelen et al., 2010a) and mannitol (Brannan et al., 2003; Larsson et al., 2011) were used as surrogates for exercise. That bronchial provocation with hypertonic solutions can increase the urinary excretion of inflammatory mediators implicates a hyperosmotic environment as a stimulus for inflammatory mediator release.



**Figure 2.19.** Urinary excretion of  $11\beta$ -PGF $_{2\alpha}$  (formally written as  $9\alpha,11\beta$ -PGF $_2$ ) in individuals with asthma with exercise-induced bronchoconstriction (EIB) (hatched bars) or without EIB (empty bars). Adapted from O'Sullivan et al., (1998).

Due to the potent broncho-constrictive effects of  $LTE_4$  and  $PGD_2$ , research into inflammatory mediator release in EIB has focused predominantly on these two mediators. The most common method of assessing inflammatory mediator release, enzyme-linked immunosorbent assays (ELISA), is limited to analysing a single mediator at a time; consequently, inflammatory mediator release is often measured in isolation. However, understanding the kinetics of release of a wide range of mediators, as well as their interactions is important for the future development of new targets for treating asthma and EIB. Technological advancement in mass spectrometry has opened new opportunities in the quantitative measurement of small endogenous metabolites in biological fluids.

At the time of printing this thesis, our collaborators at the Karolinska Institutet (Stockholm) have just released their first results of urinary mediator release post-

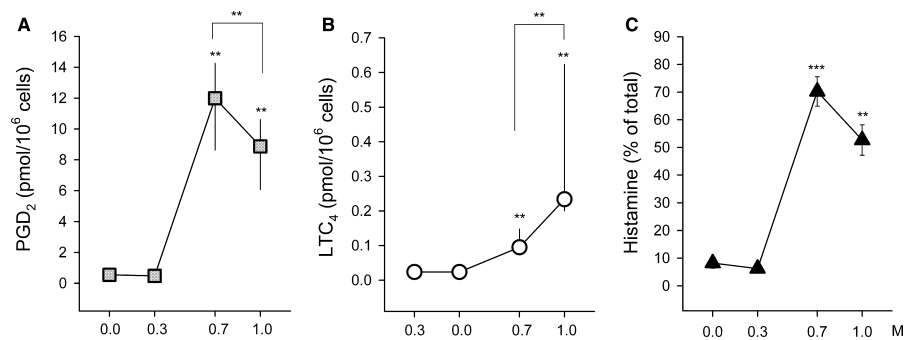
bronchial provocation with dry air based on ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). For the first time, Bood and colleagues (2015) were able to demonstrate that, alongside the commonly reported excretion of the broncho-constrictive mediators cystLTs and PGs, the broncho-protective mediators PGE<sub>2</sub> and PGI<sub>2</sub> are released in response to dry air hyperpnoea in patients with asthma (Bood et al., 2015). There is now need to confirm these findings and to further investigate the interplay of lipid mediator release in response to bronchial provocation with exercise.

### **2-4.3 Inflammatory mediator release**

The release of inflammatory mediators in 'classic', allergic asthma is caused by the immunoglobulin E (IgE)-mediated degranulation of inflammatory cells. Henceforth, asthma is more prevalent in atopic individuals than non-atopic individuals (Simpson et al., 2001). A large study of 5687 adults revealed that nearly half were atopic, and that atopy to house dust mite, cat, dog and mixed grasses were all independently associated with asthma (Simpson et al., 2001). EIB, however, seems to be equally prevalent in atopic and non-atopic individuals with asthma (Lee et al., 1984). Therefore, in EIB an alternative stimulus to IgE activation must be involved in inflammatory mediator release.

As suggested in the osmotic theory, a hyperosmotic environment is believed to be the stimulus of inflammatory mediator release (Anderson & Daviskas, 2000). *In*

*vivo*, evidence for this stems from numerous studies showing mediator release following hypertonic aerosol challenges (Anderson et al., 1997; Brannan et al., 2003; 2005; Holzer et al., 2003; Kersten et al., 2009; Larsson et al., 2011; Smith & Anderson, 1986). Furthermore, *in vitro* support comes from demonstrations that a hypertonic mannitol solution stimulates the release of cyst-LTs from eosinophils (Moloney et al., 2003), and that histamine, cyst-LTs and PGs are released from mast cells during an osmotic challenge [Figure 2.20 (Gulliksson, Palmberg, Nilsson, Ahlstedt, & Kumlin, 2006)]. That inhalation of fully conditioned air can prevent EIB (Anderson et al., 1979) is also in line with this idea, in that, by removing the osmotic stimulus, mediator release may be prevented and EIB may be blocked. However, direct evidence of an inhibition of inflammatory mediator release during warm-humid air breathing is still lacking.



**Figure 2.20.** Release of (A) Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), (B) Leukotriene C<sub>4</sub> (LTC<sub>4</sub>) and (C) Histamine from human mast cells after exposure to increasing doses of mannitol. From Gulliksson et al., (2006).



## 2-4.4 Preventing mediator release

As highlighted above, there is compelling evidence that inflammatory mediators contribute to the pathophysiology of asthma and EIB. There is, therefore, good rationale that the prevention of inflammatory mediator release would improve the prognosis of individuals with EIB.

Mast cell stabilising agents have been shown to reduce bronchoconstriction following various bronchial provocation challenges. Kippelen and colleagues (2010) demonstrated that the inhalation of a high dose (40 mg) of sodium cromoglycate reduced the fall in FEV<sub>1</sub> following EVH by 39% in athletes with EIB. In the same study, the authors demonstrated that the broncho-protection was associated with a reduction in the urinary excretion of 11β-PGF<sub>2α</sub> and LTE<sub>4</sub>. Similarly, following bronchial provocation with mannitol, a supra-therapeutic dose of sodium cromoglycate (40 mg) reduced the fall in FEV<sub>1</sub> by 61% and abolished the increase in urinary 11β-PGF<sub>2α</sub> post-challenge (Brannan et al., 2006). These data support the idea that the broncho-protective effect of cromones is mediated by a reduction in mast cell mediator release, and that inflammatory mediators are key in the development of asthma/EIB.

Inhaled corticosteroids (ICS) are a mainstay treatment for persistent asthma (GINA, 2014) and are commonly used to reduce the severity of EIB. The inhalation of a single, supra-therapeutic dose of fluticasone propionate (1 mg) has been shown to

attenuate bronchoconstriction following exercise by 50% in children with EIB (Thio et al., 2001). Similarly, a supra-therapeutic dose of inhaled beclomethasone (1500 µg of QVAR) has been shown to reduce the severity of bronchoconstriction following EVH by 34% and 44% in athletes with EIB and untrained individuals with asthma, respectively (Kippelen et al., 2010a). In the latter study, this broncho-protective effect was associated with the attenuation of the rise in urinary excretion of  $11\beta$ -PGF<sub>2α</sub> and LTE<sub>4</sub> following EVH. This indicates that supra-therapeutic doses of ICS may attenuate EIB by reducing inflammatory mediator release.

The GINA guidelines suggest that β<sub>2</sub>-adrenoceptor agonists should be prescribed to all individuals with asthma for use “as needed reliever” (GINA, 2014). The main mechanism of β<sub>2</sub>-adrenoceptor agonists is believed to be relaxation of the bronchial smooth muscle. However, β<sub>2</sub>-adrenoceptor agonists may also provide broncho-protection by preventing the release of inflammatory mediators (Howarth et al., 1985; Martin, Atkins, Dunskey, & Zweiman, 1980). Indeed, Church and Hiroi (1987) demonstrated *in vitro* that the β<sub>2</sub>-adrenoceptor agonist salbutamol significantly reduced the release of histamine and PGD<sub>2</sub> from human lung mast cells following an antigen challenge. Furthermore, the authors noted that the attenuation of mast cell mediator release with salbutamol was significantly greater than with the mast cell stabiliser agent sodium cromoglycate.

*In vivo*, a supra-therapeutic dose (24 µg) of the long-acting  $\beta_2$ -adrenoceptor agonist medication formoterol has been shown to reduce the release of  $11\beta$ -PGF<sub>2 $\alpha$</sub>  following bronchial provocation with a dry powder mannitol in individuals with asthma (Brannan et al., 2006). Similarly, a supra-therapeutic dose (50 µg) of the long acting  $\beta_2$ -adrenoceptor agonist salmeterol reduced the release of  $11\beta$ -PGF<sub>2 $\alpha$</sub>  following an aspirin-induced asthma challenge (Szczeklik et al., 1998). However, it is still unknown whether a single, therapeutic dose of  $\beta_2$ -agonist medication can provide a similar mast cell stabilising effect during exercise-induced bronchoconstriction.

## Summary

A hypertonic environment is believed to be the main stimulus for activation of inflammatory cells and for broncho-active mediator release during exercise, ensuing in acute bronchoconstriction in individuals with overly reactive airways. It is however not yet established whether removal of the hypertonic stimulus during exercise can inhibit inflammatory mediator release.

The prostaglandin D<sub>2</sub> metabolite 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  is currently considered the most sensitive *in vivo* marker of mast cell activation. Supra-therapeutic doses of pharmacological agents used in the treatment of asthma and EIB have been shown to reduce urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion; this supports the idea of mast cell activation in EIB. Whether mast cell blockade can be achieved with therapeutic doses of inhaled  $\beta_2$ -adrenoceptor agonists is currently unknown.

Whilst the broncho-constrictive agents PGD<sub>2</sub> and cyst-LTs are likely key players in EIB, a full profile of inflammatory mediator release post-bronchial provocation with exercise is still missing.

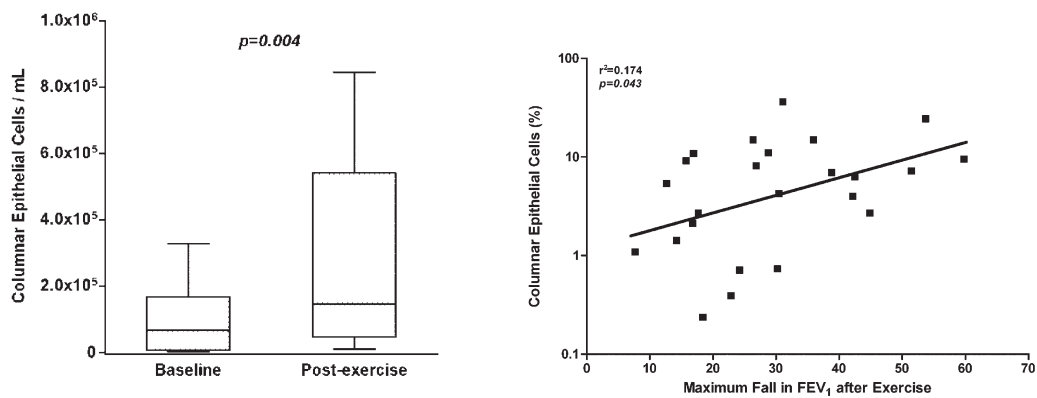
## **2-5 Aetiology of EIB in athletes**

As previously discussed, the prevalence of EIB/AHR is greater in athletes than in the general population, and athletes who participate in endurance events or compete in environments that contain noxious agents are at an increased risk for the condition. Anderson and Kippelen (2005) proposed that repeated injury and repair of the airway epithelium might be an important factor in the aetiology of EIB in athletes. In this section, investigations into epithelial injury will be discussed, followed by details of how repeated epithelial injury-repair might lead to the development of EIB/AHR. Finally, strategies aimed at preventing epithelial injury in athletes will be considered.

### **2-5.1 Airway epithelial injury**

One way to measure epithelial disruption is by examining the number and concentration of epithelial cells in induced sputum. Using this technique, Hallstrand and colleagues (2005a) demonstrated, in a resting state, an increased concentration of epithelial cells in induced sputum of individuals with asthma and EIB compared to individuals with asthma alone. Furthermore, the authors noted that individuals with asthma and EIB exhibited an increased number of eosinophils and an overproduction of  $\text{LTE}_4$  and relative underproduction in  $\text{PGE}_2$ . As  $\text{PGE}_2$  is produced by the airway epithelium (Stuart-Smith & Vanhoutte, 1988), the authors proposed that the underproduction of  $\text{PGE}_2$  could be mediated by the disruption of epithelial cells. In a follow-up study involving the individuals identified as having asthma and EIB, Hallstrand and colleagues (2005b) noted further epithelial disruption following

exercise (Figure 2.21). Moreover, in that population, the baseline concentration of epithelial cells in induced sputum correlated with the severity of bronchoconstriction following exercise (Figure 2.21). These studies indicate that individuals with asthma and EIB display epithelial disruption at baseline, and that exercise may exaggerate epithelial injury.



**Figure 2.21.** Concentration of columnar epithelial cells in induced sputum before and after exercise in individuals with asthma and exercise induced bronchoconstriction (EIB) (left). The relationship between the percentage of epithelial cells in induced sputum at baseline and the maximum fall in forced expiratory volume in 1 s (FEV<sub>1</sub>) following exercise (right). Data suggests that exercise increases epithelial disruption in individuals with EIB and that the severity of EIB is associated with epithelial disruption at baseline. From Hallstrand et al., (2005b).

Evidence that exercise-hyperpnoea can cause airway epithelial injury, independent of the presence of asthma/EIB, was first documented in animals. Davis and colleagues (2002b) demonstrated an abnormal amount of intraluminal debris in bronchoalveolar lavage fluid collected from Alaskan sled dogs 24-48 h after the completion of a multi-day, 1100 mile race across Alaska. Similarly, an increased number of epithelial cells were recovered in bronchoalveolar lavage fluid of race horses following a single bout of exercise in a cold environment (5°C) (Davis, Lockard, Marlin, & Freed, 2002a). Both of these studies involved exercise in a cold and dry environment, and dry air appears to be an important factor in epithelial injury. Indeed, mucosal damage of the canine bronchial tree caused by 5 min of exposure to high ventilation has been shown to be attenuated with warm (28°C - 34°C), humid air (98% - 100% RH) (Freed, Omori, Schofield, & Mitzner, 1994). These findings in animal models support the idea that the inhalation of dry air at high flow rates may induce dehydration injury of the airway epithelium.

Exercise-hyperpnoea *per se* (independent of the quality of the inhaled air) may also cause acute perturbations to the airway epithelium. Chimenti and colleagues (2010) observed a significant increase in the number of epithelial cells in induced sputum of athletes following completion of half-marathon races performed at different times of the year in varying environmental conditions (9.8°C and 7.4 mg H<sub>2</sub>O·l<sup>-1</sup> to 20.5°C and 10.3 mg H<sub>2</sub>O·l<sup>-1</sup>). Similar observations were made following runs varying in distance from 10 to 21 km, and in environmental conditions (from 10°C and 4.9 mg H<sub>2</sub>O·l<sup>-1</sup> to 27.5°C and 17.2 mg H<sub>2</sub>O·l<sup>-1</sup>) (Chimenti et al., 2009). An increase in

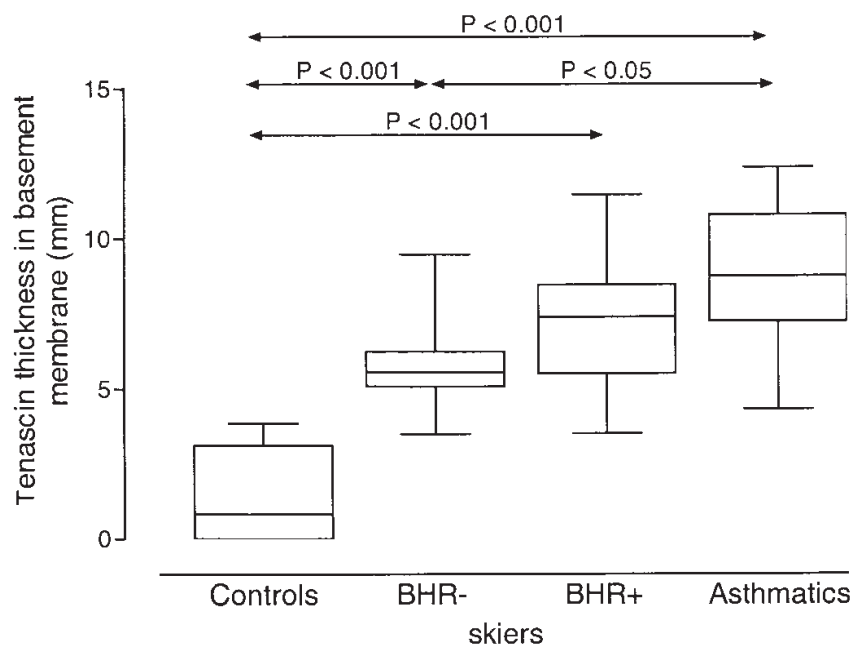
concentration of epithelial cells was also noticed following a laboratory-based 1000 m high intensity rowing challenge in athletes (Morici et al., 2004). In the latter investigation the concentration of epithelial cells correlated with the level of ventilation achieved during exercise (Morici et al., 2004). As previously discussed, respiratory water losses can occur during the inhalation of large volumes of dry air [Figure 2.11, (Daviskas et al., 1990)]; athletes achieving high levels of ventilation may therefore experience dehydration injury of the distal airways.

Direct evidence of repeated epithelial injury and repair in elite athletes come also from bronchial biopsy analysis, with evidence of airway remodelling observed in various athletic populations. Lung biopsies of elite cross-country skiers revealed an increased tenascin deposition on the basement membrane of the airways [Figure 2.22 (Karjalainen et al., 2000)]. Similarly, lung biopsies from elite swimmers obtained at rest displayed an increase in collagen thickness and tenascin deposition (Bougault et al., 2012). Swimmers, in contrast to cross-country skiers, breathe warm, moist air during exercise. It is therefore unlikely that epithelial injury in swimmers is a consequence of dehydration injury. Instead, it is generally accepted that chlorination by-products (such as tri-chloramines) contribute to airway epithelial injury in swimmers. In agreement with this idea, an animal study demonstrated that mice exposed to chlorine developed a patchy loss of epithelial cells, increased airway inflammation and increased reactivity to methacholine (Martin et al., 2003). Epithelial disruption in swimmers has, however, been noted in the absence of chlorine by products. Indeed, Carbonnelle and colleagues (2002) noted epithelial disruption in trained swimmers following exercise completed in



chlorinated and non-chlorinated swimming pools. The authors noted no such change in recreational swimmers and therefore proposed that the mechanical stress associated with high ventilation may also contribute to airway epithelial perturbations in swimmers.

Taken together these results indicate that repeated airway epithelial injury and repair may lead to airway remodelling in elite athletes, and that swimmers may experience epithelial injury in response to exposure to chlorination by products and/or due to the mechanical stress associated with high ventilation.



**Figure 2.22.** Tenascin thickness in the basement membrane of healthy individuals (Control), cross-country skiers with and without bronchial hyper-responsiveness (BHR) and individuals with asthma. Data demonstrates an increased tenascin thickness in cross country skiers and individuals with asthma. From Karjalainen et al., (2000).

The airway epithelium is constantly exposed to varying levels of mechanical stress and of shear stress throughout the cyclic deformation during the respiratory cycle. During exercise, as lung volumes increase and breathing frequency becomes more frequent, the mechanical and shear stress exerted on the airway epithelium is increased (Waters, Navajas, & Roan, 2011). The forces acting on the airway epithelium may also increase during bronchoconstriction. Indeed, the contraction of the airway smooth muscle can cause compressive stress on the airway epithelium (Waters et al., 2011). It is likely that the shear stress and compressive stress associated with exercise and/or bronchoconstriction may cause epithelial perturbation during exercise.

### **2-5.2 Non-invasive measurement of airway epithelial injury**

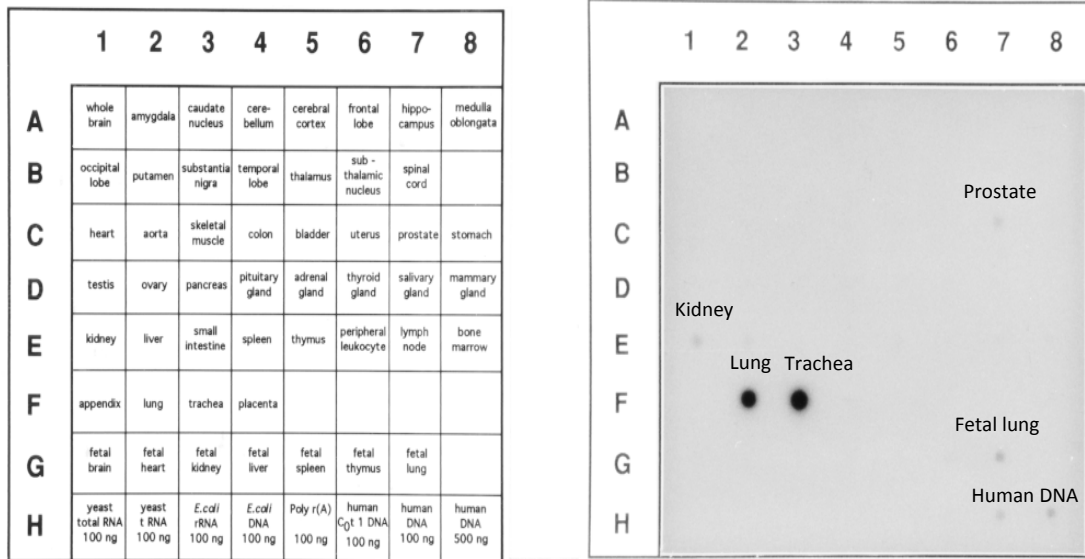
The composition of sputum may change during sputum induction (Gershman, Liu, Wong, Liu, & Fahy, 1999) and lung biopsies are invasive. As a consequence, neither method is suitable when repeated measurements are required over a relatively short period of time. Therefore, non-invasive techniques to measure epithelial injury have been developed to indirectly, but non-invasively, assess airway epithelial perturbation. In relation to exercise-induced epithelial injury, the measurement of club cell protein (CC16), formally known as Clara cell protein (Winkelmann & Noack, 2010), in extra pulmonary fluids is the most commonly employed method.

CC16 is secreted from the non-ciliated bronchiolar club cells located predominantly in the peripheral airways (Singh et al., 1988). The club cells are facultative progenitor cells and are able to alter their structure in response to cellular damage (Reynolds & Malkinson, 2010). In the presence of epithelial injury, club cells are able to proliferate and differentiate in order to maintain a ciliated epithelium (Reynolds & Malkinson, 2010). Club cells secrete CC16, which has been proposed to have anti-inflammatory properties. *In vitro* CC16 has been shown to inhibit phospholipase A2 (PLA<sub>2</sub>) activity (Lesur et al., 1995). Since PLA<sub>2</sub> is required to form eicosanoids (Figure 2.16), its inhibition could result in a reduction in the release of inflammatory mediators. CC16 may therefore have an important role in the regulation of airway inflammation.

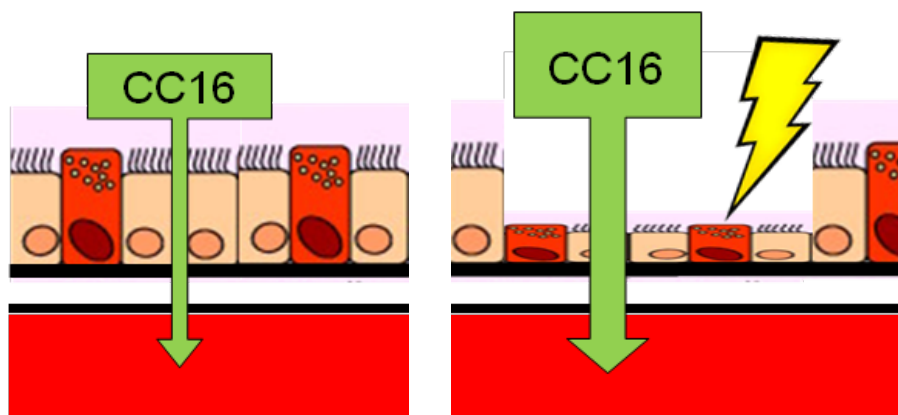
In humans CC16 is almost exclusively expressed in the airways. Indeed, a northern blot analysis of RNA from 50 human tissues demonstrated that, with the exception of a weak signal in the prostate and kidney (approx. 20 times less than in the lung), CC16 was only detected in the airways (Figure 2.23) (Hermans & Bernard, 1999). Due to the predominance of CC16 in the airways, an increase in the concentration of CC16 in extra-pulmonary fluids has been proposed to be used as a marker of epithelial disruption (Hermans & Bernard, 1999).

The idea behind the use of CC16 as a marker of airway epithelial disruption is that, with an intact epithelium, only a small amount of CC16 will passively diffuse into the bloodstream. When the epithelial barrier is compromised additional CC16 will leak into the bloodstream, where it can be measured non-invasively as a marker of epithelial barrier integrity (Figure 2.24). An example of the use of CC16 to assess epithelial perturbation is provided by Aarsalane and colleagues (2000). The authors demonstrated that when acute lung injury was induced in rats by infusion of lipopolysaccharide, the concentration of CC16 in the bronchoalveolar lavage fluid was reduced, and that this reduction was associated with a concurrent increase in serum CC16 concentration.

From the bloodstream, CC16 passes into the bladder *via* glomerular filtration (Doyle, Hermans, Bernard, Nicholas, & Bersten, 1998) and can then be measured non-invasively in urine. When appropriate precautions are taken, such as elimination of the first 100 ml to avoid prostate contamination and accounting for the rate of glomerular filtration (Andersson, Lundberg, & Barregard, 2007), changes in urinary CC16 concentrations can be used as a non-invasive marker of epithelial perturbation (Andersson et al., 2007; Hermans & Bernard, 1999).



**Figure 2.23.** Master dot plot indicating the presence of club cell protein (CC16) in 50 human tissues. Data suggests that CC16 is predominant in the airways, with small traces detected in the kidney and prostate. Adapted from Hermans and Bernard (1999).



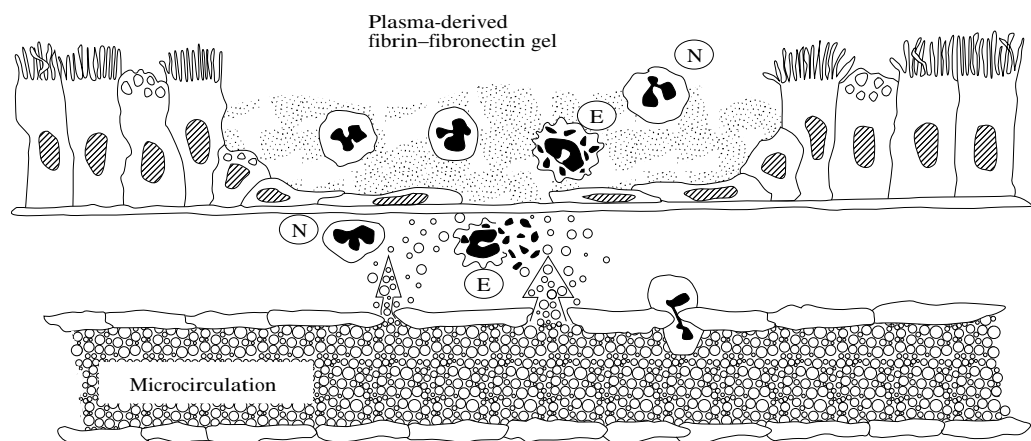
**Figure 2.24.** Representation of the concept of club cell protein (CC16) being used as a marker of epithelial integrity. With an intact epithelium (left), a small amount of CC16 passively diffuses into the bloodstream. In the presence of epithelial injury (right), a larger amount of CC16 enters the bloodstream.

A change in CC16 concentrations in extra-pulmonary fluids has been used following various sporting activities to support the occurrence of exercise-induced epithelial perturbation. Indeed, increases in CC16 has been shown, alongside an increase in epithelial cells in induced sputum, following a half marathon in amateur runners (Chimenti et al., 2010). An acute increase in urinary CC16 was also demonstrated in male athletes with and without EIB, following short-duration high-intensity exercise in a laboratory-controlled environment (4°C and 37% RH) (Bolger, Tufvesson, Anderson, et al., 2011a). Further, the adverse effects of hyperpnoea of dry air and of chlorination by-products on epithelial integrity is supported by demonstrations of an increase in urinary CC16 following EVH and swimming, respectively (Bolger, Tufvesson, Sue-Chu, et al., 2011b; Romberg, Bjermer, & Tufvesson, 2011). CC16 has been used to provide evidence that ozone exposure may also enhance airway epithelial damage. Broeckaert and colleagues (2000) demonstrated that ozone exposure increases serum CC16 in a dose-response manner following 2 h of cycling. As CC16 has been implicated in the modulation of airway inflammation (Jorens et al., 1995; Lesur et al., 1995), an increase in CC16 in extra-pulmonary fluids in athletes may also reflect an increase in CC16 production in response to airway inflammation. These studies therefore highlight the applicability of CC16 as a non-invasive marker of epithelial injury and/or inflammation in athletes.

### **2-5.3 Airway epithelial repair**

The repair process of the airway epithelium is likely to be initiated rapidly following injury. Using an *in vivo* animal model, Erjefalt and colleagues (1994) documented

the immediate responses to epithelial injury. The authors noted that the removal of epithelial cells from a 800  $\mu\text{m}$  wide section of guinea pig tracheas initiated prompt entry of bulk plasma from the microvasculature and caused the formation of an extracellular matrix rich in fibrin-fibroconectin gel across the damaged area [Figure 2.25 (Erjefält, Erjefält, Sundler, & Persson, 1994)]. Beneath this extracellular matrix evidence of epithelial cell flattening, proliferation, and migration was observed (Erjefält et al., 1994). In this study, the restoration of the damaged area was quick. Indeed, a patchy distribution of extracellular matrix fibres was evident at 10 min following epithelial injury, and the damaged area was completely covered by 30 min (Erjefält et al., 1994). *In vivo* human models support the quick restoration of epithelial barrier integrity. Indeed, in female athletes hyperpnoea-induced increases in CC16 have been shown to return to baseline values within 90 min post-challenge (Bolger, Tufvesson, Sue-Chu, et al., 2011b).



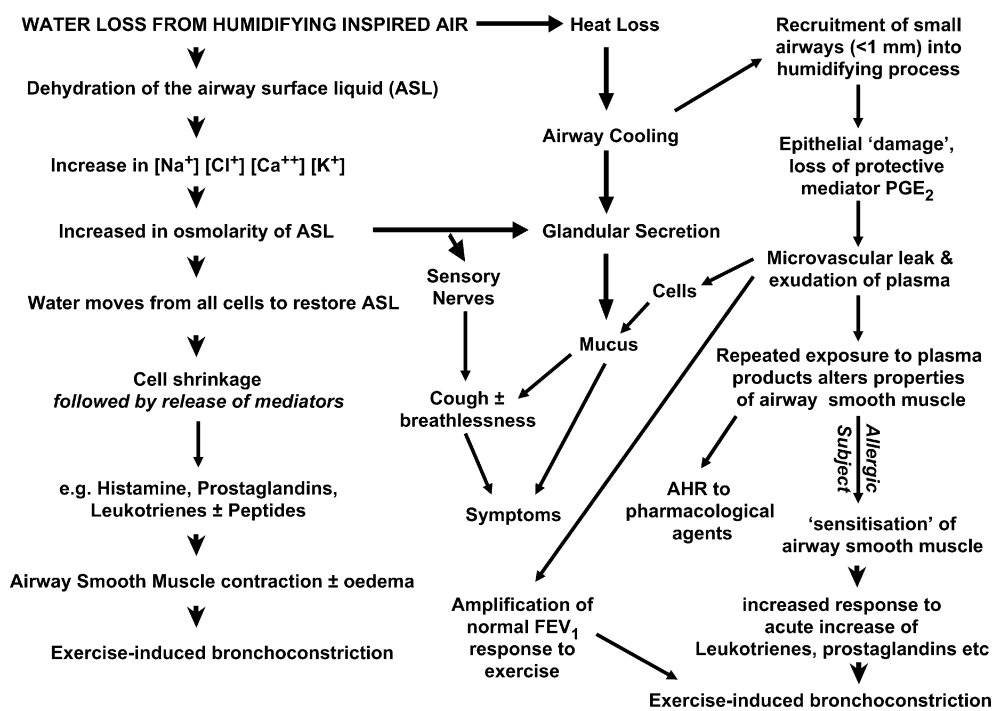
**Figure 2.25.** Illustration of the epithelial restoration process involving bulk plasma entry, formation of an extracellular matrix containing fibrin-fibroconectin gel across the damaged area and the flattening, proliferation and migration of epithelial cells. From Persson et al., (1996).

Importantly, it has been suggested that repeated epithelial injury in athletes may cause pathological changes in the airways that lead, over time, to the development of EIB/AHR [Figure 2.26 (Anderson & Kippelen, 2005)]. The extravasation of bulk plasma during acute phases of injury may expose the airway smooth muscle to cytokines and growth factors. Repeated exposure of the ASM to these substances may alter, in the long term, its contractile properties and increase its responsiveness (Anderson & Kippelen, 2005). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) contained in the plasma, is known to increase following exercise (Mickleborough et al., 2003; Pedersen, 2000). *In vitro* the responsiveness of ASM has been shown to increase following incubation with TNF- $\alpha$  (Anticevich, Hughes, Black, & Armour, 1995). Over time, repeated exposure to cytokines could increase the responsiveness of the ASM, leaving it hyper-responsive to various airborne substances (Anderson & Kippelen, 2005). This concept would explain the high prevalence of AHR in swimmers and in ice rink users who are repeatedly exposed to noxious inhaled agents (such as trichloramines from chlorinated swimming pools or fine and ultrafine particles emitted from ice-rink resurfacing machines) (Dickinson, 2005; Langdeau et al., 2000; Lumme et al., 2003; Mannix et al., 1999; Zwick et al., 1990).

The close proximity of mast cells to the ASM (Carroll et al., 2002) could lead to the passive sensitisation of the ASM to mast cell mediators in the presence of epithelial injury (Anderson & Kippelen, 2005). The sensitisation of the ASM to inflammatory mediators could particularly be enhanced in atopic individuals. Indeed, individuals



with season allergic rhinitis (hay fever) display increased levels of circulating LTs during the hay fever season (Cáp, Pehal, Chládek, & Malý, 2005); extravasation of bulk plasma during the injury-repair process in atopic athletes could therefore cause sensitisation of the ASM to LTs and similar circulating mediators (Anderson & Kippelen, 2005). Given that inflammatory mediators are released following bronchial provocation challenges, even in individuals without EIB (Brannan et al., 2003; Caillaud, Le Creff, Legros, & Denjean, 2003; Kippelen et al., 2010a; Mickleborough et al., 2003), it is possible to envisage that a gradual increase in responsiveness of the ASM could lead to the development of AHR/EIB. This concept is in keeping with the relatively late (i.e., past 25 yr of age) development of AHR/EIB witnessed in athletic populations (Fitch, 2006).



**Figure 2.26.** Acute events leading to EIB in subjects with asthma (left) and the events leading to the development of exercise-induced bronchoconstriction in athletes (right). From Anderson and Kippelen (2005).

## **2-5.4 Preventing dehydration injury of the airways**

To protect the long-term respiratory health of elite athletes and avoid progressive development of AHR/EIB, appropriate measures should be taken to reduce airway epithelial injury. These measures can include modifying the environmental conditions of practice/competition, or the use of pharmacological and non-pharmacological prevention strategies.

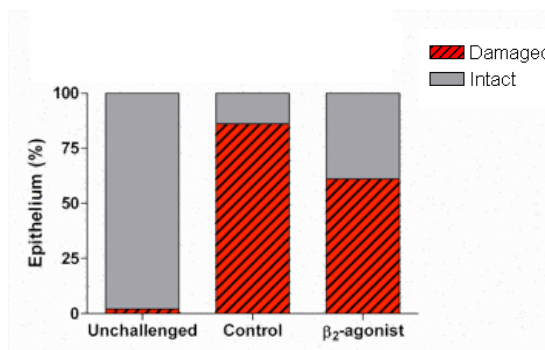
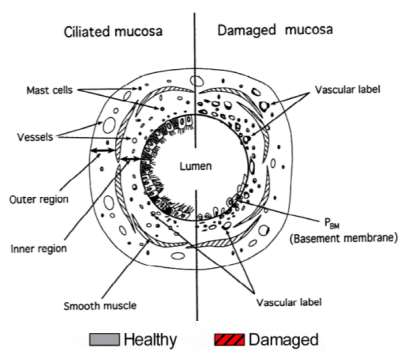
The most effective strategy for the prevention of airway epithelial injury is to remove the environmental stimulus responsible for the damage. In a laboratory-based study conducted in male athletes with and without EIB, it was demonstrated that an increase in temperature and humidity of the inspired air, from 4°C and 37% RH to 25°C and 94% RH, inhibited epithelial perturbation induced by 8 min of high intensity treadmill running (Bolger, Tufvesson, Anderson, et al., 2011a). Since athletes have limited options in the choice of environments in which they train, some sporting governing bodies have developed regulations in an attempt to protect athletes from adverse environmental effects during competitions. For example, the Federation Internationale de Ski (FIS) implemented a rule for the minimum temperature for cross-country ski competitions (FIS, 2013). Currently, the FIS states that a race must not begin if the air temperature at the coldest part of the track is <-20°C (FIS, 2013). However, this rule was not developed specifically to protect athletes against airway epithelial injury; therefore, respiratory water loss and dehydration injury of the epithelium are still likely to occur during competition held in cold and sub-freezing conditions (Sue-Chu, 2012). As athletes have little

control over the environmental conditions in which they train and compete, non-pharmacological and pharmacological intervention strategies may be more practical.

Non-pharmacological interventions may include nasal breathing, warm-up exercises or the use of heat and moisture exchange devices (Kippelen et al., 2012). Nasal breathing allows for the humidification of the inspired air *via* the nasal mucosa, which would reduce the demand of the lower airways to contribute to the conditioning process. However, during exercise, when ventilation exceeds 35-45 l·min<sup>-1</sup>, mouth breathing predominates in order to accommodate an increased tidal volume and respiratory frequency (Niinimaa et al., 1980; Saibene et al., 1978). Nasal breathing is therefore not practical during exercise. A warm-up may reduce epithelial injury by increasing bronchial blood flow, which may enhance the return of water to the airway epithelium at the start of exercise (Kippelen et al., 2012). However, the efficacy of a warm-up at attenuating epithelial injury is still unknown. The use of a heat and moisture exchange devices has also been proposed to prevent airway epithelial injury (Kippelen et al., 2012). These devices utilise exhaled heat and moisture to help condition the inspired air. These devices have been demonstrated to inhibit bronchoconstriction following exercise (Millqvist, Bake, Bengtsson, & Löwhagen, 1995); however, their efficacy of attenuating epithelial injury has not yet been tested. Furthermore, these devices may increase the work of breathing, which would make them undesirable for competition. Taken together the efficacy for non-pharmacological interventions to prevent epithelial injury is yet

to be established, and practicality issues in the context of sport performance need to be considered.

There is also limited data regarding the efficacy of pharmacological interventions at reducing airway epithelial injury. Wang and colleagues (1992) demonstrated that intravenous infusion of the short-acting  $\beta_2$ -agonist terbutaline reduced the number of epithelial cells in bronchial lavage fluid following a dry air challenge in anaesthetised canine airways. Similarly, the intravenous infusion of salbutamol reduced the damage of the canine bronchi by 30% following exposure to dry air [Figure 2.27, (Omori, Schofield, Mitzner, & Freed, 1995)]. The mechanism underlying the protective action of  $\beta_2$ -agonist is not fully understood, but might include ion-mediated water secretion towards the airway surface (Davis, Marin, Yee, & Nadel, 1979). These animal-based investigations suggest that short-acting  $\beta_2$ -agonist medication has the potential to inhibit dry air-induced epithelial injury, which may constitute a useful prevention strategy for athletes. However, the efficacy of inhaled short-acting  $\beta_2$ -agonist treatment to inhibit airway epithelial injury has yet to be demonstrated in humans.



**Figure 2.27.** (A) Schematic representation of bronchiole showing ciliated mucosa (intact) and damaged mucosa. Percentage of airway perimeter occupied by each mucosal category was used to determine epithelial injury. (B) Effect of infusion of salbutamol ( $\beta_2$ -agonist) and saline (control) on dry air induced epithelial injury. Percentage of airway perimeter categorised by presence of normal ciliated mucosa (intact; grey bar) and damaged mucosa (hatched bar). Adapted from Omori et al., (1995)

## Summary

Prophylactic administration of short-acting  $\beta_2$ -agonist medication is widely used to prevent acute post-exercise bronchoconstriction. Whether short-acting  $\beta_2$ -agonists, *via* ion mediated water secretion, may also impact on hyperpnoea-induced dehydration-injury within the airways – which is thought to contribute to long-term development of EIB in athletes – remains to be established.

The applicability of urinary CC16 as a marker of airway epithelial perturbation has been demonstrated in athletes. The quantification of urinary CC16 as a marker of epithelial barrier integrity allows for non-invasive, multiple time point, measurement of epithelial barrier integrity, and is therefore an ideal tool to investigate the effects of treatments on airway epithelial injury.

## **2-6 Aims and hypotheses**

Exercise-induced bronchoconstriction is highly prevalent in elite athletes and has been the subject of intense research over the past half a century. Despite this, our understanding of the pathophysiology of EIB in athletes remains incomplete. A better understanding of the pathophysiology EIB in athletes may improve the management of the condition and lead to the development of new preventative strategies. The general aims of this thesis were therefore to i) investigate factors that could influence the severity of EIB, ii) further our understanding of the pathophysiology of the condition and iii) identify treatment strategies that could improve the management and/or prevent the development of EIB in athletes.

### **2-6.1 Specific aims and hypotheses**

***Study 1 - Effect of whole-body dehydration on lung function and airway responsiveness in athletes.***

#### ***Aims***

- i) Determine the effect of whole-body dehydration on airway responsiveness to dry air in symptomatic, recreational athletes.
- ii) Determine whether whole-body dehydration affects basal lung function values.

#### ***Hypotheses***

- i) The fall in FEV<sub>1</sub> following EVH with dry air will be exaggerated in a condition of induced dehydration compared to euhydration.

- ii) Resting lung function parameters, especially those representing the small airways, will be reduced in a state of whole-body dehydration.

***Study 2 - Effect of terbutaline on hyperpnoea-induced airway epithelial injury in athletes.***

***Aims***

- i) Assess the efficacy of an inhaled short-acting  $\beta_2$ -adrenoceptor agonist at reducing hyperpnoea-induced airway epithelial injury in athletes.
- ii) Ascertain the broncho-protective effect of terbutaline in athletes.

***Hypotheses***

- i) A single 0.5 mg dose of terbutaline will attenuate the rise in urinary CC16 following 8 min of EVH with dry air in athletes with EIB.
- ii) Terbutaline will offer significant broncho-protection following EVH in athletes.

***Study 3 - Effect of terbutaline on mast cell activation in athletes with exercise-induced bronchoconstriction.***

***Aim***

Test the efficacy of a therapeutic dose of an inhaled short-acting  $\beta_2$ -adrenoceptor agonist at inhibiting mast cell mediator release in athletes with EIB.

### ***Hypothesis***

A single 0.5 mg dose of inhaled terbutaline will attenuate the rise in urinary  $11\beta$ -PGF<sub>2α</sub> following 8 min of EVH in athletes with EIB.

### ***Study 4 - Effect of warm-humid air on exercise-induced bronchoconstriction and inflammatory mediator release***

#### ***Aim***

- i) Determine the feasibility of using mass spectrometry to detect a range of airway-derived inflammatory mediators in urine following bronchial provocation with exercise.
- ii) Determine whether warm-humid air breathing during exercise prevents EIB through the inhibition of inflammatory mediator release.

#### ***Hypotheses***

- i) Mass spectrometry will enable the detection of a range of broncho-active mediators (not limited to PGD<sub>2</sub> and cyst-LTs metabolites) following exercise provocation in individuals with EIB.
- ii) The inhibition of airway narrowing post-exercise when warm-humid air is inhaled will be associated with a change in the balance between broncho-constricting and broncho-protective inflammatory mediator release



## **CHAPTER 3**

### **General Methods**

The following sections detail the general methods used in this thesis. The study protocols and a succinct description of the methods are presented in the relevant study chapters.

### **3-1 Ethical approval**

The research ethics committee (REC) of the School of Sport and Education at Brunel University approved Study 1 and Study 4 of this thesis. The National Health Service (NHS) Research Ethics Committee approved Study 2 and Study 3. Evidence of ethics approval is provided in appendix.

### **3-2 Participants**

The study population differs between studies and is presented in the specific methodology section of each experimental chapter.

#### **3-2.1 Recruitment**

The research was publicised *via* posters placed around Brunel University, local gyms and at various sports centres. Posts were placed on social media (incl. Facebook, Twitter and online chat forums for sports events) and announcements were made in lectures and at sports events. For Study 4 only, letters and study adverts were sent to local GP centres and a study advert was placed with Asthma UK.

### **3-2.2 Pre-participation**

Prior to enrolment, all participants were given a detailed participant information sheet that outlined the testing procedures along with the associated risks and benefits of taking part. Participants were given appropriate time to comprehend the information and were encouraged to ask any questions of the investigator. Participants were asked to complete a general health questionnaire (example provided in appendix) and any participant who reported adverse health issues was omitted from the study. Eligible participants provided written informed consent (example provided in appendix).

## **3-3 Participant characteristics**

### **3-3.1 Anthropometry**

Freestanding stature and body mass were measured using a stadiometer and electronic scales (SECA model 798, Hamburg, Germany). The measurements were made with the participant stood barefoot with their heels, buttocks and back touching the stadiometer. Their head was orientated in the Frankfort plane (i.e., the lower border of the eye socket and the upper border of the ear opening in a horizontal plane). The participants were instructed to take a full breath and their height was recorded to the nearest 0.5 cm. Body mass was recorded to the nearest 0.1 kg, with the participant wearing minimal clothing. For the dehydration study (Study 1), to improve accuracy of the measurement, nude body mass was recorded by the participant.

### **3-3.2 Atopic status**

Skin prick testing (SPT) was used to assess the atopic status of the participants. In Study 2 and Study 3 participants were tested for sensitivity to timothy grass, house dust mite, and cat hair. In addition, for Study 1 and Study 4, following recommendations from a consultant allergist (Prof Adnan Custovic, personal communication, 2012), participants were also tested against silver birch and dog hair. Standard guidelines for skin prick testing were adhered to (Bousquet et al., 2012).

The forearm of the participant was cleansed. A single drop of each allergen extract and a positive and negative control (ALK-Abello, Reading, England) was then placed on the forearm of the participant, ensuring each drop of extract was >2 cm apart. The skin was pricked through the drop of allergen using the tip of a lancet (ALK-Abello, Reading, England). After 15 min the diameter of the reaction wheal at the longest point was measured. A test was defined positive if the reaction wheal was  $\geq 3$  mm compared with the negative control.

***General principle.*** Skin prick testing uses the presence and size of the reaction wheal as a marker for sensitisation to a given allergen; in sensitised individuals the introduction of allergen to the skin causes the degranulation of mast cells and the production of histamine and other mediators which causes the flare response (Heinzerling et al., 2013).

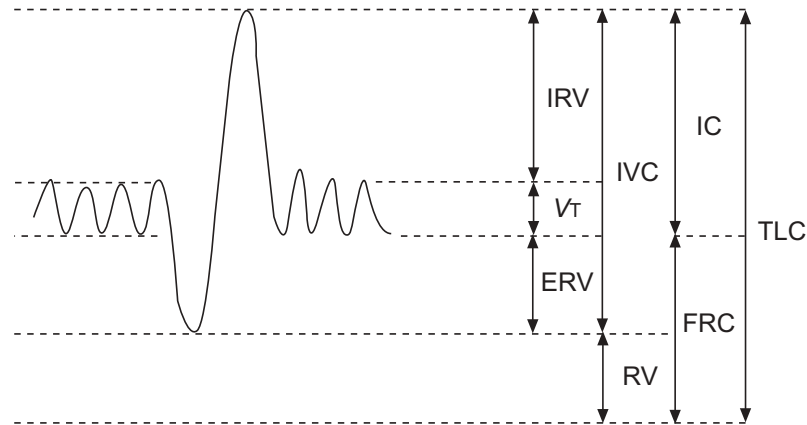
## 3-4 Lung function measurements

### 3-4.1 Lung volumes and flow rates

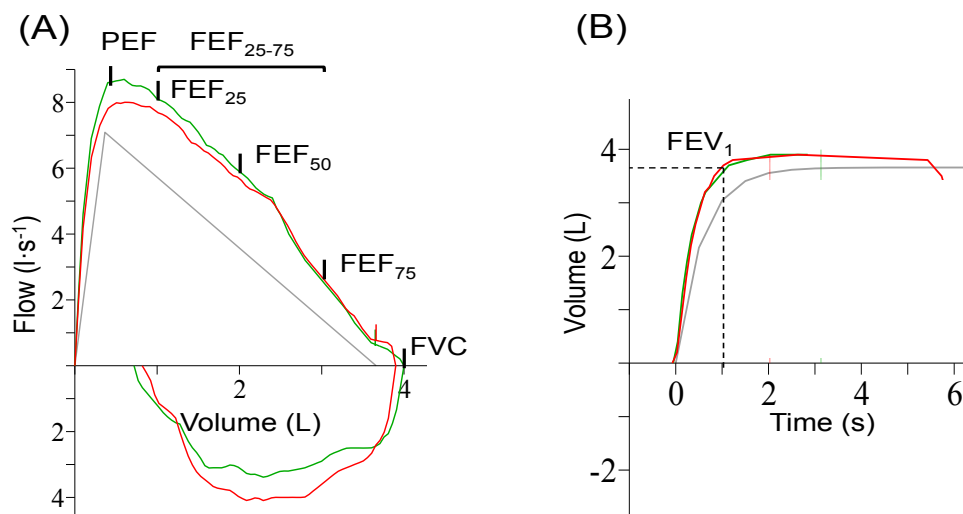
A summary of the lung volumes and flow rates measured in this thesis and the lung function test used to make the measurements is presented in Table 3.1. A graphical representation of the static lung volumes is presented in Figure 3.1 and for dynamic “forced” lung volumes in Figure 3.2.

**Table 3.1.** Summary of lung volumes and flow rates presented in this thesis and the various lung function tests used to make the measurements.

Measurement	Abbreviation	Lung function test
Forced Expiratory Volume in 1 second	FEV <sub>1</sub> (l)	Spirometry
Forced Vital Capacity	FVC (l)	Spirometry
Mean forced expiratory flow between 25% and 75% of FVC	FEF <sub>25-75</sub> (l·s <sup>-1</sup> )	Spirometry
Instantaneous forced expiratory flow when 25% of FVC has been expired	FEF <sub>25</sub> (l·s <sup>-1</sup> )	Spirometry
Instantaneous forced expiratory flow when 50% of FVC has been expired	FEF <sub>50</sub> (l·s <sup>-1</sup> )	Spirometry
Instantaneous forced expiratory flow when 75% of FVC has been expired	FEF <sub>75</sub> (l·s <sup>-1</sup> )	Spirometry
Peak expiratory flow	PEF (l·s <sup>-1</sup> )	Spirometry
Total lung Capacity	TLC <sub>pleth</sub> (l)	Whole-body plethysmography
	TLC <sub>He</sub> (l)	Helium dilution
Residual Volume	RV <sub>pleth</sub> (l)	Whole-body plethysmography
	RV <sub>He</sub> (l)	Helium dilution
Functional residual capacity	FRC <sub>pleth</sub> (l)	Whole-body plethysmography
	FRC <sub>He</sub> (l)	Helium dilution



**Figure 3.1.** Static lung volumes based on volume-time graph of an inspiratory capacity manoeuvre. IRV, inspiratory reserve volume;  $V_T$ , tidal volume; ERV, expiratory reserve volume; IVC, inspiratory vital capacity; RV, residual volume; IC, inspiratory capacity; FRC functional residual capacity; TLC total lung capacity. From Wanger et al., (2005).



**Figure 3.2.** Flow-volume loop (A) and volume-time graph (B) displaying lung volumes and flow rates from a forced expiratory manoeuvre. FVC, forced vital capacity; PEF, peak expiratory flow;  $FEF_x$ , forced expiratory flow at X % of FVC;  $FEF_{25-75}$ , mean forced expiratory flow between 25 and 75% of FVC; Forced expiratory volume in 1 second ( $FEV_1$ ).

### **3-4.2 Spirometry – Forced vital capacity manoeuvres**

Spirometry is used to measure the flow and volume of air as it is inhaled and exhaled. For all the studies presented in this thesis forced vital capacity manoeuvres were conducted to determine a variety of lung function parameters (Table 3.1, Figure 3.2). Two different spirometers were used to record these measurements. In Study 1 spirometry was conducted using the MasterScreen PFT system (Carefusion, Hoechberg Germany). In Study 2, Study 3 and Study 4, spirometry was conducted using a Microloop spirometer (MicroLoop, Micromedical Limited, Kent, England). Both spirometers meet the ATS/ERS requirements (Miller et al., 2005). The calibration of the spirometer was checked on the day of testing using a 3 l calibration syringe (Carefusion Calibration Pump, Carefusion, Germany). Dedicated software was used to manage lung function data, i.e., Spida V5 (Micromedical Limited, Kent, UK) and JLab (Carefusion, Germany) for the Microloop and MasterScreen PFT spirometers, respectively.

Forced vital capacity manoeuvres were conducted in accordance with ATS/ERS guidelines (Miller et al., 2005). The manoeuvres were performed with the participants seated, with their head slightly elevated and their nose occluded. Following an explanation of the procedure, participants were required to breathe into the spirometer *via* a mouth-piece and bacterial filter (MicroGard, Carefusion, Germany). Participants were then asked to inhale completely and rapidly to TLC and to “blast” all the air out of their lungs as fast and as powerfully as they could and to continue breathing out until no more air could be expelled. Participants were

encouraged by the investigator to continue breathing out until a plateau in the volume-time curve was observed (or a minimum of 6 s). The manoeuvres were inspected against the within-manoevre acceptability criteria presented in Table 3.2. To ensure repeatability, the between-manoevre repeatability criteria presented in Table 3.2 were adhered to. The greatest FEV<sub>1</sub>, FVC and PEF from acceptable manoeuvres were selected for analysis. The FEF<sub>25-75</sub>, FEF<sub>25</sub>, FEF<sub>50</sub> and FEF<sub>75</sub> were selected from the manoeuvre with the greatest sum of FEV<sub>1</sub> and FVC.

### **3-4.3 Airway response to bronchial provocation**

Where spirometry was used to assess airway response to a bronchial provocation challenge, results are displayed as either maximum fall from baseline or area under the FEV<sub>1</sub> time curve (FEV<sub>1</sub>-AUC). Where maximum fall in FEV<sub>1</sub> or FVC are presented, results are expressed as a percentage of the measurement taken immediately prior to provocation. FEV<sub>1</sub>-AUC was calculated using the trapezoidal method and calculated from the FEV<sub>1</sub> value recorded immediately prior to bronchial provocation.



**Table 3.2.** Within and between manoeuvre acceptability criteria for forced vital capacity manoeuvres. Reproduced from Miller et al., (2005).

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<b>Within-manoevrue criteria</b>
Individual FVC manoeuvres are acceptable if:
They are free from artefacts
Cough during the first second of exhalation
Glottis closure that influences measurement
Early termination or cut-off
Effort that is not maximal throughout
Leak
Obstructed mouthpiece
They have good starts
Extrapolated volume <5% of FVC or 0.15L, whichever is greater
They show satisfactory exhalation
Duration $\geq 6$ s or a plateau in the volume-time curve

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<b>Between-manoevrue criteria</b>
After three acceptable manoeuvres have been performed apply the following tests
The two largest values of FVC must be within 0.150 L of each other
The two largest values of FEV <sub>1</sub> must be within 0.150 L of each other
If both of these criteria are met, the test session may be concluded
If both these criteria are not met, continue testing until
Both criteria are met with analysis of additional acceptable manoeuvres or
A total of eight manoeuvres have been performed or
The patient/subject cannot continue

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### 3-4.4 Helium dilution

For the purpose of Study 1, helium dilution was used to measure FRC, RV and TLC (i.e., lung volumes that cannot be measured by simple spirometry). The helium dilution test was conducted according to the manufacturer's protocol (Carefusion, Hoechberg, Germany) and in adherence with the ATS/ERS guidelines (Wanger et al., 2005).

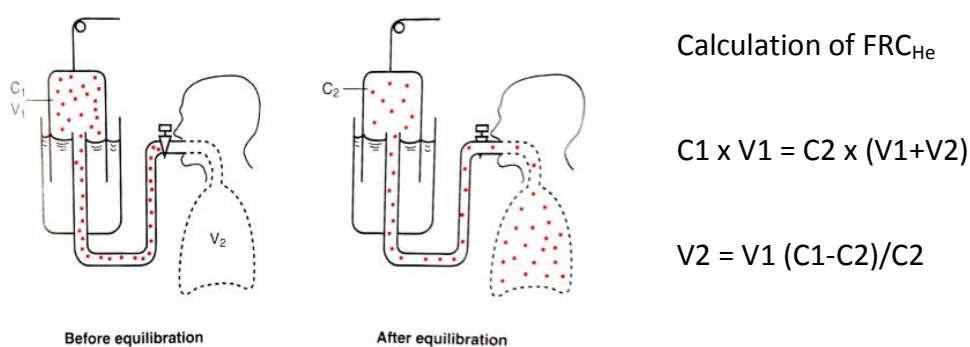
The helium dilution test was conducted using the MasterScreen PFT system (Carefusion, Hoechberg, Germany). Participants were required to breathe into the system through a mouthpiece with a nose clip in place. After steady tidal breaths (and FRC) were observed for approximately 30 s, participants were connected to the test gas that contained 9% He and 32% O<sub>2</sub>. Whilst breathing the new gas mixture, participants were instructed to exhale to RV before returning to normal tidal breathing. Participants were then requested to continue to breathe normally until an equilibration of the concentration of He was achieved; equilibrium was considered when the change in helium concentration was <0.02% for 30 s. Once this equilibrium was achieved, the participants were disconnected from the test gas and were required to exhale to RV and then to inspire to TLC. FRC<sub>He</sub> was calculated by the JLab software (Carefusion, Hoechberg, Germany).

Helium dilution tests were repeated at least twice to ensure repeatability (i.e., FRC<sub>He</sub> variation <10%), with at least a 10 min interval between trials. The mean FRC<sub>He</sub> from reproducible manoeuvres was selected for analysis. RV<sub>He</sub> was calculated

as mean  $FRC_{He}$  – largest ERV and  $TLC_{He}$  was derived from  $RV_{He}$  + the largest  $VC_{He}$ .

The equations of Quanjer et al., (1993) were used for calculation of predicted values. Of importance, helium dilution measures FRC from the communicating gas in the lungs and gas trapped behind closed airways is not measured.

**General principle.** The helium dilution technique calculates  $FRC_{He}$  by having the participant breathe through a closed circuit of a known volume ( $V_1$ ) and by using a gas mixture containing a tracer gas (i.e., helium) of a known concentration ( $C_1$ ). The participant breathes the test gas until an equilibration of the tracer gas is achieved with the gas in the lungs and the new concentration of the tracer gas is noted ( $C_2$ ). As no helium is lost, the change in concentration of the tracer gas can be used to calculate  $FRC_{He}$  ( $V_2$ ), Figure 3.3. In practice the calculations are more complex to adjust for the utilisation of oxygen and made *via* specialist software. With the addition of a maximum expiratory and inspiratory manoeuvre, the lung volumes  $TLC_{He}$  and  $RV_{He}$  can be obtained.



**Figure 3.3.** Calculation of  $FRC_{He}$  using the helium dilution technique. From West (2012). See text for details.

### 3-4.5 Whole-body plethysmography

In contrast to the helium dilution technique, whole-body plethysmography measures FRC inclusive of trapped air; therefore, complementary information can be obtained by conducting both tests. Whole-body plethysmography was used in Study 1 of this thesis. Manoeuvres were conducted using the MasterScreen Body/Diff system (Carefusion, Hoechberg Germany) according to the manufacturer's "normal breathing" protocol; this protocol is in adherence with the ATS/ERS guidelines (Wanger et al., 2005).

In brief, following an explanation of the procedure, participants sat inside the MasterScreen plethysmograph box for 2 minutes to allow for the temperature inside the box to stabilise. Participants were then asked to breathe at a rate of 25 breaths *per* minute into a mouthpiece, with a nose clip in place, whilst specific airway resistance measurements were made. At the end of a normal expiration a shutter was closed for ~2-3 s (until the mouth pressure sum equalled 7 kPa). The participants were required to breathe normally against the closed shutter without increasing their effort. Once the shutter opened, the participants were asked to exhale fully to RV and then to inspire to TLC. A minimum of three manoeuvres were conducted to ensure reproducibility (i.e.,  $FRC_{pleth}$  varied by <5% across the 3 trials).  $FRC_{pleth}$  was calculated by the JLab software and the mean  $FRC_{pleth}$  was selected for analysis.  $RV_{pleth}$  was derived from the mean  $FRC_{pleth}$  – mean ERV, and  $TLC_{pleth}$  was calculated as the maximum VC +  $RV_{pleth}$ . The equations of Quanjer et al., (1993) were used for calculation of predicted values for lung volumes.

**General principle.** Whole-body plethysmography detects changes in box pressures whilst simultaneously measuring changes in mouth pressure and lung volumes. FRC is then calculated based on Boyle's law (i.e., pressure x volume is constant at given temperature) (Wanger et al., 2005).

***Airway resistance measurements.***

Airway resistance is the pressure difference between the alveolar pressure and the mouth pressure *per* unit of airflow. Airway resistance is measured concomitantly with the procedure described above. Specific airway resistance (sRaw) is calculated as the difference between the box pressure (alveolar pressure) and the mouth pressure, and divided by the airflow. Total airway resistance (Raw) is derived from the measurements of sRaw and FRC<sub>pleth</sub> ( $Raw = sRaw \times FRC_{pleth}$ ). All airway resistance measurements were calculated as the mean of 10 breaths. Airway resistance predicted values were derived from the equations of Quanjer (1983).

### **3-4.6 CO diffusion capacity (DLCO): single breath method**

DLCO was measured in Study 1 of this thesis using the single breath technique. The measurements were conducted using the MasterScreen PFT system (Carefusion, Hoechberg, Germany) according to the ERS/ATS guidelines (MacIntyre et al., 2005). Participants were sat upright, with a nose clip in place, and attached to the system *via* a bacterial filter (MicroGard, Carefusion, Germany). After several tidal breaths participants were instructed to completely empty their lungs, with an unforced exhalation to RV. Once at RV, the participants were asked to perform a rapid inhalation of a test gas (which contained 0.3% CO, 9.7% He, 20.9% O<sub>2</sub> and balanced N<sub>2</sub>) to TLC. The participants were then required to hold their breath for 10 s. At the end of the breath-hold period the participants were prompted to smoothly empty their lungs, without hesitation or interruption, in no more than 4 seconds. This procedure was repeated at least twice, with a 4 min interval (to allow adequate elimination of the test gas from the lungs), and repeatability was ensured (i.e., <10% variation in DLCO). The mean DLCO, KCO and VA values were calculated from two reproducible manoeuvres and kept for analysis. The equations of Quanjer et al., (1993) were used for calculation of predicted values.

***General principle.*** The transfer of CO into the pulmonary capillaries is diffusion limited. Therefore, the change in concentration of CO from inspiration of the test gas to expiration is a reflection of the diffusing capacity of the lungs (DLCO being determined by the uptake of CO over the breath-holding period) (MacIntyre et al., 2005).

***Haemoglobin (Hb) correction.*** The transfer of CO from the airspace into the blood is affected by the concentration of Hb in the blood. Hb was therefore measured in Study 1 prior to the DLCO measurements and values corrected for the level of haemoglobin. A fingertip capillary sample was collected using a specialised micro-cuvette and measured using a photometer (Hemocue Hb201+, HemoCue AB, Angelholm, Sweden).

### **3-5 Bronchial provocation**

In Study 1 and Study 4, exercise challenge tests were used for bronchial provocation during a screening visit. These exercise provocation challenges were identical and described in the following section (3-5.1 Exercise provocation –screening visit).

Exercise provocation was also used during the experimental visits in Study 4. In those experimental conditions, a more complex set-up was utilised to allow for the delivery of different inspired air conditions (as described in section 3-5.2 Exercise - experimental visits).

#### **3-5.1 Exercise provocation - screening visit**

For the screening visits of Study 1 and Study 4 exercise was used to determine the presence and severity of EIB. This was used to establish eligibility of the participants for the studies. The exercise provocation was conducted according to the ATS guidelines (Crapo et al., 2000). The exercise consisted of 8 minutes of cycling on a cycle ergometer (Lode Excalibur, Lode B.V, Groningen, The Netherlands). The

exercise was incremental, with the final target workload calculated as follows:  
 $\text{Watts} = (53.76 \times \text{measured FEV}_1) - 11.07$ . Participants achieved this workload after 3 minutes of exercise at lower intensities. The workload was set at 60%, 75%, 90% and 100% of the target workload for the first, second, third and fourth minute of exercise, respectively. The workload was adjusted at the discretion of the investigator to ensure that all participants could achieve the full duration of exercise. Previous research has demonstrated that some participants are unable to maintain the target workload (Anderson, Lambert, Brannan, Wood, Koskela, et al., 2001b). Any change in the workload was recorded and used to inform the exercise challenges in the experimental conditions for Study 4.

The exercise was conducted in a climate-controlled environmental chamber (Procema Ltd, Twickenham, UK) in dry air conditions (target temperature = 16°C; target relative humidity <50%). Low humidity was achieved using a portable desiccant dehumidifier (ELA Model DD822, DIO Ltd, Powys, UK). The temperature and humidity were measured using a hygrometer (RH32, Omega, Manchester, UK) every minute and presented as the mean during the 8 min challenge. The heart rate (HR) of the participants was monitored by short-range radio-telemetry using a chest belt (Polar H7, Polar Electro (UK) Ltd, Warwick, UK). Oxygen saturation (SpO<sub>2</sub>) was monitored continuously by pulse oximetry (Model 2500A, Nonin Medical Inc, Minnesota, USA). Ventilatory and pulmonary gas exchange indices were obtained breath-by-breath using an online system (Quark b<sup>2</sup>, Cosmed, Rome, Italy) and managed with dedicated software (CPET Software suite 9.1b, COSMED, Rome,



Italy). HR, VE, SpO<sub>2</sub> and pulmonary gas indices are presented as the means of the final four minutes of exercise.

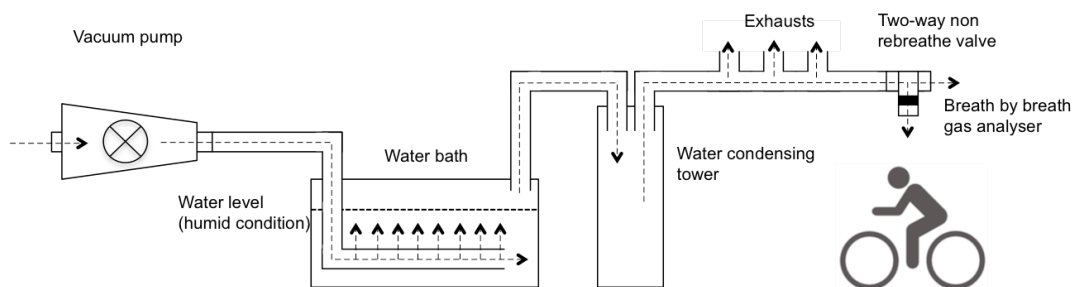
Spirometry was performed at baseline and again (in duplicate) at 3, 5, 10, 15, and 20 min post-challenge. The largest FEV<sub>1</sub> from each pair of acceptable manoeuvres post-exercise was used for analysis. The severity of EIB was determined by the largest change in FEV<sub>1</sub> from the baseline measurement and expressed as a percentage change from baseline [i.e., (baseline pre-exercise FEV<sub>1</sub> – lowest post-exercise FEV<sub>1</sub>) / baseline pre-exercise FEV<sub>1</sub> x 100].

### **3-5.2 Exercise - experimental visits**

For experimental visits in Study 4, the same guidelines were followed (Crapo et al., 2000). The workload was however adjusted, based on the workload achieved by each participant during the screening visit, and was standardised across experimental visits.

To allow for the inhalation of pre-conditioned air (37°C and 100% RH) during exercise, ambient air was pumped through a custom-built system [Figure 3.4, as reproduced from the original research by Anderson and colleagues (1982)]. Air was pumped through a water bath (Labmaster, Annex Pty Ltd, Melbourne, Australia), that was either empty (for the temperate-dry condition) or contained 18 l of distilled water heated to 44°C (for the warm-humid condition). From the water

bath, air was passed through insulated tubing into a condensing tower (to remove excess water). Additional insulated tubes delivered the air to a two-way non-rebreathing valve (custom made) attached to a mouthpiece (Hans Rudolph Inc, Kansas, USA). Exhausts made from additional tubing were located between the condensing tower and the two-way valve to prevent positive pressure.



**Figure 3.4.** Diagram of the custom-built system used to deliver warm-humid air (with water in water bath) and temperate-dry air (without water in water bath) in Study 4.

Temperature and humidity were measured 5 cm from the mouthpiece using a hygrometer (RH32, Omega, Manchester, UK) in the temperate-dry condition.

Temperature was measured from the same location using RS digital thermometer 1319A (RS Components Ltd, Corby, UK) in the warm-humid condition. Humidity could not be measured in the warm-humid condition as it caused damage to the hygrometer; the humidity was assumed to be 100% based on visible condensation on the inspiratory tube, as has been done previously (Hahn, Nogrady, Burton, &

Morton, 1985). The relative humidity (RH%) and temperature were recorded every minute and the mean data over the entire 8 min of exercise are presented in the result section.

Participants breathed through the system for 4 minutes prior to the start of exercise and remained connected to the system for 2 min after termination of the exercise; the latter prevented the occurrence of a thermal gradient at the end of exercise that could have contributed to airway narrowing through vascular events (McFadden et al., 1986). As described in the previous section, HR, VE, SpO<sub>2</sub> and pulmonary gas exchange indices were measured during exercise and results are expressed as mean from the final 4 min of exercise.

### **3-5.3 Eucapnic voluntary hyperpnoea (EVH)**

EVH was used for bronchial provocation in Study 1, Study 2 and Study 3 of this thesis. The duration of the EVH challenge and timing of spirometry measurements post-EVH varied between studies. Details are contained in the methodology section of the relevant experimental chapters.

The EVH challenges were conducted according to an established protocol (Anderson, Argyros, Magnussen, & Holzer, 2001a; Argyros, Roach, Hurwitz, Eliasson, & Phillips, 1996) using the Eucapsys system (SMTEC, Nyon, Switzerland). From a seated position with nose occluded, participants were required to breathe through

a two-way non-rebreathing valve and a mouthpiece (Hans Rudolph Inc, Kansas, USA Hans Rudolph). A dry gas mixture containing 5% CO<sub>2</sub>, 21% O<sub>2</sub> and balance N<sub>2</sub> was delivered from compressed medical gas tanks into the EucapSys, and to the participants. The target ventilation for the initial visit was 85% MVV [calculated as 30 times baseline FEV<sub>1</sub> (Anderson, Argyros, Magnussen, & Holzer, 2001a)]. Participants received continuous visual feedback on their current and average ventilation, and were encouraged to try and match the target ventilation the best they could. The tidal volume and breathing frequency were self-selected by the participants. The mean achieved VE was recorded from the Eucapsys system, and the VE achieved during the first visit was used as the target ventilation for any subsequent visits.

## **3.6 Urinary analysis**

### **3.6.1 CC16**

In Study 2, CC16 was measured in urine and used as a non-invasive marker of airway epithelial integrity. Urine was collected at baseline and at 30 and 60 min post-EVH. At each time point, participants were instructed to completely empty their bladder. In males the first 100 ml was systematically discarded to avoid prostatic contamination (Andersson et al., 2007). Samples were transferred into 2 ml Eppendorfs and kept at -80°C prior to analysis. As recommended, samples were analysed within 2 months of collection (Andersson et al., 2007).

The analysis of CC16 was conducted by trained biochemical scientists at the Department of Respiratory Medicine and Allergology, University Hospital, Lund, Sweden. CC16 was measured using the Human Club Cell Protein ELISA kit from BioVendor (Modrice, Czech Republic) according to the manufacturer's instructions.

The human club cell protein ELISA kit is a sandwich enzyme immunoassay for the quantitative measurement of human club cell protein. In short, 100  $\mu\text{l}$  of each urine sample, along with the quality control solutions and a blank buffer solution, were added to wells pre-coated with polyclonal anti-human club cell protein antibody. After 60 minutes incubation the samples were washed with the wash solution and the biotin labelled polyclonal anti-human club cell protein antibody was added. Following another 60 minutes of incubation and another washing, streptavidin-horseradish peroxidase conjugate was added for a further 60 min of incubation. Following further washing, 100  $\mu\text{l}$  of substrate solution containing tetramethylbenzidine (TMB) was added to each well and left to incubate for 10 min before the addition of the stop solution. The absorbance of each well was measured spectrophotometrically at 450 nm within 5 min of the addition of the stop solution. The absorbance of the samples was compared against the standard curve to calculate the concentration of CC16 with a detection limit of 20  $\mu\text{g}\cdot\text{ml}^{-1}$ .

### 3-6.2 Inflammatory mediators

In Study 3 and Study 4, the release of airway-derived inflammatory mediators was measured non-invasively in urine samples. The collection time points differ between Study 3 and Study 4 and are described in the relevant experimental chapters. At each time point participants were instructed to completely empty their bladder. The urine was immediately measured and stored at -80°C.

Urine was selected as the most appropriate specimen for several reasons (Kupczyk et al., 2011). Firstly, the collection of urine at regular intervals integrates the amount of the studied compound that is released into the circulation, and then excreted into the urine during that time period. Secondly, the resting levels of urinary eicosanoids are very low and, therefore, increases are easily detected. Finally, urine samples are non-invasive, thus biosynthesis during sampling is avoided.

In this thesis the urinary analysis of inflammatory mediators was conducted using two methods, i.e., *via* commonly used (and commercially available) enzyme linked immunosorbent assays, and also *via* the more advanced technique of ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The latter analysis was performed on a newly developed platform for mass spectrometry at the Karolinska Institutet (Stockholm, Sweden), and its use in the context of exercise provocation constitutes one of the novel aspects this thesis.

### ***Enzyme Linked Immunosorbent Assays (ELISA)***

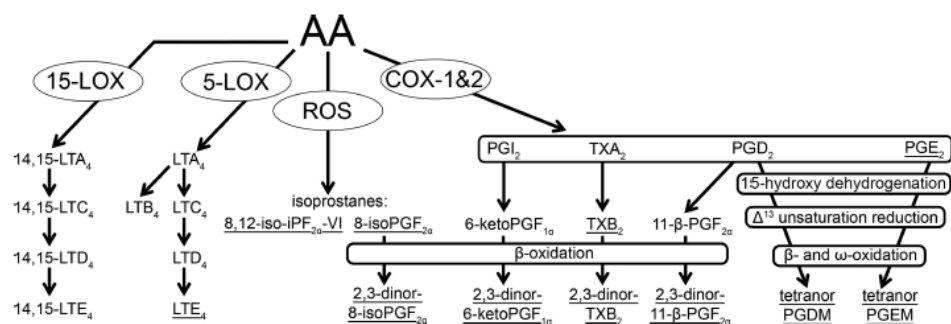
A trained biochemical scientist at the Karolinska Institutet conducted analysis of the inflammatory mediators. Analysis of  $11\beta$ -PGF<sub>2 $\alpha$</sub>  (Study 3) and LTE<sub>4</sub> (Study 3 and 4) was conducted using commercially available competitive ELISA kits (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. The competitive ELISA is based on the competition between the inflammatory mediator metabolite (i.e.,  $11\beta$ -PGF<sub>2 $\alpha$</sub>  and LTE<sub>4</sub>) and the provided tracer agents (i.e.,  $11\beta$ -PGF<sub>2 $\alpha$</sub>  acetylcholinesterase (AChE) conjugate and LTE<sub>4</sub> AChE conjugate) for binding sites on wells pre-coated with a specific antibody. Urine samples are first mixed with the tracer agents, before being added to the wells. Following 18 h of incubation the wells were washed and the Ellman's reagent added. The Ellman's reagent contains the substrate to AChE, and the product of the enzymatic reaction is the production of colour. Following 60-90 min of incubation with the Ellman's reagent, the plate was read spectrophotometrically at 405 nm. The absorbance of the samples was compared against the standard curves to calculate concentration of  $11\beta$ -PGF<sub>2 $\alpha$</sub>  and LTE<sub>4</sub>. The detection limit for  $11\beta$ -PGF<sub>2 $\alpha$</sub>  and LTE<sub>4</sub> was 5.5 pg·mL<sup>-1</sup> and 25pg·mL<sup>-1</sup> respectively.

### ***Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).***

In Study 4, the analysis of inflammatory mediators was conducted using both ELISA (as above) and UPLC-MS/MS. UPLC-MS/MS is a technique that combines the physical separation capabilities of liquid chromatography with the mass analysis

capabilities of mass spectrometry. The development and validation of this method, and the technical specification and methodology are described in detail by (Balgoma et al., 2013). Briefly, to account for the large matrix effect (background noise) in urine, the extracted volume was normalised by the ratio of its absorbance to a reference material. A standard control compound was then added to each urine sample. Following the preparation of the sample using solid phase extraction, the samples were analysed using the UPLC-MS/MS platform on an Acquity-Xevo TQ mass spectrometer system (Waters, Sweden).

The development of a new UPLC-MS/MS platform by our collaborators at the Karolinska Institutet allowed for the first time the simultaneous measurement of numerous lipid mediator metabolites in urine [incl. metabolites of PGD<sub>2</sub>, PGE<sub>2</sub> PGI<sub>2</sub>, Thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and various isoprostanes species (Figure 3.5)] after bronchial provocation with exercise.



**Figure 3.5.** Schematic of eicosanoid metabolic cascade leading to urinary metabolites from Balgoma et al., (2013).



### **3-6.3 Creatinine**

To adjust for glomerular filtration, the concentrations of urinary metabolites and CC16 were corrected for the production creatinine in the urine. For Study 2 and Study 3, the analysis of creatinine was performed by the department of Klinisk Kemi, Labmedicin Skåne (part of University Hospital of Scania), Lund, Sweden, using the enzymatic, colorimetric method with an automated COBAS 6000 analyser (Roche Diagnostics, Bromma, Sweden). This method has been used in previous investigations (Bolger, Tufvesson, Anderson, et al., 2011a; Bolger, Tufvesson, Sue-Chu, et al., 2011b; Kippelen, Anderson, Tufvesson, Ali, & Bjermer, 2013; Tufvesson, Svensson, Ankerst, & Bjermer, 2013). In Study 4, creatinine was analysed manually by a trained biochemical scientist at the Karolinska Institutet following the modified Jaffe's method (full protocol is presented in the appendix). This method has successfully been used by our group and by collaborators to this thesis (Kippelen, et al., 2010a; 2010b; Larsson et al., 2011).

## **CHAPTER 4**

### **STUDY 1 – Effect of whole-body dehydration on lung function and airway responsiveness in athletes**

## 4-1 Introduction

During exercise individuals sweat to dissipate heat and regulate their body temperature. During prolonged exercise, particularly in a hot environment, the fluid lost through sweating can be substantial. Athletes whose fluid intake is less than their sweat loss can become dehydrated, which is reflected in a post-exercise loss of body mass of >2% (Sawka et al., 2007). Whole-body dehydration of >2% is common in athletes completing endurance events, with some athletes recording a loss of body mass of up to 8% (Rüst et al., 2012; Zouhal et al., 2011). Interestingly, sweat secretion induced by the muscarinic agonist, pilocarpine, appears diminished in individuals with asthma, possibly reflecting abnormal fluid secretions at both skin and airway epithelial level (Park, Stafford, & Lockette, 2008). The effects of whole-body dehydration on haemodynamic responses, cardiac function and exercise performance has been investigated in depth (Cheuvront & Kenefick, 2014), however there is little information regarding its effects on lung function.

The bronchial circulation is the primary source of nourishment and of hydration of the airway epithelium. According to Starling's equation, the movement of water and proteins across the capillary wall is governed by the permeability of the capillary membrane and differences in hydrostatic and oncotic (osmotic) pressures across the capillary wall (Charan & Carvalho, 2002). Dehydration induced by exercise is well known to cause hypovolemia and to increase blood plasma osmolarity (Cheuvront & Kenefick, 2014). These two factors could limit the movement of water towards the airway surface and reduce the volume of ASL.

Whole-body dehydration could, therefore, interfere with the hydration at the airway surface.

During exercise or periods of high ventilation, water is lost from the airway surface to condition the inspired air (Daviskas et al., 1991). Dehydration of the airways, with consequential increase in osmolarity of the ASL, is the main stimulus for EIB during periods of increased ventilation (Anderson & Daviskas, 2000). It is conceivable that during periods of whole-body dehydration, an airway surface already partially depleted of liquid could become more sensitive and/or responsive to acute osmotic changes induced by exercise-hyperventilation, thus leading to enhanced airway narrowing.

This study therefore investigated the effect of whole-body dehydration on airway responsiveness in recreational athletes with symptoms of EIB. Our hypothesis was that whole-body dehydration would increase airway responsiveness induced by hyperpnoea of dry air (as assessed by the change in FEV<sub>1</sub> post-EVH). Since the effect of whole-body dehydration on lung function (irrespective of EIB) remains controversial (Govindaraj, 1972; Javaheri et al., 1987), we also investigated the effects of exercise-induced whole-body dehydration on resting lung function parameters.

## 4-2 Methods

### 4-2.1 Subjects

The study population consisted of 10 recreational athletes, aged 18 to 35 years, who experienced respiratory symptoms (i.e., chest tightness, wheeze, mucus hypersecretion or cough) during and/or following exercise. Participants were non-smokers, free from respiratory infections for 4 weeks prior to the study, and had no known chronic medical condition other than asthma or EIB.

As this was the first study to investigate the effect of whole-body dehydration on airway responsiveness, as a precaution, participants with moderate-to-severe EIB (determined by a reduction of  $\geq 15\%$  in FEV<sub>1</sub> following a standard bronchial provocation challenge with exercise performed at a screening visit, see general methods) were excluded. Participants taking any asthma medication other than inhaled short acting  $\beta_2$ -agonists or anti-histamines were excluded. Participants were asked to withhold alcohol, caffeine and exercise on the day of testing, inhaled short acting  $\beta_2$ -agonist medication for a minimum of 8 h and anti-histamine medication for 72 h. The School of Sport and Education REC reference number for this study was RE52-12.

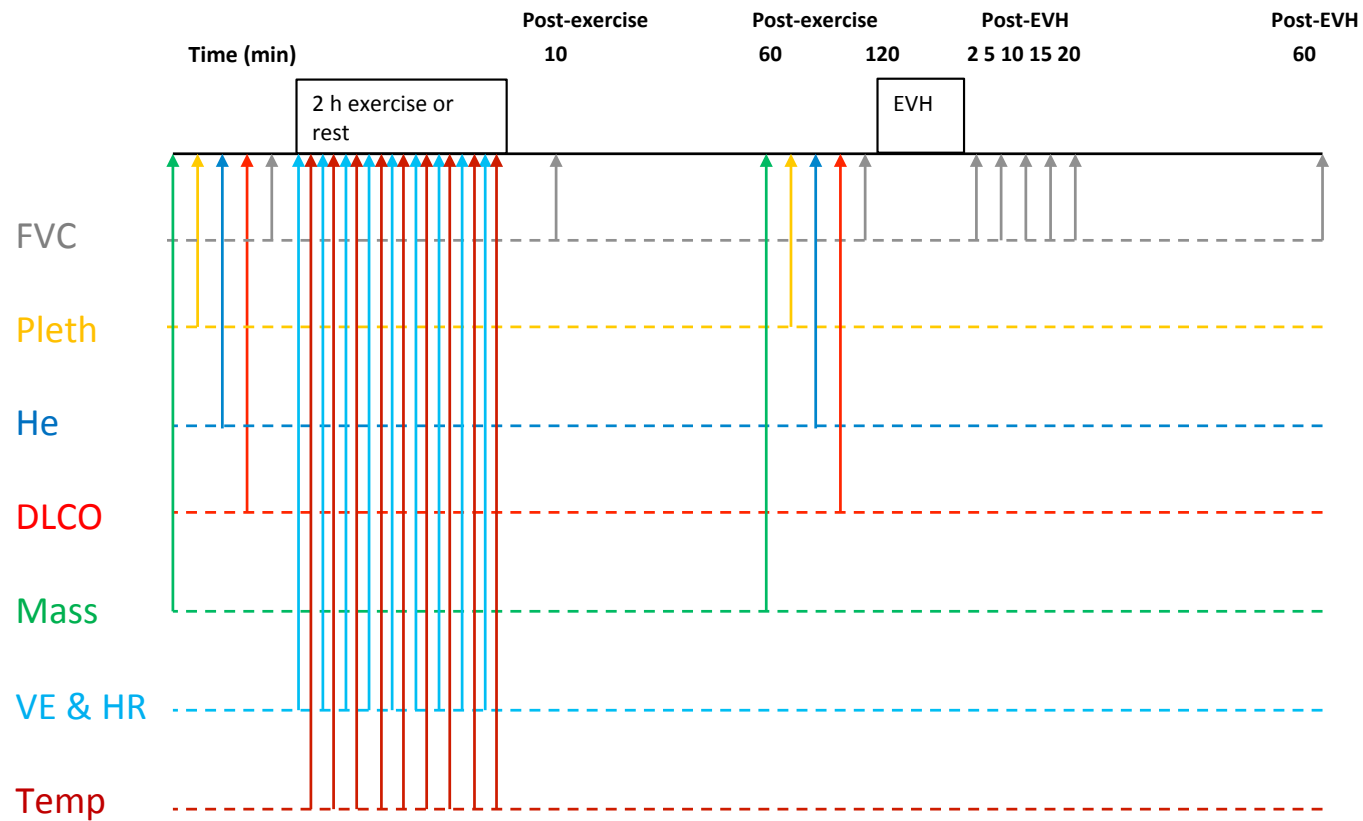
#### 4-2.1 Protocol

The study used a randomised crossover design with three experimental visits. Lung function and airway responsiveness were assessed twice in a euhydrated state (euhydration and control condition) and once in a dehydrated state (dehydration condition). Lung function was assessed by whole-body plethysmography, helium dilution, DLCO and spirometry (as described in the general methods) at baseline, and 1 h following the exercise / resting period. Airway responsiveness to dry air was assessed by the maximum change in FEV<sub>1</sub> following a standard 6 minute EVH challenge (see general methods). A schematic of the experimental protocol is presented in Figure 4.1.

All experimental visits commenced in the morning so as to standardise for variability in lung function throughout the day (Lebowitz, Krzyzanowski, Quackenboss, & O'Rourke, 1997). To ensure participants were euhydrated prior to the commencement of any lung function measurements, urine osmolality was checked using a portable refractive index osmometer (Osmocheck, Vitech scientific Ltd, UK). Euhydration was defined by a urine osmolality of less than 700 mOsmol·kgH<sub>2</sub>O<sup>-1</sup> (Sawka et al., 2007).

The experimental visits commenced with baseline lung function measurements. Participants then completed either 2 h of low intensity exercise or 2 h of rest (for the control condition). Exercise was performed in the heat with fluid restriction (to induce mild dehydration), or in temperate conditions with voluntary fluid consumption (euhydrated condition). Participants completed forced vital capacity

manoeuvres 10 min after finishing the exercise bouts (or the matched resting period for the control condition). One hour after exercise / rest the body mass of the participants was recorded, with the change in body mass from the baseline value being used as an index of dehydration. Lung function measurements were then repeated. An EVH test commenced 2 h following the exercise / rest period, with recovery FVC manoeuvres performed at 2, 5, 10, 15, 20 and 60 min post-EVH. In the dehydration condition, participants were allowed to drink water *ad-libitum* between 20 and 60 min of recovery; thereafter, final FVC manoeuvres were performed.



**Figure 4.1.** Schematic of the experimental protocol used in Study 1. FVC, forced vital capacity manoeuvre; Pleth, whole-body plethysmography; He, FRC-He re-breathing; DLCO, carbon monoxide diffusing capacity; Mass, nude body mass; VE & HR, Ventilation and heart rate.



#### **4-2.2 Exercise (or matched resting period)**

Dehydration was achieved by a 2 h light-intensity exercise session in the heat with fluid restriction. The exercise involved four bouts of 20 min of cycling, followed by 10 min of stepping, and was performed in a heat chamber set at 37°C and 50% RH. The workload for the cycling was set at 25% of estimated peak power (Hansen, Sue, & Wasserman, 1984). Stepping was conducted on a 20 cm step at a rate of 45 steps *per* minute (the pace was set by a metronome). HR and VE was measured mid-way through each phase of exercise (i.e., 10 min into each bout of cycling and 5 min into each bout of stepping) using respectively, short-range radio telemetry (Polar H7, Polar Electro (UK) Ltd, Warwick, UK) and expired gas analysis (in Douglas bags). In the euhydration condition the same exercise was performed in temperate conditions (~20°C and ambient humidity) with voluntary fluid consumption. In the control condition, participants did not carry out any exercise and consumed fluid *ad-libitum*.

#### **4-2.3 Sample size and data analysis**

Sample size calculation was based on previous publications including EVH testing in healthy, normally active individuals (Bolger, Tufvesson, Sue-Chu, et al., 2011b; Kippelen et al., 2010a). We predicted a maximal fall in FEV<sub>1</sub> post-EVH of 5 ± 3% in the euhydrated state and of 10 ± 3% in the dehydrated state. With a risk alpha of 5% and a risk beta of 80%, and with a difference in population means of 5% and a standard deviation of the difference in the response of matched pairs of 5%, our

sample size calculation determined that 10 participants should be included in the study.

The primary outcome measure was maximum fall in FEV<sub>1</sub> post-EVH (in %) and FEV<sub>1</sub>-AUC<sub>0-60</sub>; these parameters were non-normally distributed and therefore compared between conditions using a Friedman 2 way ANOVA by ranks and Wilcoxon-signed rank test and data displayed as median and interquartile range. Resting lung function measurements were our secondary outcomes. Differences in lung function data between conditions and times were analysed using repeated-measures ANOVAs. In cases of statistical significance, pairwise comparisons with Bonferroni adjustments were conducted. Pearson's correlation tests were run to test for relationships between change in body mass and change in lung function following exercise in the dehydration condition. All data were analysed using a statistical software package (SPSS 20, Chicago, IL, US).

## 4-3 Results

### 4-3.1 Participant characteristics

Ten recreational athletes (5 females) completed the study. Mean age, height and body mass were  $21 \pm 2$  yr,  $170 \pm 12$  cm and  $63 \pm 10$  kg, respectively. Participants were involved in summer sports and trained for  $6 \pm 4$  h *per* week in aerobic activities. All participants had a previous diagnosis of asthma and/or of EIB (5 had childhood asthma), and 8 were using short-acting  $\beta_2$ -agonist medication at the time of the study. All participants had FEV<sub>1</sub>, FVC and FEV<sub>1</sub>/FVC above the LLN (Quanjer et al., 2012). Only one participant had a positive response to the exercise challenge during the screening visit (i.e., max fall in FEV<sub>1</sub> post-exercise of 11%), consistent with current mild EIB.

### 4-3.2 Dehydration protocol

During exercise in the heat with fluid restriction (dehydration condition) participants had significantly higher heart rate values than during exercise in the euhydration condition:  $148 \pm 16$  bpm vs  $118 \pm 20$  bpm ( $P < 0.001$ ). There was also a trend for ventilation to be higher in the dehydration condition compared to the euhydration condition:  $42 \pm 15$  l·min<sup>-1</sup> vs  $34 \pm 6$  l·min<sup>-1</sup> ( $P = 0.084$ ).

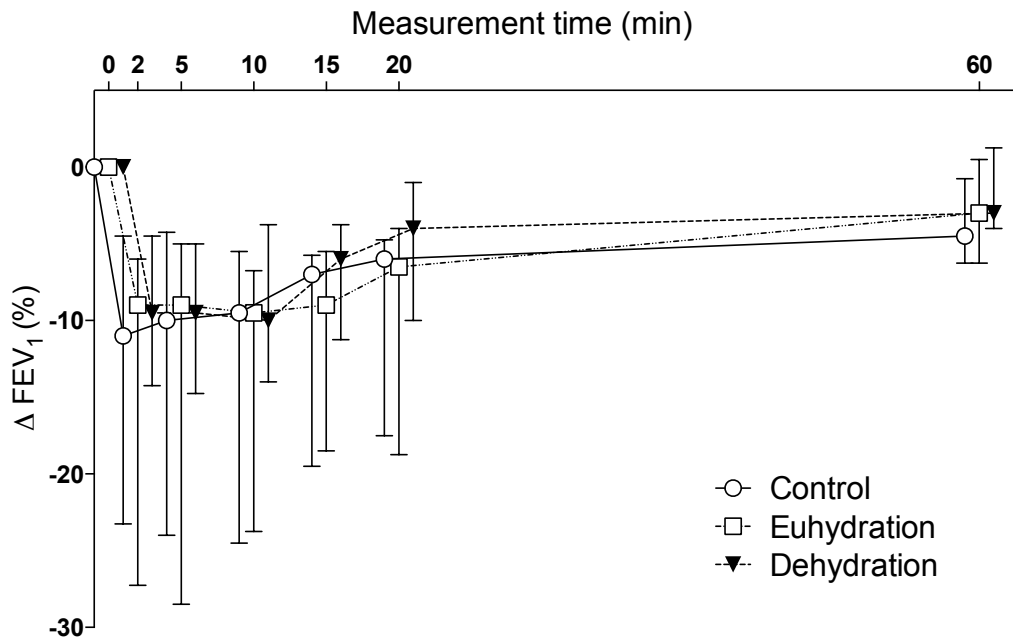
There was no significant difference in nude body mass at baseline between the conditions ( $P = 0.742$ ). Exercise in the dehydration condition caused significant reduction in nude body mass, from  $63.3 \pm 10.4$  kg at baseline to  $61.8 \pm 10.1$  kg post-exercise ( $P < 0.001$ ). There was no change in nude body mass following exercise in

the euhydration condition or following the resting period in the control condition ( $P>0.05$ ). The reduction in nude body mass in the dehydration condition of  $2.3 \pm 0.8\%$  (95% CI, 1.7 to 2.9%) was significantly greater than in the euhydration and control condition:  $0.3 \pm 0.5\%$  (95% CI; -0.1 to 0.6%) and  $0.2 \pm 0.5\%$  (95% CI; -0.1 to 0.7%), respectively ( $P<0.001$ ). In the dehydration condition, participants drank  $830 \pm 190$  ml during the rehydration phase, which equated to approximately  $61 \pm 19\%$  of their loss in body mass.

#### **4-3.3 Airway response to EVH**

Participants achieved a mean ventilation of  $104 \pm 29$  l·min<sup>-1</sup> during EVH over the three experimental visits, which corresponds to  $70 \pm 9\%$  of their predicted MMV. No between-condition differences were noticed for ventilation ( $P=0.639$ ).

The median and interquartile range (Q1 – Q3) for maximum reduction in FEV<sub>1</sub> post-EVH was, 12% (7 - 20%), 11% (9 - 24%) and 13% (7 – 15%) in the control, euhydration and dehydration conditions respectively. These values were not different between conditions ( $P=0.196$ ). Similarly, there was no significant difference between conditions for FEV<sub>1</sub>-AUC<sub>0-60</sub> (Figure 4.2,  $P=0.150$ ), indicating that dehydration had no effect on the severity of bronchoconstriction following EVH.



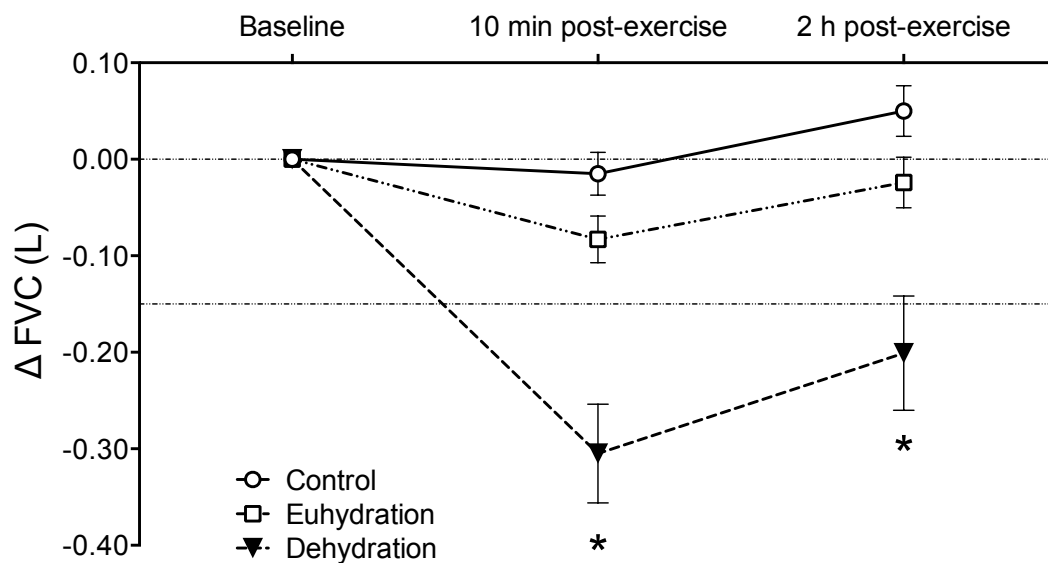
**Figure 4.2.** Change in forced expiratory volume in 1 sec (FEV<sub>1</sub>) following 6 min of hyperpnoea of dry air in a dehydrated condition (i.e., 2 h of exercise in the heat with fluid restriction), a euhydrated condition (i.e., 2 h of exercise in ambient conditions with voluntary fluid consumption) and a control condition (i.e., 2 h of rest with voluntary fluid consumption) in symptomatic recreational athletes. Results displayed are median and inter-quartile range. There was no difference in the area under the FEV<sub>1</sub> AUC<sub>0-60</sub> between conditions ( $P=0.150$ ).

#### 4-3.4 Lung function response to dehydration

##### *Spirometry*

Dehydration caused a significant reduction in FVC, from  $5.09 \pm 1.22$  l at baseline to  $4.79 \pm 1.10$  l at 10 min post-exercise ( $P=0.001$ , Table 4.1); this equates to a reduction of  $310 \pm 160$  ml (95% CI; 190 to 420 mL). FVC remained lower than baseline after 2 h of recovery ( $4.89 \pm 1.10$  l,  $P=0.024$ ) and showed a reduction of  $200 \pm 190$  ml (95% CI; 70 to 340 ml) from baseline level. A smaller, yet statistically significant ( $P=0.022$ ) reduction in FVC was noted in the euhydration condition at 10

min post-exercise: the reduction from baseline was  $80 \pm 80$  ml (95% CI; 30 to 140 ml). This difference did not remain at 1 h post-exercise ( $P=1.000$ , Table 4.1). The reduction in FVC was significantly greater in the dehydration condition compared to the control and the euhydration conditions at both, 10 min ( $P=0.001$ ) and 1 h ( $P=0.024$ ) post-exercise (Figure 4.3). Following rehydration, FVC remained slightly, but significantly lower ( $P=0.022$ ) than baseline: the reduction from baseline was  $90 \pm 100$  ml (95% CI; 20 to 160, Table 4.1). Interestingly, dehydration had no significant effect on FEV<sub>1</sub> or PEF (Table 4.1).



**Figure 4.3.** Change in forced vital capacity (FVC) from baseline following 2 h of exercise in the heat with fluid restriction (dehydration condition), 2 h of exercise in ambient conditions with voluntary fluid consumption (euhydration condition) and 2 h of rest with voluntary fluid consumption (control condition) in symptomatic recreational athletes; Results displayed are mean  $\pm$  SD; \*  $P<0.05$ , significantly different from control and euhydration condition.

**Table 4.1.** Spirometry measurements at baseline, 10 min post-exercise, 2 h post-exercise and post-rehydration (1 h following eucapnic voluntary hyperpnoea).

		Control	Euhydration	Dehydration
FEV <sub>1</sub> (l)	Baseline	4.18 ± 0.85	4.17 ± 0.87	4.21 ± 0.89
	10 min post-exercise	4.28 ± 0.90	4.20 ± 0.96	4.24 ± 0.90
	2 h post-exercise	4.30 ± 0.92	4.24 ± 0.93	4.23 ± 0.89
	Rehydration (1 h post-EVH)	4.10 ± 0.81	4.10 ± 0.90	4.19 ± 0.94
FVC (l)	Baseline	5.12 ± 1.19	5.09 ± 1.23	5.09 ± 1.22
	10 min post-exercise	5.10 ± 1.17	5.00 ± 1.21*	4.79 ± 1.10*, <sup>C,E</sup>
	2 h post-exercise	5.17 ± 1.25	5.06 ± 1.20	4.89 ± 1.10*, <sup>C,E</sup>
	Rehydration (1 h post-EVH)	5.06 ± 1.21	5.03 ± 1.25	5.00 ± 1.20*
PEF (l·s <sup>-1</sup> )	Baseline	9.20 ± 2.10	9.12 ± 2.12	9.13 ± 2.25
	10 min post-exercise	9.64 ± 2.44	9.47 ± 2.47	9.16 ± 2.01
	2 h post-exercise	9.62 ± 2.32	9.36 ± 2.31	9.12 ± 2.16
	Rehydration (1 h post-EVH)	8.89 ± 1.94	9.10 ± 2.40	8.90 ± 2.17

Mean ± SD; FEV<sub>1</sub>, Forced expiratory volume in 1 s; FVC, forced vital capacity; PEF, peak expiratory flow; \* different to baseline  $P < 0.05$ , <sup>C</sup> different to control  $P < 0.05$ ; <sup>E</sup> different to euhydration  $P < 0.05$ .

#### 4-3.5 Whole-body plethysmography, DLCO and airway resistance

A small, yet statistically significant time effect was noticed for VC ( $P = 0.011$ ), indicating a reduction in this parameter after 2 h of exercise / rest in all conditions (Table 4.2). However, only in the dehydration condition did the difference with baseline appear of clinical significance, with a delta value of  $220 \pm 170$  mL (95% CI 100 to 350 mL, Table 4.2). Moreover, this reduction in VC at 1 h post-exercise was significantly correlated with the change in FVC at 2 h post-exercise ( $r = 0.645$ ,  $P = 0.044$ ).

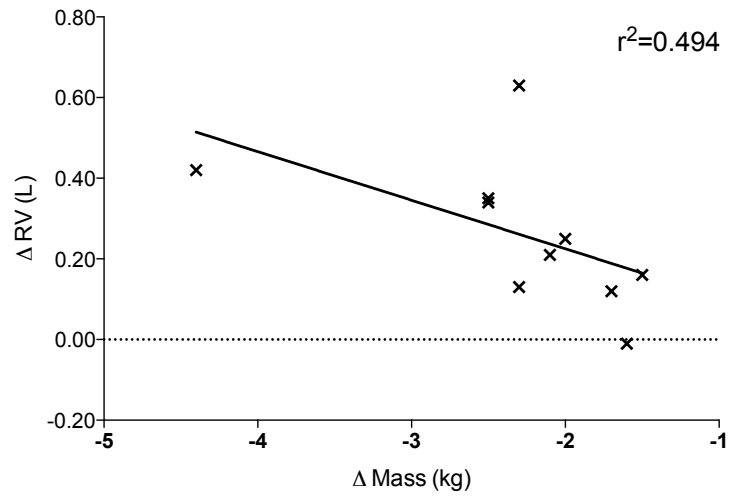
Dehydration caused a significant increase ( $P=0.011$ ) in FRC of  $260 \pm 250$  ml (95% CI; 80 to 440), as well as a significant increase ( $P=0.001$ ) in RV of  $260 \pm 180$  ml (95% CI; 130 to 390, Table 4.2). A significant time effect was noticed for the difference in TLC measured by plethysmography and helium dilution ( $P=0.013$ , Table 4.2).

A significant time effect ( $P=0.014$ ) was noticed for Raw, indicating a reduction from baseline to post-exercise/rest that was not significantly different between conditions (Table 4.2). sRaw, on the other hand, showed a significant interaction effect ( $P=0.010$ ). Post-exercise sRaw in the dehydration condition was significantly greater than in the control condition ( $P=0.030$ ). Furthermore, a similar trend was noticed when comparing the dehydrated and euhydration conditions ( $P=0.077$ ), with higher sRaw values observed in the dehydrated state (Table 4.2). Dehydration had no effect on the diffusing capacity for carbon monoxide (Table 4.2).

### ***Correlation between changes in body mass and changes in lung volumes***

There was a significant negative correlation ( $r=-0.703$ ,  $P=0.023$ ) between the change in body mass and the change in RV (Figure 4.4) at 1 h post-exercise. No other significant correlations were found.





**Figure 4.4.** Relationship between change in nude body mass and residual volume following 2 h of exercise in the heat with fluid restriction.

**Table 4.2.** Lung function data following 2 h of exercise in the heat with fluid restriction (dehydration condition), 2 h of exercise in ambient conditions with voluntary fluid consumption (euhydration condition) and 2 h of rest with voluntary fluid consumption (control condition) in symptomatic recreational athletes.

	Control		Euhydration		Dehydration	
	Baseline	Post-rest	Baseline	Post-exercise	Baseline	Post-exercise
FRC (l)	3.49 ± 0.97	3.55 ± 1.02	3.46 ± 1.02	3.35 ± 0.95 <sup>C</sup>	3.40 ± 0.99	3.65 ± 0.90 <sup>*E</sup>
RV (l)	1.77 ± 0.55	1.81 ± 0.59	1.76 ± 0.45	1.74 ± 0.51	1.73 ± 0.46	1.99 ± 0.57 <sup>*E</sup>
ERV (l)	1.72 ± 0.61	1.74 ± 0.66	1.71 ± 0.67	1.61 ± 0.56	1.67 ± 0.64	1.67 ± 0.48
VC (l)	4.94 ± 1.20	4.91 ± 1.18 <sup>t</sup>	4.96 ± 1.17	4.92 ± 1.19 <sup>t</sup>	4.97 ± 1.21	4.75 ± 1.12 <sup>t</sup>
TLC <sub>pleth</sub> (l)	6.72 ± 1.66	6.71 ± 1.59	6.72 ± 1.55	6.66 ± 1.62	6.70 ± 1.58	6.74 ± 1.61
TLC <sub>pleth</sub> -TLC <sub>he</sub> (l)	0.09 ± 0.64	0.40 ± 0.40 <sup>t</sup>	0.26 ± 0.58	0.43 ± 0.53 <sup>t</sup>	0.30 ± 0.67	0.61 ± 0.48 <sup>t</sup>
DLCOC (mmol·min <sup>-1</sup> ·kPa <sup>-1</sup> )	10.16 ± 2.87	9.71 ± 2.61	9.92 ± 2.69	9.72 ± 2.53	10.14 ± 2.81	10.07 ± 2.85
KCOc (mmol·min <sup>-1</sup> ·kPa <sup>-1</sup> ·l <sup>-1</sup> )	1.63 ± 0.27	1.57 ± 0.25	1.65 ± 0.25	1.60 ± 0.22	1.65 ± 0.22	1.63 ± 0.20
Raw (kPa·s·l <sup>-1</sup> )	0.26 ± 0.09	0.24 ± 0.07 <sup>t</sup>	0.28 ± 0.09	0.25 ± 0.07 <sup>t</sup>	0.25 ± 0.06	0.25 ± 0.06 <sup>t</sup>
sRaw (kPa·s <sup>-1</sup> )	0.96 ± 0.21	0.89 ± 0.17	1.05 ± 0.23	0.90 ± 0.14 <sup>*</sup>	0.91 ± 0.13	0.98 ± 0.17 <sup>Ce</sup>

Mean ± SD. FRC, functional residual capacity; RV, residual volume; ERV, expiratory reserve volume; VC, vital capacity; TLC<sub>pleth</sub>, Total lung capacity; TLC<sub>pleth</sub>-TLC<sub>he</sub>, difference between TLC measure by whole-body plethysmography and helium dilution, DLCOC, diffusing capacity for carbon monoxide; KCOc, transfer coefficient; Raw, airway resistance; sRaw specific airway resistance; \*  $P < 0.05$ , significantly different from baseline; <sup>C</sup> different to control  $P < 0.05$ ; <sup>E</sup> different to euhydration condition  $P < 0.05$ ; <sup>e</sup> trend towards different from euhydration condition  $P = 0.077$ ; <sup>t</sup> significant time effect ( $P < 0.05$ ).

## 4-4 Discussion

The aim of this study was to determine the effect of whole-body dehydration on airway responsiveness and lung function in recreational athletes with respiratory symptoms on exertion. We showed that exercise-induced dehydration (~2.3%) had no effect on the airway response to dry air hyperpnoea. We did however note a reduction in FVC and an increase in  $FRC_{pleth}$  and  $RV_{pleth}$  in the mild dehydrated state, suggesting that dehydration has a negative impact on small airway function.

This is the first study to assess the effect of whole-body dehydration on airway responsiveness to dry air. We reasoned that whole-body dehydration may have the potential to affect the volume and composition of ASL and, consequently, could enhance the osmotic stimulus responsible for hyperpnoea-induced bronchoconstriction (Anderson & Daviskas, 2000). That no difference in the severity of airway narrowing was noticed following EVH in the dehydration condition contradicts our hypothesis.

In this study, we induced a state of dehydration that was only mild in nature; hence, this may not have been sufficient to interfere with local hydration of the airways. It is also possible that, in our population consisting of individuals with a previous diagnosis of asthma and/or EIB, the regulation of ASL was altered by their medical conditions. The glands of the respiratory epithelium and the sweat glands epithelium share an identical mechanism for water secretion, which is mediated by muscarinic receptor (m3 subtype) activation. Park and colleagues (2008)

demonstrated a reduced pilocarpine-induced sweat secretion in individuals with asthma, which negatively correlated with the severity of bronchoconstriction following a methacholine challenge. The authors proposed that the observed reduction in sweat secretion might also reflect a reduction in fluid secretion at the airway surface in individuals with asthma. If water secretion towards the airway surface is already reduced in individuals with asthma, the potential for whole-body dehydration (*via* increased plasma osmolarity and decreased plasma volume) to further reduce water secretion may be limited. This may explain the lack of difference in the airway response to dry air hyperpnoea in a dehydrated and euhydrated state in our subjects. It should however be acknowledged that the findings of a diminished sweat response in individuals with asthma have been contested (Laitano et al., 2008).

The volume of ASL is very small, indeed calculations from Anderson (1984) suggest that the volume of ASL over the first 10 generations of airways is <1 ml. Relative to this small volume of ASL, the volume of water lost during hyperpnoea of dry air can be very high. In a mathematical model developed by Daviskas and colleagues (1991), it was proposed that water loss can exceed 1 ml *per* minute during ventilation at 60 l·min<sup>-1</sup> in temperate conditions. In the present study, ventilation averaged 104 l·min<sup>-1</sup> and inspired air, delivered through medical air canisters, was very dry (i.e., ~0% RH). It is therefore possible that any change to the volume of the ASL, caused by whole body dehydration, was relatively small in comparison to the

water loss during hyperpnoea of dry air. This could account for the absence of any effect of whole-body dehydration on airway responsiveness.

An alternative explanation is that 6 min of hyperpnoea of dry air provoked a maximal airway response. A maximum response plateau is a common feature of non- and mildly-asthmatic individuals during methacholine and histamine challenges (James, Loughed, Pearce-Pinto, Ryan, & Musk, 1992; Woolcock, Salome, & Yan, 1984). Similarly, a maximum response plateau has been shown to occur following bronchial provocation with exercise in asthmatic children, with no further increase in the severity of bronchoconstriction past 6 min of exercise (Godfrey, Silverman, & Anderson, 1975). If, in the present study, 6 minutes of hyperpnoea of dry air produced a maximum airway response in the euhydrated condition, it is likely that in the dehydrated state the effect of our intervention was masked. Since a maximal response plateau occurs less frequently in individuals with a greater degree of airway responsiveness (Woolcock et al., 1984), our findings should not be generalised to individuals with moderate-to-severe EIB.

Given that 50% of individuals with asthma display a refractory response to a second exercise challenge when completed within 1 h of the initial challenge (Larsson, Dahlén, Dahlén, & Anderson, 2013), one could argue that the exercise intervention itself may have acted as a confounding factor on our airway responsiveness results. However we do not believe this to be the case for a number of reasons. Firstly, a 2

h period was given between the end of the exercise and the start of the EVH challenge; the majority of individuals will not be refractory past 2 h of recovery (Edmunds, Tooley, & Godfrey, 1978). Secondly, the intensity of the exercise was very light; the mean VE over both visits was  $38 \pm 12 \text{ l}\cdot\text{min}^{-1}$ . Therefore it is unlikely that the level of osmotic stress applied to the airways during the exercise period was enough to elicit any significant change in airway calibre; that we did not observe any change in FEV<sub>1</sub> or PEF at 10 min post-exercise supports this idea. Finally, if we did induce a state of refractoriness following exercise, we would have expected to observe a smaller airway response in the euhydration condition compared to the control condition; however such was not the case (median fall in FEV<sub>1</sub> was 11% and 12% in the control and euhydration condition, respectively). Therefore we can be confident that the exercise protocol we used to induce a state of dehydration did not cause airway refractoriness.

The secondary aim of this study was to establish the effect of whole-body dehydration on resting lung function. In contrast to previous research (Govindaraj, 1972; Javaheri et al., 1987), our results suggest that dehydration caused a sustained reduction in FVC, with no associated change in FEV<sub>1</sub>. Previously, induced dehydration by either 16 h of fluid deprivation (Govindaraj, 1972) or diuretic drug administration (Javaheri et al., 1987) had no effect on FVC. However both types of interventions caused either a decrease (Govindaraj, 1972) or an increase (Javaheri et al., 1987) in FEV<sub>1</sub>. The divergence in the results may be due to the various dehydration protocols employed. Fluid deprivation for 16 h resulted in much

smaller decreases in body mass [range: 0.0 to 2.5%, (Javaheri et al., 1987)] than the present study (range: 1.5 to 4.4%). Conversely, whilst a larger reduction in body mass was observed after diuretic administration (~4.5 %), the different mechanism of water loss may have resulted in different osmotic gradients across the airway epithelium. Indeed, whilst sweat loss during exercise mainly drives intracellular water loss, diuretic administration causes extracellular dehydration (Cheuvront & Kenefick, 2014); this latter condition could therefore result in limited loss of water at the surface of the airways, and therefore have limited impact on small airway lung function.

The reduction in FVC measured in the present study by spirometry fits well with the concomitant increase in RV and FRC noted through whole-body plethysmography. Taken together these results suggest that dehydration has an adverse effect on small airway function. We propose that the primary underlying mechanism for these changes is reduced peripheral airway stability caused by a change in the properties and/or volume of the airway surface liquid during periods of dehydration. Airway surface liquid has low surface tension that inhibits small airway closure at low lung volumes (Macklem, Proctor, & Hogg, 1970). If whole body-dehydration increases airway surface tension, it would explain the reduction in FVC. At present, there is no data pertaining to the effect of airway surface dehydration on surface tension; however, cystic fibrosis (a condition that causes depletion of ASL) may offer a reasonable model. Inhalation of a dry powder of mannitol – a treatment known to increase the secretion of fluid to the airway surface (Daviskas,

Anderson, & Eberl, 1999) – has been shown to reduce surface tension (Daviskas, Anderson, Jaques, & Charlton, 2010) and to increase FVC in individuals with cystic fibrosis (Teper, Jaques, & Charlton, 2011). Therefore, a reduction in FVC and increase in RV in the present study could be caused by increased surface tension induced by changes in the volume and/or properties of the ASL during exercise induced whole-body dehydration.

Dehydration levels of 2.3% and 2.9% loss of body mass have previously been reported following marathon (Zouhal et al., 2011) and ultra marathon events (Rüst et al., 2012), respectively, with some individuals losing up to 8% body mass (Rüst et al., 2012; Zouhal et al., 2011). Our data suggests that dehydration of 2.3% can result in >200 ml reduction in FVC; a value that the ERS/ATS regard as clinically significant (Pellegrino, 2005). The clinical significance of a reduced FVC in an exercise context has yet to be determined. Given that end expiratory lung volume (EELV) decreases with exercise, and given that whole-body dehydration may affect peripheral airway stability at low lung volumes, we propose that whole body dehydration may lead to the cyclic opening and closure of peripheral airways. *In vitro* the re-opening of closed airways can cause epithelial injury (Bilek et al., 2003). As repeated epithelial injury in athletes has been proposed as a key factor in the pathogenesis of EIB (Anderson & Kippelen, 2005), these findings could be of importance to athletes who regularly experience whole-body dehydration during exercise.



In conclusion, with this study we demonstrated that whole-body dehydration does not exacerbate airway responsiveness in symptomatic, recreational athletes. These results suggest that whole-body dehydration has limited impact on local airway dehydration during hyperpnoea of dry air. We also documented for the first time that exercise-induced whole-body dehydration causes a decrease in FVC and an increase in RV. Whole-body dehydration may therefore affect peripheral airway stability and further investigations are warranted to determine the functional and clinical relevance of these findings.

## **CHAPTER 5**

### **STUDY 2 - Effect of terbutaline on hyperpnoea-induced bronchoconstriction and urinary CC16 release in athletes**

## 5-1 Introduction

The main stimulus for EIB is evaporative water and heat loss from the airway surface as a consequence of heating and humidifying large volumes of unconditioned air (Anderson & Daviskas, 2000). While EIB can be found in both, the general population and in elite athletes, the latter are more at risk (Carlsen et al., 2008) and often develop EIB later in life (Fitch, 2006). It has therefore been proposed that, in athletes, EIB is a consequence of repeated injury and repair of the airway epithelium in response to long hours of strenuous training (Anderson & Kippelen, 2005).

During exercise, as ventilation increases, the conditioning of inspired air extends progressively towards the peripheral airways. If the replacement of water to the airway surface is insufficient, dehydration injury of the distal airways may occur (Anderson & Kippelen, 2005). In addition, mechanical stress associated with high airflow and / or bronchoconstriction may accelerate disruption of the epithelial cell layer (Kippelen & Anderson, 2012). Following epithelial injury, bulk plasma may leak from the microcirculation to repair the damage to the epithelial cells (Persson et al., 1996). The process of plasma exudation could then expose the airway smooth muscle to substances that alter its growth and affect its contractile properties, leading to the development of AHR and EIB in susceptible individuals (Anderson & Kippelen, 2005).

In support of this idea, increased levels of bronchial epithelial cells have been found in induced sputum of asthmatic patients with EIB (Hallstrand, Moody, Aitken, &

Henderson, 2005a), as well as in various athletic populations [elite swimmers (Bougault, Turmel, St-Laurent, Bertrand, & Boulet, 2009) and recreational road-runners (Chimenti et al., 2010)]. Increases in tenascin expression and inflammatory cell counts in the lung biopsies of elite cross-country skiers (Karjalainen et al., 2000) and of elite swimmers (Bougault et al., 2012) have also provided direct evidence of repeated injury-repair within the airways of athletes.

Using the concentration of the lung specific club cell protein (CC16) in extra-pulmonary fluids, our group recently established that dry air hyperpnoea causes an acute perturbation of the airway epithelium (Bolger, Tufvesson, Sue-Chu, et al., 2011b). Consistent with this finding, serum and urinary CC16 concentrations have also been shown to increase following bouts of cycling (Broeckaert et al., 2000), running (Chimenti et al., 2010; Nanson, Burgess, Robin, & Bernard, 2001) and swimming (Carbonnelle et al., 2002; Font-Ribera et al., 2010; Romberg et al., 2011). In a recent study on running (Bolger, Tufvesson, Anderson, et al., 2011a), inhalation of warm-humid air attenuated the rise in urinary CC16 post-exercise (likely as a result of a reduced water loss from the airway surface). We are now aiming to establish whether pharmacological agents also have the potency to blunt, or even to completely abolish, the CC16 response associated with exercise hyperpnoea.

In a healthy human epithelium, airway surface liquid is mainly regulated *via* apical Cl<sup>-</sup> secretion and Na<sup>+</sup> absorption; passive flow of water occurring along the osmotic gradient (Hollenhorst, Richter, & Fronius, 2011). Some pharmacological agents have the potential to modify ion transport. *In vitro*, the  $\beta_2$ -agonist terbutaline has

been shown to increase the transport of  $\text{Cl}^-$  ions towards the airway lumen (Davis et al., 1979). Moreover, in canine peripheral airways challenged with dry air, infusion of terbutaline attenuated airway narrowing and reduced epithelial cell shedding (Wang et al., 1992). To date, the efficacy of pharmacological agents administered directly to the airways has not been tested in relation to preventing epithelial injury. We reasoned that if terbutaline facilitates replacement of water at the airway surface level during exposure to dry air, pre-treatment with inhaled terbutaline may attenuate hyperpnoea-induced airway injury in humans.

In this study, we tested the efficacy of a single, therapeutic dose of inhaled terbutaline at reducing hyperpnoea-induced airway epithelial perturbation in athletes. We also aimed to confirm the broncho-protective effect of terbutaline in athletes with EIB. Our hypothesis was that 0.5 mg terbutaline will attenuate the increase in urinary concentration of CC16 and the fall in forced expiratory volume in 1 s ( $\text{FEV}_1$ ) following 8 min of eucapnic voluntary hyperpnoea (EVH) with dry air in athletes with EIB.

## 5-2 Methods

### 5-2.1 Subjects

The study population consisted of 27 athletes with EIB. EIB was confirmed by a fall of  $\geq 10\%$  in FEV<sub>1</sub> following an 8 min EVH challenge during a screening visit.

Participants were non-smokers, free from respiratory infections for 4 weeks prior to the study, and with no known chronic medical condition other than asthma and/or EIB. Regular swimmers ( $>1$  h *per week*) were excluded. Participants abstained from alcohol, caffeine and exercise on the day of testing, and medication was withheld as follows: short acting  $\beta_2$ -agonist treatments were withheld for a minimum of 8 h, long acting  $\beta_2$ -agonist treatments for 24 h, inhaled corticosteroid treatments for 12 h, and combination therapies of long acting  $\beta_2$ -agonist + inhaled corticosteroid treatments for 24 h (Miller et al., 2005). The study was approved by the UK National Health Service Research Ethics Committee (NHS REC reference number: 10/H0716/30).

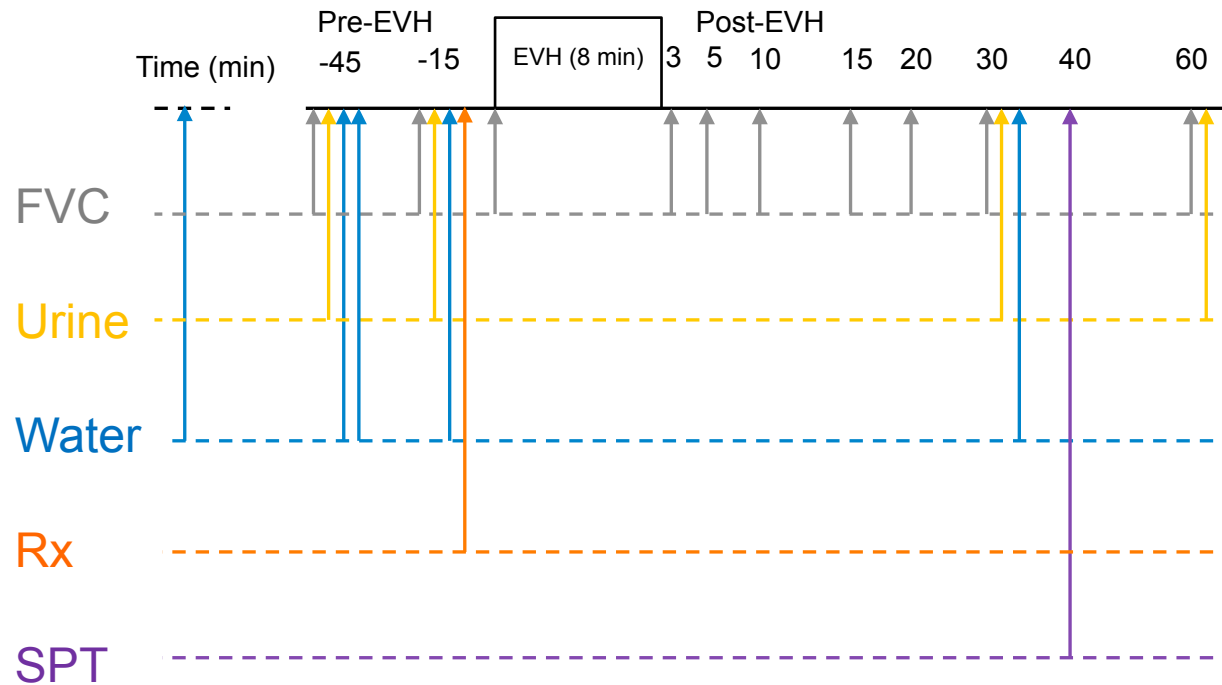
### 5-2.2 Experimental design

The study used a randomised, double-blind, placebo-controlled, crossover experimental design. All participants attended two experimental visits, separated by at least 2 days, but no more than 3 weeks. During the visits participants were administered either 0.5 mg of terbutaline or a placebo 15 min prior to completion of an 8 min EVH challenge (see general methods). The primary end points were

changes in urinary CC16 concentration and the maximum fall in FEV<sub>1</sub>. A schematic of the study protocol is presented in Figure 5.1.

Experimental visits were conducted in the morning between 8.00 and 11.00 am [to standardise for fluctuations in lung function (Lebowitz et al., 1997) and CC16 (Andersson et al., 2007) throughout the day] and started with the recording of baseline lung function. FVC manoeuvres were performed on a spirometer and followed international recommendation (see general methods). The active drug (i.e., 0.5 mg of terbutaline) was administered *via* a dry powder inhaler (Bricanyl Turbohaler®, Astra Zeneca, London, England). An empty demonstration Turbohaler® was used for administration of the placebo. Subjects were instructed to take one deep, hard inhalation of the drug or placebo, and to hold their breath for 10 s. Post-treatment lung function manoeuvres were repeated at 10 min. The EVH challenge started 15 min after treatment. Spontaneous recovery of FEV<sub>1</sub> to baseline levels following the EVH challenge was measured at 2, 5, 10, 15, 20, 30 and 60 min.

Subjects ingested 200 ml of water 1 h before each visit. They ingested a further 400 ml upon arrival at the laboratory, and then 200 ml every 30 min. Two baseline urine samples were obtained: the first, on arrival at the laboratory, which was discarded; the second, 30-60 min later and immediately before administration of the drug, which was used as baseline. Further urine samples were collected 30 and 60 min after the EVH challenge. All samples were stored without preservatives at -80°C. The atopic status of the participants was determined by a skin prick test conducted during the first experimental visit (as described in general methods).



**Figure 5.1.** Schematic of the experimental protocol used in Study 2. FVC, forced vital capacity manoeuvre; Urine, urine sample collected; Water, 200 ml of water consumed; Rx; treatment given; SPT, skin prick test (first experimental visit only).



### ***CC16 analysis***

Urine samples were analysed for CC16 using a commercially available sandwich ELISA kit from BioVendor (Modrice, Czech Republic) as described in the general methods section. Urine samples were also analysed for creatinine (see general methods) and results are expressed as picograms of excreted mediator *per* micromole of creatinine.

### ***Lung function measurements***

Spirometry was conducted according to the ATS/ERS guidelines (Wanger et al., 2005). The maximum fall in FEV<sub>1</sub> was expressed as a percentage from the post-treatment value. The degree of broncho-protection afforded by terbutaline was calculated by subtracting the maximum fall in FEV<sub>1</sub> on the drug treatment day from the maximum fall in FEV<sub>1</sub> on the placebo day, and expressing it as a percentage of the placebo.

### ***Data Analysis***

Sample size requirement was dictated by previous work (Bolger, Tufvesson, Anderson, et al., 2011a). With 21 participants Bolger and colleagues (2011a) were previously able to detect a between-condition effect in urinary CC16 excretion following bronchial provocation challenge. To accommodate for the likely loss of some samples (due to CC16 being below the level of detection) 27 participants were recruited for this study.

All data were checked for normality using Shapiro-Wilk test. CC16 data were not normally distributed; therefore, natural logarithmic transformation was applied. Differences between conditions for FEV<sub>1</sub> (in l) and CC16 were analysed using paired sample t-test. The maximum fall in FEV<sub>1</sub> and FVC were expressed as a percentage from the post-treatment value. The trapezoidal method was used to calculate the area under the FEV<sub>1</sub> time curve (FEV<sub>1</sub>-AUC<sub>0-60</sub>) and was calculated from the post-treatment FEV<sub>1</sub> value. FEV<sub>1</sub> maximum fall post-EVH (in %) and FEV<sub>1</sub>-AUC<sub>0-60</sub> were non-normally distributed and therefore analysed using a Wilcoxon-signed rank test.

Differences between conditions and times were analysed using a repeated-measures ANOVA. In cases of statistical significance, least significance difference pairwise comparisons were conducted post-hoc. Additional analyses were carried out to determine potential confounding factors: i.e., gender, asthma treatment, age, severity of bronchoconstriction, degree of bronchoprotection and bronchodilator response. Between-subject factors for gender and for inhaled  $\beta_2$ -agonist or inhaled corticosteroid use were entered into the repeated-measures ANOVA. Furthermore, Pearson's correlation coefficient and multiple regression

analyses for within-subject repeated measures (Bland & Altman, 1995) were conducted to determine relationships between CC16 excretion and age, severity of bronchoconstriction following EVH, broncho-protection afforded by terbutaline, and bronchodilator response to terbutaline. All data were analysed using a statistical software package (SPSS 20, Chicago, IL, US).

## **5-3 Results**

### **5-3.1 Participant characteristics**

Twenty-seven participants (11 females), aged 18 to 49 (mean:  $27 \pm 9$  yr), completed the study. Mean height and body mass were  $174 \pm 8$  cm and  $74 \pm 13$  kg, respectively. Participants were involved in summer sports (athletics, cycling, football, rugby or rowing) and trained for  $8 \pm 3$  h *per* week in aerobic activities.

Twenty three participants competed at a local level and three competed at national or international level. Participants had  $9 \pm 6$  yr of experience in their sport.

Participants' medical diagnosis, current treatment and atopic status are presented in Table 5.1. One participant had an underlying diagnosis of Crohn's disease.

However, at the time of the study, he was asymptomatic and was not treated for this condition. During the screening visit baseline FEV<sub>1</sub> was >80% predicted in all participants and the fall in FEV<sub>1</sub> post-EVH averaged  $19 \pm 10\%$  (Table 5.1).

### 5-3.2 Baseline lung function and ventilation level during EVH

There was no difference between experimental visits in baseline lung function parameters (Table 5.2). Terbutaline had a small ( $5 \pm 3\%$  increase in  $FEV_1$ ), but statistically significant bronchodilator effect ( $P < 0.001$ , Table 5.2).  $FEF_{25-75}$ ,  $FEF_{25}$ ,  $FEF_{50}$ ,  $FEF_{75}$  and PEF significantly increased post-administration of terbutaline ( $P < 0.001$ ), whilst FVC remained unchanged (Table 5.2). No significant change in lung function was observed following administration of the placebo (Table 5.2). Minute ventilation was slightly but significantly larger in the terbutaline condition compared to the placebo condition:  $102 \pm 20$  L vs  $101 \pm 20$  L ( $P = 0.047$ ). Participants reached  $80 \pm 8\%$  MVV during the terbutaline visit and  $78 \pm 7\%$  MVV during the placebo visit ( $P = 0.022$ ).

### 5-3.3 Airway Response to EVH

Terbutaline significantly inhibited the airway response to EVH. The maximum fall in  $FEV_1$  was reduced from  $17 \pm 8\%$  in the placebo condition to  $8 \pm 5\%$  in the terbutaline condition ( $P < 0.001$ , Table 5.2).  $FEV_1$ -AUC<sub>0-60</sub> was significantly reduced following terbutaline administration: from  $425 \pm 283\% \cdot \text{min}$  to  $163 \pm 146\% \cdot \text{min}$  ( $P < 0.001$ , Figure 5.2). The broncho-protection offered by terbutaline for max % fall in  $FEV_1$  and  $FEV_1$  AUC<sub>0-60</sub> was  $54 \pm 28\%$  (range 0-94%) and  $60 \pm 30\%$  (range 0-100%), respectively. Terbutaline provided complete protection (i.e., post-EVH max % fall in  $FEV_1 < 10\%$ ) to 22 of the 27 participants (81%).

EVH caused a significant decrease in FVC and in all expiratory flow values in both conditions ( $P < 0.001$ , Table 5.2). The decrease in FVC post-EVH was significantly

attenuated with the administration of terbutaline (from  $0.28 \pm 0.19$  l to  $0.12 \pm 0.11$  l,  $P < 0.001$ ).

**Table 5.1.** Participant characteristics

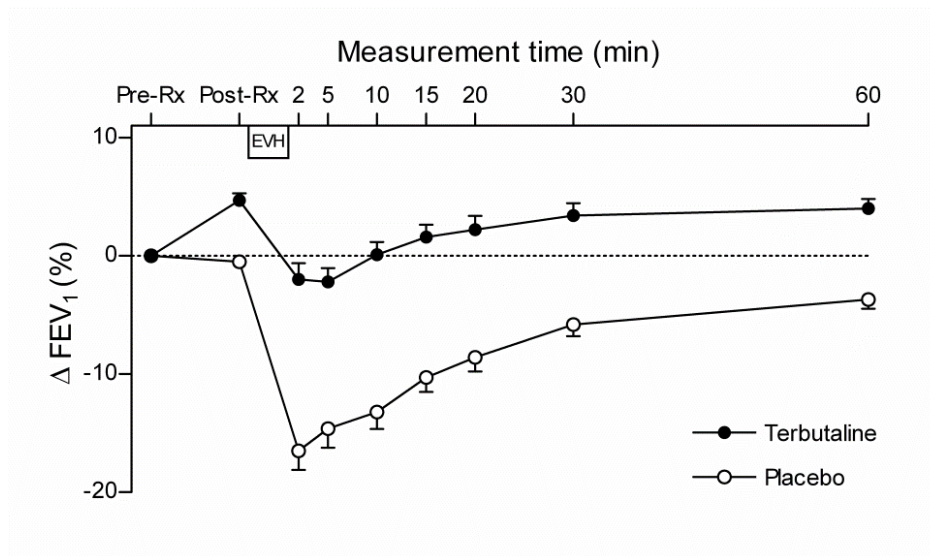
ID	Gender	Atopic status	Baseline FEV <sub>1</sub> (% pred.)	Previous diagnosis	Treatment	Prescribed ICS dose (µg/day)	Post-EVH max. FEV <sub>1</sub> fall (%)			Protection (%)
							Screening	Placebo	Terbutaline	
1	M	+	95	Asthma + EIB	Combination, SABA	1600	17	18	15	17
2	M	+	92	Asthma	SABA, ICS	200	50	13	5	62
3	M	+	99	Asthma + EIB	Combination	400	16	12	18	0
4	F	+	108	EIB	SABA, ICS	200	21	13	12	8
5	M	+	97	-	-	-	16	15	4	73
6	F	+	92	Asthma	SABA, ICS	200	10	14	8	43
7	M	+	93	Asthma + EIB	SABA	-	23	21	8	63
8	M	+	102	Asthma	SABA	-	21	12	5	58
9	F	+	93	Asthma	Combination	400	10	13	2	85
10	M	+	99	Asthma	SABA, ICS	600	36	26	25	4
11	M	-	99	-	-	-	11	11	6	45
12	M	+	85	Asthma	-	-	9	14	6	57
13	F	+	103	Asthma	-	-	27	29	9	69
14	M	+	109	EIB	SABA	-	14	11	7	36
15	F	-	99	Asthma + EIB	Combination	400	19	16	7	56
16	F	-	109	-	-	-	12	20	11	45
17	M	+	94	Asthma	SABA	-	18	17	1	94
18	M	-	85	Asthma	Combination	200	14	13	6	54
19	M	+	109	Asthma + EIB	ICS	100	14	15	9	40
20	F	-	96	Asthma	SABA	-	12	10	8	20
21	M	+	94	Asthma	SABA	-	15	13	7	46
22	M	+	111	Asthma	SABA, ICS	200	16	16	1	94
23	F	+	102	EIB	SABA, ICS	200	23	28	6	79
24	F	+	86	Asthma	SABA	-	28	22	8	64
25	F	-	88	EIB	-	-	10	11	1	91
26	F	+	82	Asthma + EIB	SABA	-	46	47	6	87
27	M	+	91	Asthma	SABA	-	16	11	2	81
Mean ± SD			97±8				19±10	17±8	8±5***	54±28

Atopic status: +, positive skin prick response to house dust mite, timothy grass and/or cat hair; FEV<sub>1</sub> % pred., baseline forced expiratory volume in 1 s expressed relative to the predicted value (Quanjer et al., 1993); EIB, exercise-induced bronchoconstriction; SABA, short acting β<sub>2</sub>-agonist; LABA, long acting β<sub>2</sub>-agonist; ICS, inhaled corticosteroids; combination, combination therapy of LABA and ICS; post-EVH max. FEV<sub>1</sub> fall, maximal fall in FEV<sub>1</sub> after eucapnic voluntary hyperpnoea; protection, % broncho-protection afforded by terbutaline. \*\*\* Significantly different from placebo (*P*<0.001)

**Table 5.2.** Lung function results

Lung function measurement	Pre-Rx	Post-Rx	Post-EVH (min value)	Pre-Rx vs Post-Rx ( <i>P</i> value)	Post-Rx vs Post-EVH ( <i>P</i> value)
<b>Placebo</b>					
FEV <sub>1</sub> (L)	3.68 ± 0.65	3.67 ± 0.67	3.06 ± 0.68	0.316	<0.001
FVC (L)	4.74 ± 0.93	4.76 ± 0.95	4.48 ± 1.04	0.798	<0.001
FEF <sub>25-75</sub> (L·s <sup>-1</sup> )	3.27 ± 0.90	3.27 ± 0.90	2.17 ± 0.69	0.741	<0.001
FEF <sub>25</sub> (L·s <sup>-1</sup> )	6.18 ± 1.23	6.12 ± 1.23	4.39 ± 1.30	0.495	<0.001
FEF <sub>50</sub> (L·s <sup>-1</sup> )	3.57 ± 0.86	3.71 ± 0.98	2.47 ± 0.78	0.478	<0.001
FEF <sub>75</sub> (L·s <sup>-1</sup> )	1.70 ± 0.90	1.63 ± 0.58	1.02 ± 0.39	0.866	<0.001
PEF (L·s <sup>-1</sup> )	8.15 ± 1.48	8.15 ± 1.44	6.45 ± 1.65	0.968	<0.001
<b>Terbutaline</b>					
FEV <sub>1</sub> (L)	3.65 ± 0.64	3.82 ± 0.68	3.43 ± 0.63	<0.001	<0.001
FVC (L)	4.74 ± 0.94	4.73 ± 0.95	4.62 ± 0.95	0.796	<0.001
FEF <sub>25-75</sub> (L·s <sup>-1</sup> )	3.26 ± 0.93	3.37 ± 0.97	3.07 ± 0.86	<0.001	<0.001
FEF <sub>25</sub> (L·s <sup>-1</sup> )	6.15 ± 1.32	6.78 ± 1.41	5.57 ± 1.32	<0.001	<0.001
FEF <sub>50</sub> (L·s <sup>-1</sup> )	3.77 ± 1.06	4.22 ± 1.33	3.45 ± 0.84	<0.001	<0.001
FEF <sub>75</sub> (L·s <sup>-1</sup> )	1.62 ± 0.61	2.16 ± 1.43	1.47 ± 0.50	<0.001	<0.001
PEF (L·s <sup>-1</sup> )	7.95 ± 1.41	8.27 ± 1.50	7.38 ± 1.31	<0.001	<0.001

Pre-Rx, pre-treatment with either 0.5 mg terbutaline or placebo; Post-Rx, post-treatment; Post-EVH (min value), lowest value recorded after 8 min of eucapnic voluntary hyperpnoea of dry air; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; FEF<sub>25-75</sub> mean forced expiratory flow between 25 and 75% of FVC; FEF<sub>x</sub>, forced expiratory flow at X% of FVC; PEF, peak expiratory flow.



**Figure 5.2.** Mean  $\pm$  SEM percentage changes from baseline (Pre-Rx) in forced expiratory volume in 1 s (FEV<sub>1</sub>) after inhalation (Post-Rx) of 0.5 mg terbutaline (closed circles) or placebo (open circles), and up to 60 min after eucapnic voluntary hyperpnoea (EVH) of dry air in athletes with exercise-induced bronchoconstriction. Post-EVH FEV<sub>1</sub> AUC<sub>0-60</sub> (measured from post-Rx) was significantly reduced following administration of terbutaline ( $P < 0.001$ ).

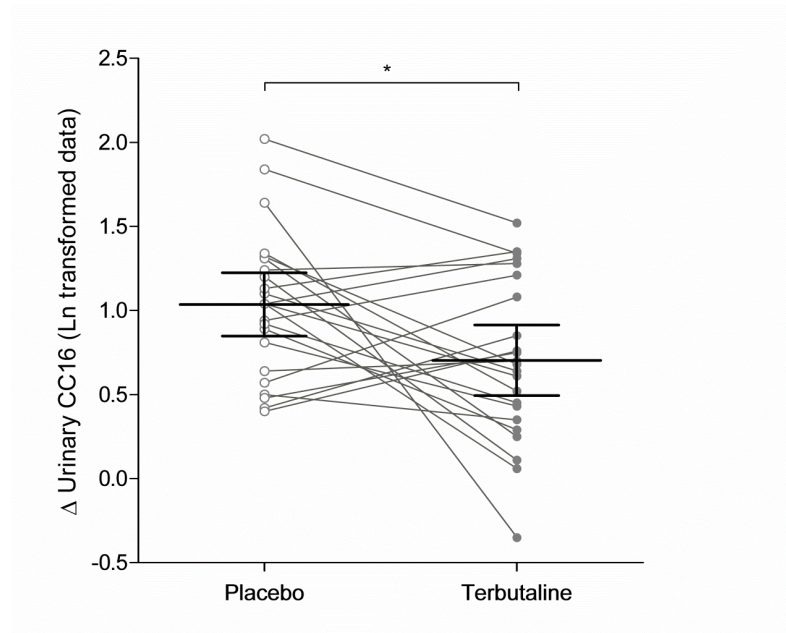
### 5-3.4 Urinary CC16

Three participants were excluded from the urinary CC16 statistical analysis as their CC16 concentrations were below detection point. In addition, one participant was excluded on the grounds of being an outlier. His CC16 measurements were highly variable at baseline ( $2457 \mu\text{g} \cdot \mu\text{mol creatinine}^{-1}$  and  $1055 \mu\text{g} \cdot \mu\text{mol creatinine}^{-1}$  in the



placebo and terbutaline condition, respectively) and he was the only participant to display a reduction in urinary CC16 excretion post-challenge in both the placebo and terbutaline conditions ( $-1582 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  and  $-261 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$ , respectively). The total number of data analysed for CC16 was therefore twenty three.

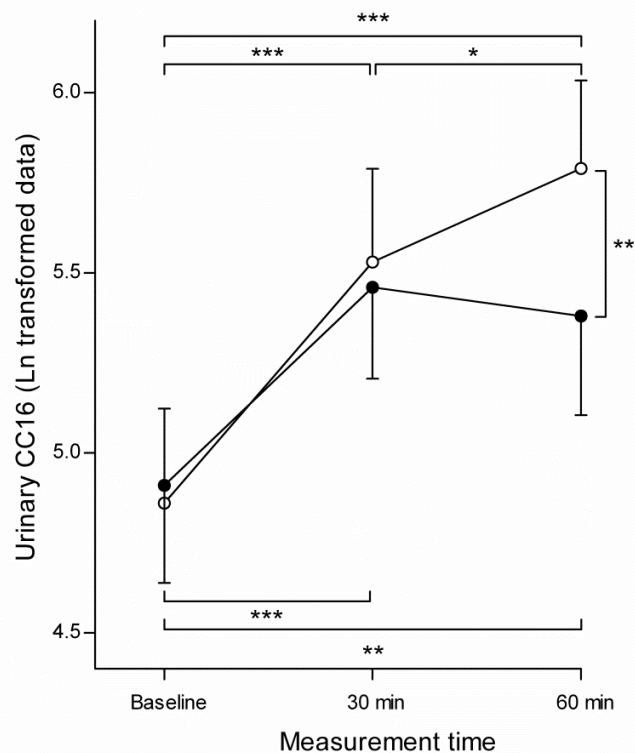
Baseline urinary CC16 was not significantly different between conditions:  $266 \pm 329 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  in the placebo condition vs  $267 \pm 292 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  in the terbutaline condition ( $P=0.695$ ). EVH caused a significant increase in urinary excretion of CC16 in both conditions ( $P<0.001$ ), but the peak urinary release of CC16 post-challenge was significantly attenuated by terbutaline: from  $682 \pm 788 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  to  $582 \pm 741 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  ( $P=0.032$ ). The magnitude of the change in urinary CC16 (pre- to max post-challenge CC16) was also significantly reduced after pre-medication with terbutaline (from  $416 \pm 495 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$  to  $315 \pm 523 \text{ pg}\cdot\mu\text{mol creatinine}^{-1}$ ,  $P=0.016$ ; Figure 5.3).



**Figure 5.3.** Maximum change in urinary excretion of CC16 following eucapnic voluntary hyperpnoea of dry air after pre-treatment with placebo or 0.5 mg of terbutaline in athletes with exercise-induced bronchoconstriction. Individual values with means (95% confidence intervals). \*  $P < 0.05$ .

Repeated measures ANOVA revealed significant time ( $P < 0.001$ ) and interaction effects ( $P < 0.01$ ), which indicate that terbutaline altered the kinetics of urinary CC16 excretion. Post-hoc analysis revealed that urinary CC16 increased significantly from baseline to 30 and 60 min post-EVH in both conditions ( $P < 0.01$ ). However, in the placebo condition, urinary CC16 continued to increase between 30 and 60 min of recovery ( $P < 0.05$ ), whereas it started to plateau at 30 min of recovery in the terbutaline condition. As a result, urinary concentrations of CC16 were significantly lower in the terbutaline condition at 60 min recovery compared to placebo ( $P < 0.01$ , Figure 5.4).

No significant between-subject effects for gender or current asthma treatment were found, suggesting that neither gender nor asthma treatment interfered with our CC16 results. Moreover, no significant relationships were detected between urinary CC16 excretion and age, severity of bronchoconstriction following EVH, bronchoprotection afforded by terbutaline or bronchodilator response to terbutaline.



**Figure 5.4.** Mean  $\pm$  SEM urinary CC16 concentrations at baseline, 30 and 60 min post-eucapnic voluntary hyperpnoea of dry air following pre-treatment with 0.5 mg terbutaline (closed circles) or placebo (open circles) in athletes with exercise-induced bronchoconstriction. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

## 5-4 Discussion

The aim of this study was to test the efficacy of inhaled terbutaline at reducing hyperpnoea-induced airway epithelial injury and bronchoconstriction in athletes.

We showed that a single therapeutic dose of terbutaline of 0.5 mg was able to blunt the rise in urinary CC16 concentration following hyperpnoea of dry air. We also demonstrated that the level of broncho-protection afforded to athletes with EIB is in line with that afforded to individuals with asthma (Bonini et al., 2013).

These results demonstrate the potential for inhaled  $\beta_2$ -agonists to attenuate acute airway epithelial perturbation in athletes with EIB.

This study supports previous findings that suggested that hyperpnoea of dry air causes perturbation to the airway epithelium in athletes (Bolger, Tufvesson, Sue-Chu, et al., 2011b). It is also in line with work that showed that exercise-hyperpnoea is associated with an increase in serum and urinary concentration of CC16 in athletes (Bolger, Tufvesson, Anderson, et al., 2011a; Broeckeaert et al., 2000; Carbonnelle et al., 2002; Romberg et al., 2011). The ability of parenteral  $\beta_2$ -agonists to reduce dry air-induced epithelial injury was previously demonstrated in animals (Omori et al., 1995; Wang et al., 1992). We have now extended these findings by demonstrating that terbutaline can attenuate airway epithelial perturbation when administered by inhalation in the human lungs.

Airway epithelial perturbation in this study was assessed through changes in urinary CC16 concentration. CC16 is a protein secreted from the club cells located primarily in the distal airways (Singh et al., 1988). Acute increases in the concentration of

CC16 in extra-pulmonary fluids have previously been proposed to reflect a transient loss of the lung epithelial barrier integrity (Hermans & Bernard, 1999). An increase in urinary CC16 may also represent an increase in CC16 production / secretion by club cells in an attempt to modulate local inflammatory reactions (Jorens et al., 1995). Hyperpnoea of dry air is associated with mast cell activation and inflammatory mediator release in athletes (Kippelen et al., 2010a; 2010b). Therefore, the rise in urinary CC16 following EVH in the current study may be due to a combination of increased leakage of CC16 across the airway epithelium and increased production and / or secretion of the protein at the club cell level.

The use of a non-invasive marker (with multiple sampling time points) enabled us to test, for the first time, the efficacy of a pharmacological agent at attenuating hyperpnoea-induced airway perturbation in athletes. We showed that the rise in urinary CC16 concentration following a short period of hyperpnoea of dry air was significantly blunted with pre-medication with terbutaline. *In vitro*, terbutaline has been shown to increase the flow of Cl<sup>-</sup> towards the airway lumen (Davis et al., 1979); an action likely to be mediated through binding of the drug with  $\beta_2$ -receptors on epithelial cells and subsequent release of cAMP (Smith, Welsh, Stoff, & Frizzell, 1982). A reduction of the dehydration stress to the airways through inhalation of warm-humid air limits epithelial cell perturbation after exercise (Bolger, Tufvesson, Anderson, et al., 2011a). Similarly, in the current study, we propose that the reduction in urinary CC16 excretion post-EVH was due to reduced dehydration of the airway epithelium mediated through terbutaline-enhanced water secretion.

An alternative interpretation of the results is that terbutaline did not affect the severity of epithelial perturbation *per se*, but rather enhanced the speed of epithelial cell repair. Perkins and colleagues (2008) showed *in vitro* that salbutamol stimulated both wound repair, and spreading and proliferation of human lung epithelial cells. The potential of  $\beta_2$ -agonists to stimulate epithelial cell repair may explain why, in our study, urinary CC16 continued to increase between 30 and 60 min of recovery in the placebo condition, whilst it started to plateau in the terbutaline condition. We propose that terbutaline was able to stimulate the repair of the epithelial cells before the later time point (at 60 min), and therefore reduced leakage of CC16 occurred across the airway epithelium.

A third contributing factor to our results could be the effect of terbutaline on the bronchial vascular system. In a murine model, terbutaline given in the instillate (intra-tracheally) reduced microvascular permeability during high-volume ventilation (de Prost, Dreyfuss, Ricard, & Saumon, 2008).  $\beta_2$ -agonists are thought to mediate vascular permeability, either directly [by relaxing the endothelial contractile proteins and thereby reducing gaps between endothelial cells (Warren, Wilson, Loi, & Coughlan, 1993)], or indirectly [by inhibiting vaso-active mediator release from the lung mast cells (Church & Hiroi, 1987)]. We therefore cannot exclude that terbutaline, by reducing vascular leakage, limited the passage of CC16 into the bloodstream post-EVH.

Terbutaline in the present study was delivered through a dry powder inhaler (Turbuhaler®). When delivered *via* Turbuhaler®, terbutaline is known to reach all

levels of the tracheobronchial tree, including the small airways (Newman, Morén, Trofast, Talae, & Clarke, 1989). In our study, we noticed a significant increase in all forced expiratory flow parameters (including FEF<sub>50</sub> and FEF<sub>75</sub>) post-administration of terbutaline. This is relevant in that the recruitment of the smaller airways in the conditioning of inspired air has been highlighted as the main mechanism for exercise-induced airway epithelial injury (Anderson & Kippelen, 2005). Terbutaline has the potential to enhance osmotic driven water flux to the airway lumen (Davis et al., 1979). Enhancement of water secretion to the larger airways may have therefore reduced the necessity for the smaller airways to be recruited in the conditioning process and may have protected those smaller airways against epithelial injury. The dispersion of terbutaline within the airways is known to be dependent upon the inspiratory flow achieved with the Turbuhaler® (Newman, Morén, Trofast, Talae, & Clarke, 1991). Since inspiratory flow was not controlled in our study, inter-individual differences in dispersion of the medication may account for the variation in the effectiveness of the terbutaline in attenuating the rise in CC16 post-EVH.

Whilst there was little difference in ventilation rates achieved by the athletes between the terbutaline and the placebo conditions, bronchoconstriction was more severe after placebo inhalation. Therefore, in our control condition, significant compressive stress may have occurred within the airways (Nucci, Suki, & Lutchen, 2003), which may have further compromised the integrity of the airway epithelial barrier. However, we did not find any significant correlation between the changes in urinary excretion of CC16 and the fall in FEV<sub>1</sub> post-EVH under our two

experimental conditions (data not shown). Similarly, in previous publications (Bolger, Tufvesson, Anderson, et al., 2011a; Bolger, Tufvesson, Sue-Chu, et al., 2011b), there was no difference in magnitude of urinary CC16 increase following exercise or EVH challenge tests in individuals with and without EIB. This therefore suggests that mild-to-moderate bronchoconstriction *per se* is unlikely to affect the extent of perturbation to the airway epithelium.

These findings are relevant to endurance athletes who are thought to repeatedly damage their airways through dehydration stress (Anderson & Kippelen, 2005). Endurance athletes have an increased prevalence of EIB compared to their counterparts who perform anaerobic exercise and to the general population (Carlsen et al., 2008). Repeated airway epithelial injury and repair has been highlighted as a key factor contributing to the increased prevalence of airway hyper-responsiveness in elite endurance athletes (Anderson & Kippelen, 2005). Strategies aiming at preventing hyperpnoea-induced epithelial perturbations may therefore be beneficial in the prevention of EIB (Kippelen et al., 2012). Here, we highlighted that inhaled  $\beta_2$ -agonist medication can acutely reduce airway epithelial perturbation. Due to the known side-effects associated with the chronic use of inhaled  $\beta_2$ -agonists (Hancox et al., 2002), other interventions that have the potential to modify ion transport across the airway epithelium should be tested to try to counteract the long term development of EIB in elite athletes.



In conclusion, this study demonstrates for the first time that pre-medication with a single, inhaled dose of terbutaline reduces hyperpnoea-induced airway epithelial perturbation in athletes. We propose that terbutaline reduces epithelial perturbation mainly by enhancing water movement toward the airway lumen. Because inhaled  $\beta_2$ -agonist medications have a wide range of effects, the next chapter will investigate the efficacy of terbutaline at attenuating mast cell mediator release during induced bronchoconstriction.

## **CHAPTER 6**

### **STUDY 3 - Effect of terbutaline on hyperpnoea-induced bronchoconstriction and mast cell activation in athletes**

## 6-1 Introduction

Inhaled  $\beta_2$ -adrenoceptor agonists are the cornerstone of asthma treatment. The Global Initiative for Asthma (GINA) guidelines suggest that  $\beta_2$ -adrenoceptor agonists should be prescribed to all individuals with asthma for use “as needed reliever” and recommends their use for the short-term prevention of EIB (GINA, 2014). A recent Cochrane review of 45 studies revealed that inhaled  $\beta_2$ -adrenoceptor agonists, when taken prophylactically, reduce the post-exercise fall in FEV<sub>1</sub> by 66% (range 29 - 91%) (Bonini et al., 2013). The primary mechanism of inhaled  $\beta_2$ -adrenoceptor agonists is believed to be relaxation of the bronchial smooth muscle, through the functional antagonism, whereby  $\beta_2$ -adrenoceptor agonists oppose the contractile effects of the various mediators of bronchoconstriction (Anderson, Caillaud, & Brannan, 2006a). Mast cells are abundant in the airways of individuals with EIB (Lai et al., 2014) and the release of their inflammatory mediators has been highlighted as a key contributing factor to airway narrowing (Anderson & Daviskas, 2000).

Evidence of mast cell activation has been published previously, based on biomarkers investigations following various bronchial provocation challenges. Prostaglandin (PG)D<sub>2</sub> is a potent constrictive mediator produced almost exclusively from mast cells (O'Sullivan, 1999). O'Sullivan and colleagues (1998) noted an increase of urinary excretion of 11 $\beta$ -PGF<sub>2 $\alpha$</sub> , an early metabolite of PGD<sub>2</sub>, following exercise in individuals with asthma. Similar reports following exercise (Haverkamp et al., 2005; Mickleborough et al., 2003; Nagakura et al., 1998) and following

various indirect bronchial provocation challenges (Brannan et al., 2003; Kippelen et al., 2010a; 2010b; Larsson et al., 2011) have also been documented.

Mast cells express  $\beta_2$ -adrenoceptors (Chong, Chess-Williams, & Peachell, 2002), and  $\beta_2$ -adrenoceptor agonists may have an important secondary action of mast cell stabilisation. *In vitro*,  $\beta_2$ -adrenoceptor agonists are effective mast cell stabilisers following antigen challenges, causing a significant reduction in histamine and  $\text{PGD}_2$  release (Church & Hiroi, 1987). Anderson and colleagues (1981) previously demonstrated that nebulisation of 5 mg of terbutaline inhibits the increase in plasma histamine levels induced by exercise in individuals with asthma. Histamine release, however, is not limited to mast cell activation and elevations in plasma histamine may also reflect basophilia (Howarth et al., 1984). Furthermore, plasma histamine has a very short half life and sampling is problematic, which makes its usefulness as a marker of mast cell activation uncertain (Ind, Brown, Lhoste, Macquin, & Dollery, 1982; Ind et al., 1983).

Large doses of long acting  $\beta_2$ -adrenoceptor agonist have been shown to reduce the release of  $11\beta\text{-PGF}_{2\alpha}$  following bronchial provocation with dry powder of mannitol (Brannan et al., 2006). Mannitol acts as an osmotic agent during bronchial provocation challenge and creates a hyperosmotic environment similar to that of exercise. Brannan and colleagues (2006) noted a reduction in the urinary excretion

of  $11\beta$ -PGF<sub>2α</sub> following mannitol challenge when preceded by inhalation of a supra-therapeutic dose (24 μg) of the long acting β<sub>2</sub>-adrenoceptor agonist formoterol.

The aim of this investigation was to test the efficacy of a single, therapeutic dose of an inhaled short acting β<sub>2</sub>-adrenoceptor agonist at inhibiting mast cell mediator release following hyperpnoea with dry air. Our hypothesis was that pre-medication with 0.5 mg of terbutaline would attenuate the increase in urinary excretion of  $11\beta$ -PGF<sub>2α</sub> following 8 min of eucapnic voluntary hyperpnoea (EVH) in athletes with EIB.

## 6-2 Methods

This study was conducted concurrently with Study 2. A detailed description of the study protocol can be found in the previous chapter. Briefly, 27 athletes with confirmed EIB were recruited. Urine samples were collected at baseline and at 30 and 60 minutes following EVH with dry air on two separate days: once following treatment with 0.5 mg of terbutaline, and once after administration of a placebo. Urine samples were analysed for  $11\beta$ -PGF<sub>2α</sub> using a commercially available competitive ELISA kit (Cayman Chemical, Ann Arbor, MI), as described in the general methods. Urine samples were also analysed for creatinine (see general methods) and results are expressed as nanogram of  $11\beta$ -PGF<sub>2α</sub> *per* micromole of creatinine. Lung function was measured at baseline, post-treatment and following EVH at 2, 5, 10, 15, 20, 30 and 60 min. The study was approved by the UK National

Health Service Research Ethics Committee (NHS REC reference number: 10/H0716/30).

### ***Data analysis***

Sample size was based on a previous study in which the mast cell stabilising effect of sodium cromoglycate was investigated during EVH in athletes with EIB (Kippelen et al., 2010b). With a risk alpha of 5%, a risk beta of 95% and using the effect size from Kippelen et al. (2010b), analysis of 16 urine samples was required [G\*Power3 software (Faul et al., 2007)].

Data were tested for normality using the Shapiro-Wilk test. Baseline FEV<sub>1</sub> (l), changes in FEV<sub>1</sub> following treatment (%), ventilation during EVH (l), level of bronchoprotection afforded by terbutaline (%), and participant mass and height were normally distributed and data are presented as mean ± SD. Differences between conditions for these variables were analysed using paired sample t-tests. All other data were non-normally distributed and are therefore presented as median and interquartile range (Q1-Q3). Differences between conditions for the maximum changes in FEV<sub>1</sub> (%) and in urinary 11β-PGF<sub>2α</sub> concentrations post-challenge were analysed using Wilcoxon signed rank tests. Differences across times were analysed using Friedman two-way ANOVA by ranks. In cases of statistical significance, Wilcoxon signed ranks tests were used to identify where differences occurred.

Additional analyses were conducted to identify potential confounding factors; i.e., gender, asthma treatment, severity of bronchoconstriction, degree of bronchoprotection and bronchodilator response. Differences in the change in urinary  $11\beta$ -PGF<sub>2α</sub> excretion (baseline to peak) post-EVH were explored between groups according to gender and current asthma treatment (inhaled  $\beta_2$ -agonist or inhaled corticosteroid use) using Mann Whitney U tests. Furthermore, Spearman's rank correlation coefficient and multiple regression analysis for within-subject repeated measures (Bland & Altman, 1995) were conducted to determine relationships between urinary  $11\beta$ -PGF<sub>2α</sub> excretion and age, severity of bronchoconstriction following EVH, bronchoprotection afforded by terbutaline, and bronchodilator response to terbutaline. All data were analysed using a statistical software package (SPSS 20, Chicago, IL, US).

## **6-3 Results**

### **6-3.1 Data screening**

Three participants were excluded from the statistical analysis as their  $11\beta$ -PGF<sub>2α</sub> concentrations were below the level of detection. Of the 24 remaining participants, 18 had complete data sets (i.e.,  $11\beta$ -PGF<sub>2α</sub> was detectable in all samples) and these were used to determine the kinetics of urinary  $11\beta$ -PGF<sub>2α</sub> excretion. An additional six participants had acceptable baseline values and at least one acceptable  $11\beta$ -

PGF<sub>2α</sub> value post-EVH and were therefore included in the analysis of peak 11β-PGF<sub>2α</sub> concentrations.

### 6-3.2 Participant characteristics

The age, height and body mass of the participants were 23 (19-32) yr, 175 ± 9 cm and 75 ± 14 kg, respectively. Participants were involved in summer sports (athletics, cycling, football, rugby, cricket or rowing), trained for 8 (5-10) h *per* week in aerobic activities, and had 8 (4-12) yr experience in their sport. Participants' medical diagnosis, current treatment and atopic status are presented in Table 6.1.

**Table 6.1.** Participant characteristics

ID	Gender	Atopic status	Baseline FEV <sub>1</sub> (% pred.)	Previous diagnosis	Treatment	Prescribed ICS dose (µg/day)
1	M	+	95	Asthma + EIB	Combination, SABA	1600
2	M	+	92	Asthma	SABA, ICS	200
3	M	+	99	Asthma + EIB	Combination	400
4	F	+	108	EIB	SABA, ICS	200
5	M	+	97	-	-	-
6	F	+	92	Asthma	SABA, ICS	200
7	M	+	93	Asthma + EIB	SABA	-
8	F	+	93	Asthma	Combination	400
9	M	+	99	Asthma	SABA, ICS	600
10	M	-	99	-	-	-
11	M	+	85	Asthma	-	-
12	F	+	103	Asthma	-	-
13	M	+	109	EIB	SABA	-
14	F	-	99	Asthma + EIB	Combination	400
15	F	-	109	-	-	-
16	M	+	94	Asthma	SABA	-
17	F	-	96	Asthma	SABA	-
18	M	+	94	Asthma	SABA	-
19	M	+	111	Asthma	SABA, ICS	200
20	F	+	102	EIB	SABA, ICS	200
21	F	+	86	Asthma	SABA	-
22	F	-	88	EIB	-	-
23	F	+	82	Asthma + EIB	SABA	-
24	M	+	91	Asthma	SABA	-

Atopic status: +, positive skin prick response to house dust mite, timothy grass and/or cat hair; FEV<sub>1</sub> % pred., baseline forced expiratory volume in 1 s expressed relative to the predicted value (Quanjer et al., 1993); EIB, exercise-induced bronchoconstriction; SABA, short acting β<sub>2</sub>-agonist; LABA, long acting β<sub>2</sub>-agonist; ICS, inhaled corticosteroids; combination, combination therapy of LABA and ICS

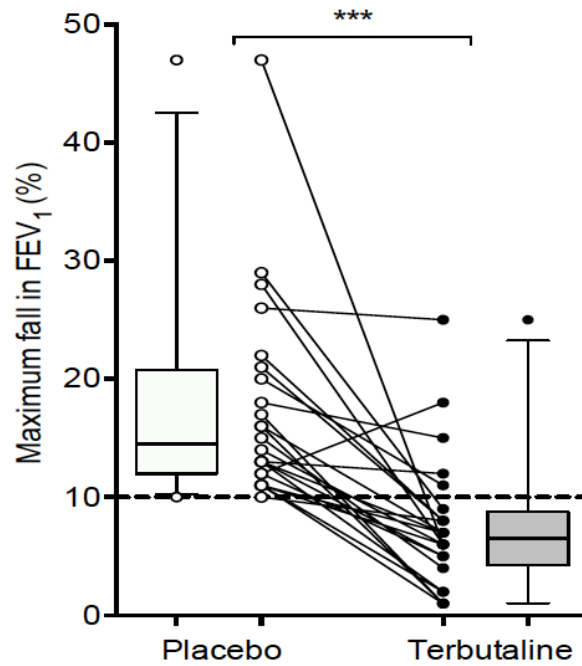


### **6-3.3 Baseline lung function and ventilation level during EVH**

Baseline FEV<sub>1</sub> was not significantly different between conditions; FEV<sub>1</sub> was 3.68 ± 0.68 l and 3.64 ± 0.66 l in the placebo and terbutaline conditions, respectively ( $P=0.160$ ). Terbutaline had a small but significant bronchodilator effect; FEV<sub>1</sub> increased by 4 ± 3% with administration of terbutaline ( $P<0.001$ ). No such bronchodilator effect was noticed after the administration of placebo ( $P=0.360$ ). The ventilation achieved during EVH was 99 ± 20 *versus* 100 ± 19 l·min<sup>-1</sup> [78 ± 7% and 80 ± 8% of predicted MVV] in the placebo and terbutaline condition, respectively ( $P=0.077$ ).

### **6-3.4 Airway response to EVH**

Terbutaline significantly inhibited the airway response to EVH. The maximum fall in FEV<sub>1</sub> was reduced from 15 (12-21)% in the placebo condition to 7 (4-9) % with administration of terbutaline ( $P<0.001$ ). The degree of broncho-protection afforded by terbutaline for maximum % fall in FEV<sub>1</sub> was 56 ± 29%, with minimum and maximum values of 0 and 94%, respectively. Terbutaline afforded complete bronchoprotection (<10% fall in FEV<sub>1</sub>) to 19 out of 24 athletes (79%) (Figure 6.1).



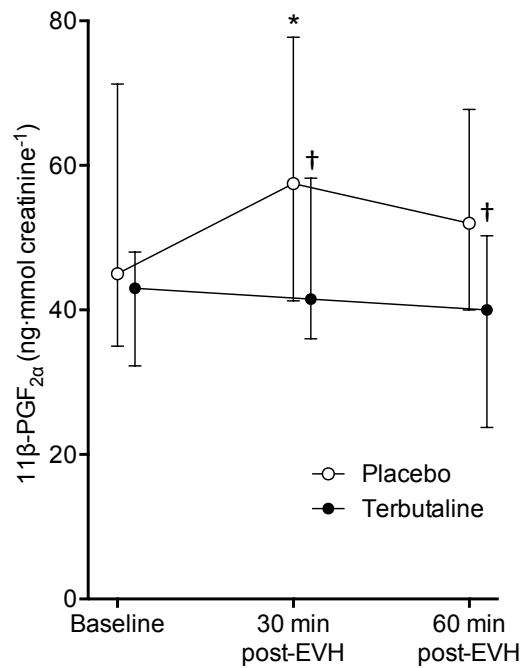
**Figure 6.1.** Maximum fall in forced expiratory volume in 1 sec ( $FEV_1$ ) following 8 min of hyperpnoea of dry air in athletes with EIB pre-treated with 0.5 mg terbutaline or placebo. Individual subject data are shown; box plots represent group median and interquartile range, with whiskers representing the 5<sup>th</sup> and 95<sup>th</sup> percentiles. Values under the broken line (10% fall in  $FEV_1$ ) represent complete bronchoprotection (n=19, 79%).

### 6-3.5 Urinary $11\beta$ -PGF<sub>2 $\alpha$</sub> analysis

#### *Kinetic of $11\beta$ -PGF<sub>2 $\alpha$</sub> excretion post-EVH (n=18)*

There was no difference in baseline  $11\beta$ -PGF<sub>2 $\alpha$</sub>  values between conditions ( $P=0.446$ ). A significant time effect was noted in the placebo condition ( $P=0.002$ ), with an increase in urinary  $11\beta$ -PGF<sub>2 $\alpha$</sub>  from 45 (35-71) ng·mmol creatinine<sup>-1</sup> at baseline to 58 (41-78) ng·mmol creatinine<sup>-1</sup> at 30 min post-challenge ( $P=0.025$ ) (Figure 2). Terbutaline inhibited the rise in urinary  $11\beta$ -PGF<sub>2 $\alpha$</sub>  post-EVH (time effect,  $P=0.446$ ). Consequently, urinary  $11\beta$ -PGF<sub>2 $\alpha$</sub>  levels were higher in the placebo

compared to the terbutaline condition at 30 min ( $P=0.018$ ) and 60 min post-EVH ( $P=0.003$ ) (Figure 6.2).



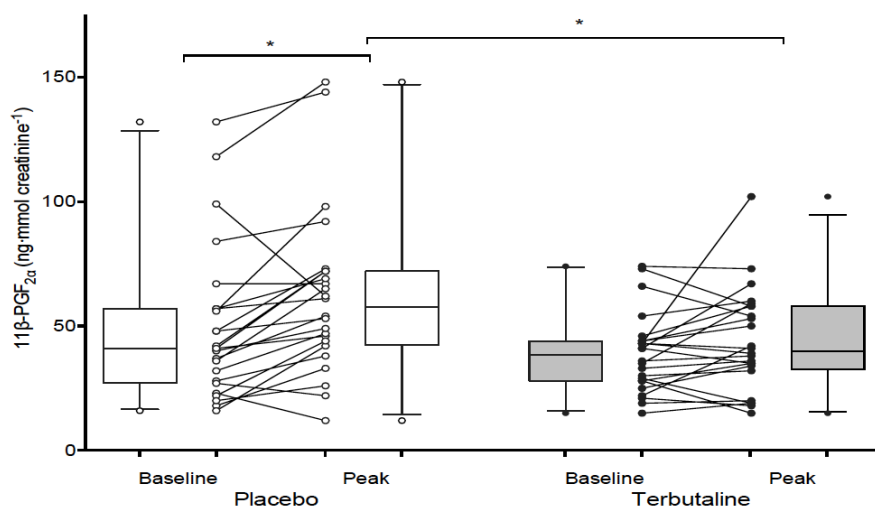
**Figure 6.2.** Median  $\pm$  interquartile range (Q1-Q3) urinary concentration of 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  at baseline, 30 and 60 min post-eucapnic voluntary hyperpnoea of dry air following pre-treatment with 0.5 mg terbutaline (closed circles) or placebo (open circles) in 18 athletes with exercise-induced bronchoconstriction. \*  $P<0.05$ , significantly different from baseline (in the placebo condition only); †  $P<0.05$ , significantly different from placebo.

**Peak 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  post-EVH (n=24)**

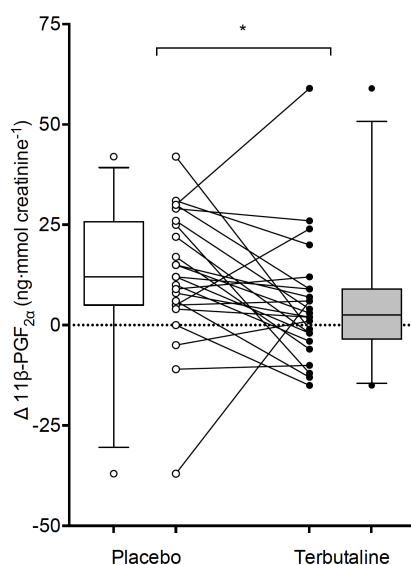
Examination of the baseline and peak 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  values revealed that EVH caused a significant increase in urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  concentration ( $P=0.002$ ) that was inhibited with the inhalation of terbutaline ( $P=0.118$ ) (Figure 6.3). The inhibition of 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion with terbutaline resulted in a significantly lower peak concentration of 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  in the terbutaline condition compared to the placebo condition ( $P=0.001$ ) (Figure 6.3). Similarly, the magnitude of the change in urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  (pre- to peak post-challenge) was significantly reduced after pre-treatment with terbutaline, from 12 (5-26) ng·mmol creatinine<sup>-1</sup> to 3 (minus 4-9) ng·mmol creatinine<sup>-1</sup> in the placebo and terbutaline condition, respectively ( $P=0.033$ ) (Figure 6.4).

There was a weak positive relationship between the fall in FEV<sub>1</sub> and the change in urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  post-EVH in the placebo condition ( $r^2= 0.251$ ,  $P=0.013$ ).

Interestingly, this relationship was absent with the administration of terbutaline ( $P=0.505$ ). Furthermore, a multiple regression analysis for within-subject repeated measures revealed no significant relationship between the maximum fall in FEV<sub>1</sub> (%) and the change in urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion post-EVH across conditions ( $P=0.117$ ). No significant between-group difference for gender or current asthma treatment was found, suggesting that gender, current inhaled corticosteroid and inhaled  $\beta_2$ -agonist use did not affect urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion. Moreover, no significant relationship was noted between urinary 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion and age bronchoprotection afforded by terbutaline or bronchodilator response to terbutaline.



**Figure 6.3.** Urinary concentrations of 11β-PGF<sub>2α</sub> at baseline and after eucapnic voluntary hyperpnoea (EVH) of dry air (peak value) following pre-treatment with 0.5 mg terbutaline (closed circles) or placebo (open circles) in 18 athletes with exercise-induced bronchoconstriction. Individual data are shown; box plots represent group median and interquartile range, with whiskers representing the 5<sup>th</sup> and 95<sup>th</sup> percentiles; \* *P*<0.05.



**Figure 6.4.** Maximal change in urinary excretion of 11β-PGF<sub>2α</sub> following eucapnic voluntary hyperpnoea of dry air after pre-treatment with 0.5 mg of terbutaline or a placebo in athletes with exercise-induced bronchoconstriction. Individual data are shown; box plots represent group median and interquartile range, with whiskers representing the 5<sup>th</sup> and 95<sup>th</sup> percentiles; \* *P*<0.05.

## 6-4 Discussion

This study shows that inhalation of 0.5 mg of terbutaline affords broncho-protection to the large majority (~80%) of athletes with EIB. This study also presents the first *in vivo* evidence of an inhibition of the release of the mast cell mediator, PGD<sub>2</sub>, by a single, therapeutic dose of a short-acting β<sub>2</sub>-adrenoceptor agonist in response to indirect bronchial provocation.

This study supports previous findings of mast cell activation following hyperpnoea of dry air in athletes (Kippelen et al., 2010a; 2010b). Previous *in vivo* evidence using 11β-PGF<sub>2α</sub> to assess the mast cell stabilising effect of β<sub>2</sub>-adrenoceptor agonist medication is limited to studies using supra-therapeutic doses of the long acting β<sub>2</sub>-adrenoceptor agonist formoterol (Brannan et al., 2006) and salmeterol (Szczeklik et al., 1998). Here, we have extended these findings by demonstrating that mast cell stabilisation can occur following inhalation of a single, therapeutic dose of the short acting β<sub>2</sub>-adrenoceptor agonist terbutaline.

Mast cell activation in this study was assessed by the change in the urinary concentration of 11β-PGF<sub>2α</sub>. PGD<sub>2</sub>, the parent molecule of 11β-PGF<sub>2α</sub>, is a major cyclooxygenase metabolite of arachidonic acid. In humans, PGD<sub>2</sub> is produced almost exclusively from mast cells (O'Sullivan, 1999). The urinary excretion rate of the metabolite 11β-PGF<sub>2α</sub> can therefore provide objective evidence of PGD<sub>2</sub> production and, hence, of mast cell activation (O'Sullivan, 1999). O'Sullivan and colleagues (1998) were the first team to utilise urinary concentrations of 11β-PGF<sub>2α</sub> to provide evidence of mast cell activation in EIB. They demonstrated an increase in urinary

excretion of  $11\beta$ -PGF<sub>2α</sub> following 5 min of heavy cycling in individuals with asthma. An increase in  $11\beta$ -PGF<sub>2α</sub> has since been demonstrated following bronchial provocation with surrogates to exercise, such as EVH (Kippelen et al., 2010a; 2010b) and mannitol (Brannan et al., 2003; 2006; Larsson et al., 2011). It is likely that mast cells are activated following indirect bronchial provocation challenges due to an osmotic stimulus. Indeed, Gulliksson and colleagues (2006) demonstrated *in vitro* that human cord mast cells stimulated with hypertonic saline solutions released, amongst other mediators, PGD<sub>2</sub>.

In the present study, an average ventilation of 100 l·min<sup>-1</sup> was achieved; this level of ventilation is deemed sufficient to cause water loss from the smaller airways (past the 9<sup>th</sup> generation) during the conditioning of inspired air (Daviskas et al., 1990). This evaporative water loss could increase the osmolarity of the ASL in these smaller airways. This is relevant, as mast cells are more densely located in the peripheral airways (Carroll et al., 2002). Furthermore, an increase in mast cell density has been noted in individuals with asthma and EIB (Lai et al., 2014), as well as in competitive swimmers (Bougault et al., 2012). An increase in mast cell density in the peripheral airways, combined with the capability of hyperpnoea of dry air to create a hyperosmotic environment, probably explains the rise in  $11\beta$ -PGF<sub>2α</sub> in the placebo condition in the studied population.

Importantly, the deposition of terbutaline from a dry powder inhaler also extends to the small airways (Newman et al., 1989). This may have facilitated the binding of terbutaline with the β<sub>2</sub>-adrenoceptors on the mast cells infiltrated in the peripheral

airways of our study participants. *In vitro*, mast cells treated with the non-selective  $\beta$ -adrenergic agonist isoprenaline displayed increased levels of cAMP (Peachell, MacGlashan, Lichtenstein, & Schleimer, 1988). Furthermore, this increase in cAMP correlated with a reduction in the release of the mast cell mediators PGD<sub>2</sub>, histamine and LTC<sub>4</sub> (Peachell et al., 1988). Increased levels of cAMP is well known to suppress mast cell secretions (Weston & Peachell, 1998). This suggests that the inhibitory effect of mast cell mediator release provided by terbutaline was likely mediated by a sustained increase in cAMP.

Mast cells are known to release a variety of bioactive substances that are associated with bronchoconstriction, airway inflammation, tissue remodelling, increased vascular permeability, mucosal gland secretion and sensitisation of airway smooth muscle (Bradding et al., 2006). These factors may contribute, either individually, or in combination, to the pathophysiology of asthma/AHR. Therefore, mast cell stabilising treatments are deemed beneficial for the treatment of asthma/AHR (Bradding et al., 2006). Athletes have an increased prevalence of EIB/AHR and mast cell activation has been observed in this population during dry air challenge, independently to the occurrence of EIB (Kippelen et al., 2010a; 2010b). Our findings are therefore of clinical relevance to individuals with asthma/AHR and to athletes who, through exercise-hyperpnoea, regularly expose their airways to a hyperosmotic environment capable of causing mast cell activation. Given that PGD<sub>2</sub> may perpetuate inflammation *via* initiation of migration of eosinophils to the airways (Emery et al., 1989), the prevention of mast cell



mediator release with  $\beta_2$ -adrenoceptor agonists may interrupt the chronic inflammatory cycle and improve the management of asthma and/or EIB.

To conclude, we have shown that the prophylactic administration of 0.5 mg of inhaled terbutaline not only offers a significant degree of broncho-protection to athletes with EIB, but is also able to inhibit the release of the mast cell mediator  $\text{PGD}_2$  following hyperpnoea of dry air. These findings support the idea of a mast cell stabilising effect of single, therapeutic doses of  $\beta_2$ -adrenoceptor agonist medication.

## **CHAPTER 7**

### **STUDY 4 - Effect of warm-humid air on exercise-induced bronchoconstriction and inflammatory mediator release**

## 7-1 Introduction

As illustrated in the previous chapter, inflammatory mediator release may be a key factor in the pathophysiology of EIB. This proposal is supported by observations of an increased urinary excretion of cyst-LTs and the PGD<sub>2</sub> metabolites (i.e., LTE<sub>4</sub> and 11β-PGF<sub>2α</sub>) following bronchial provocation challenges with exercise (Haverkamp et al., 2005; Mickleborough, Murray, Ionescu, & Lindley, 2003; O'Sullivan et al., 1998), or its surrogates (Brannan et al., 2006; Kippelen, Larsson, Anderson, Brannan, Dahlén, et al., 2010a; Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b; Larsson et al., 2011), in individuals with asthma and/or EIB. That the urinary excretion of LTE<sub>4</sub> and 11β-PGF<sub>2α</sub> following bronchial provocation with EVH and mannitol correlates with the severity of bronchoconstriction (Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b; Larsson et al., 2011), and that the mast-cell stabilising drug sodium cromoglycate concomitantly prevents bronchoconstriction and the rise in urinary 11β-PGF<sub>2α</sub> (Kippelen 2010, Brannan 2006), lend further support to this theory.

Anderson and colleagues (1979) demonstrated, over 30 years ago, that the inhalation of warm-humid air during exercise could prevent bronchoconstriction in individuals with asthma and severe EIB (i.e., post-exercise fall in FEV<sub>1</sub> 53.9 ± 11.5%). The authors postulated that this protective effect was mediated by the removal of the osmotic stimulus for mediator release. However, at present, there is no direct evidence that the prevention of EIB with inhalation of warm-humid air during exercise is caused by an inhibition of inflammatory mediator release.

Until recently, the analysis of inflammatory mediator release in the context of asthma and/or EIB has been carried out using enzyme linked immunosorbant assays, and therefore has been limited to the analysis of the two broncho-constricting mediators, cyst-LTs and PGD<sub>2</sub> (Brannan et al., 2006; Kippelen, Larsson, Anderson, Brannan, Dahlén, et al., 2010a; Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b; Larsson et al., 2011; Mickleborough et al., 2003). It is however likely that many more mediators of inflammation, capable of either constricting or dilating the airways, are released during EIB.

Technological advancement in mass spectrometry has opened new opportunities in the quantitative measurement of small endogenous metabolites in biological fluids (incl. urine). Our collaborators from the Karolinska Institutet (Stockholm, Sweden) recently developed a mass spectrometry platform to study the airway inflammatory and oxidative stress status of individuals with asthma. Using ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS), they demonstrated the release of a wide range of lipid mediators during allergen-induced (Balgoma et al., 2013; Daham et al., 2014) and dry air-induced bronchoconstriction (Bood et al., 2015). In the latter paper, they confirmed excretion of the broncho-constrictors cyst-LTs and PGD<sub>2</sub> after EVH of dry air, but also highlighted increased excretion of the broncho-protective PGE<sub>2</sub>, as well as prostacyclin (PGI<sub>2</sub>). Due to the higher potency of allergens and EVH challenges to stimulate mediator release, it remains to be established whether the same mass spectrometry technique could be utilised to characterise the profile of

inflammatory mediator release following exercise provocation in individuals with EIB.

The aim of this investigation was therefore to determine the feasibility of using mass spectrometry to detect a wide range of airway-derived inflammatory mediators in urine following bronchial provocation with exercise. The secondary aim was to determine whether warm-humid air breathing during exercise prevents EIB through the inhibition of inflammatory mediator release. Our hypotheses were that i) mass spectrometry would allow detection of a range of broncho-active mediators (not limited to PGD<sub>2</sub> and cyst-LTs metabolites) following exercise provocation in individuals with EIB, and ii) inhibition of airway narrowing post-exercise when warm-humid air is inhaled is associated by a change in the balance between broncho-constricting and broncho-protective inflammatory mediator release.

## **7-2 Methods**

### **7-2.1 Subjects**

The study population consisted of seven habitually active, 18 to 50 yr old individuals with EIB. EIB was confirmed by a  $\geq 10\%$  fall in FEV<sub>1</sub> following a standard exercise provocation challenge performed during a screening visit (see general method section). The atopic status of the participants was assessed *via* a skin prick test (which was also conducted during the screening visit). Participants were non-

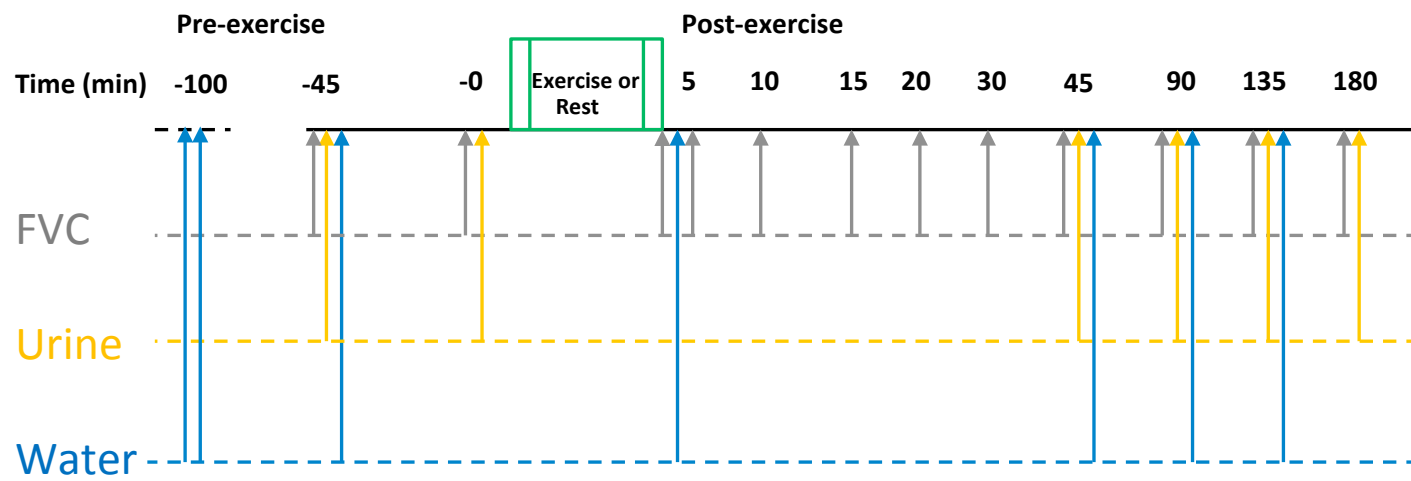
smokers, free from respiratory infections for 4 weeks prior to the study, and with no known chronic medical condition other than asthma or EIB. Participants abstained from alcohol, caffeine, exercise and niacin containing food on the day of testing, and medication was withheld as follows: short acting  $\beta_2$ -agonist treatments were withheld for a minimum of 8 h, long acting  $\beta_2$ -agonist treatments for 24 h, inhaled corticosteroid treatments for 12 h, and combination therapies for 24 h (Miller et al., 2005). Anti-histamine medication was withheld for a minimum of 72 h. Ethics approval for this study was granted by the School of Sport and Education REC (reference RE10-12).

### **7-2.2 Study design**

The study used a randomised, crossover design, with two experimental visits. Participants completed an exercise challenge while inhaling either temperate-dry or warm-humid air. Changes in FEV<sub>1</sub> and in the urinary concentrations of inflammatory mediator metabolites following exercise were the co-primary outcome measures. A schematic of the protocol is presented in Figure 7.1.

All visits commenced in the morning to standardise for the diurnal variation in lung function (Lebowitz, Krzyzanowski, Quackenboss, & O'Rourke, 1997). Participants were asked to drink 400 ml of water 1 h prior to the visit. A further 200 ml of water was given at 45 min intervals after arrival. Experimental visits commenced with the collection of baseline urine samples and lung function measurements. Urine

sampling was repeated after 45 min. Another set of lung function measurements was conducted immediately before the start of the exercise challenge test and those values served as baseline. The participants completed an 8 min exercise challenge test whilst inhaling temperate-dry or warm-humid air. Lung function measurements were made at regular intervals following the exercise challenge, and urine samples were collected at 45 min intervals over a 3 h recovery period.



**Figure 7.1.** Schematic of the experimental protocol. FVC, forced vital capacity manoeuvre; Urine, urine sample collected; Water, 200 ml of water consumed.



### **7-3.3 Exercise challenges**

Exercise challenges were conducted according to the ATS guidelines (Crapo et al., 2000). The inhaled air during the exercise challenge was either temperate-dry (16°C and  $<8 \text{ mg H}_2\text{O}\cdot\text{l}^{-1}$ ) or warm-humid (37°C and  $44 \text{ mg H}_2\text{O}\cdot\text{l}^{-1}$ , i.e., 100% relative humidity). The air was delivered through a custom built system, similar to that described by Anderson and colleagues (1982) (see general methods, section 3-5.2). Participants breathed through the system for 4 min prior to the start of exercise and for 2 min following completion of the exercise [the latter, to prevent the rapid rewarming of the airways that could contribute to airway narrowing (McFadden, Lenner, & Strohl, 1986)].

### **7-3.4 Lung function**

Forced expiratory manoeuvres were conducted in accordance with ERS/ATS guidelines (Miller et al., 2005) (see general methods for details). Manoeuvres were conducted at baseline and at 3, 5, 10, 15, 20, 30 and 45 min post-exercise, and then at 45 min intervals until 180 min of recovery.

### **7-3.5 Inflammatory mediator analysis**

At each urine collection time point, participants were instructed to completely empty their bladder. The urine was immediately measured to the nearest 2 ml and transferred into 8 ml tubes for storage at -80°C. Urinary excretion of inflammatory

mediator metabolites was measured with two methods;  $11\beta$ -PGF<sub>2 $\alpha$</sub>  and LTE<sub>4</sub> were measured using commercially available competitive ELISA kits (see general methods). Metabolites of PGD<sub>2</sub>, PGE<sub>2</sub>, prostacyclin, thromboxanes and isoprostanes were measured using UPLC-MS/MS (see general methods). The concentration of creatinine was measured in urine (see general methods) so as to allow all results to be expressed as nanogram of mediator *per* micromole of creatinine.

### **7-3.6 Statistical analysis**

Lung function. Differences between conditions and times for lung function indices (FEV<sub>1</sub>, FVC, PEF, FEV<sub>1</sub>/FVC and FEF<sub>25-75</sub>) were explored using repeated measures ANOVA. In cases of statistical significance, pairwise comparisons were conducted using repeated measure t-tests. The difference in the area under the FEV<sub>1</sub> time curve (FEV<sub>1</sub>-AUC<sub>0-45</sub>) between conditions was explored using repeated measures t-tests.

Urinary mediator excretion. The mean of the two baseline urinary mediator measurements was used for the baseline value. Due to the variability between subjects in the kinetics of urinary mediator excretion, the peak urinary mediator concentration post-exercise was selected for analysis. The inflammatory mediator metabolite data were non-normally distributed and, therefore, non-parametric tests were used. Differences between baseline and peak values were analysed using Wilcoxon signed rank tests and presented as median and interquartile range (Q1-Q3).

Since  $11\beta$ -PGF<sub>2α</sub> was undetectable by UPLC-MS/MS and prior work from our collaborators suggests that  $11\beta$ -PGF<sub>2α</sub> measured by ELISA corresponds, in fact, to 2,3DN-PGF<sub>2α</sub> (Bood et al., 2015), the agreement between the urinary excretion of  $11\beta$ -PGF<sub>2α</sub> measured by ELISA and 2,3DN-PGF<sub>2α</sub> measured by UPLC-MS/MS was assessed using Bland-Altman analysis (Bland & Altman, 1986).

## **7-4 Results**

### **7-4.1 Participant characteristics**

Seven participants (2 females) completed the study. The median (interquartile range; Q1-Q3) age, height and body mass of the participants was 22 (20-39) years, 172 (169-180) cm and 89 (72-104) kg, respectively. All participants were recreationally active, partaking in physical activity >2 h *per* week. Participants' medical diagnosis, current treatment and atopic status are presented in Table 7.1. During the screening visit, the average maximal fall in FEV<sub>1</sub> post-exercise was 20 ± 7% and all participants displayed EIB (>10% fall in FEV<sub>1</sub>, Table 7.1). All participants had at least one positive response to the allergens tested during the skin prick test.

**Table 7.1.** Participant characteristics.

ID	Gender	FEV <sub>1</sub> (% pred.)	FVC (% pred.)	Previous diagnosis	Medications	Prescribed ICS dose (µg/day)	Atopic status	EIB (max FEV <sub>1</sub> fall %)
1	M	102	117	Asthma	SABA	-	+	31
2	M	81	98	Asthma	-	-	+	14
3	M	78	102	Asthma	Combination	250	+	17
4	M	93	107	Asthma	-	-	+	16
5	F	97	109	Asthma	Combination + Anti-histamine	500	+	14
6	F	90	116	EIB	SABA	-	+	19
7	M	101	120	Asthma	SABA	-	+	27
Mean ± SD		90 ± 9	110 ± 8					20 ± 7

Lung function predicted values were taken from GLI 2012 multi-ethnic reference values (Quanjer et al., 2012). FEV<sub>1</sub> % pred., baseline forced expiratory volume in 1 s expressed relative to the predicted value; FVC % pred., baseline forced vital capacity expressed relative to the predicted value; SABA, short acting β<sub>2</sub>-agonist; ICS, inhaled corticosteroids; combination, combination therapy of long acting β<sub>2</sub>-agonist and ICS; Atopic status: +, positive skin prick response to house dust mite, cat hair, dog hair, timothy grass and/or silver birch; EIB (max FEV<sub>1</sub> fall %), exercise-induced bronchoconstriction (fall in FEV<sub>1</sub> following a screening visit).

#### 7-4.2 Inspired air conditions and ventilation during exercise

The inspired air temperature and relative humidity were 15 ± 3°C and 26 ± 5%, respectively, in the dry air condition; the mean absolute humidity was 4 ± 1 mg H<sub>2</sub>O·l<sup>-1</sup>. The inspired air in the warm-humid condition was 36 ± 1°C; assuming full saturation [based on visible condensation on the inspiratory tube (Hahn, Nogrady, Burton, & Morton, 1985)], this equates to 41 ± 3 mg H<sub>2</sub>O·l<sup>-1</sup>. During the final 4 min of exercise, participants achieved a mean ventilation of 93 ± 16 l·min<sup>-1</sup> and 91 ± 13 l·min<sup>-1</sup> (73% and 72% of predicted MVV) in the warm-humid air and dry air condition, respectively; there was no difference between conditions (*P*=0.334).

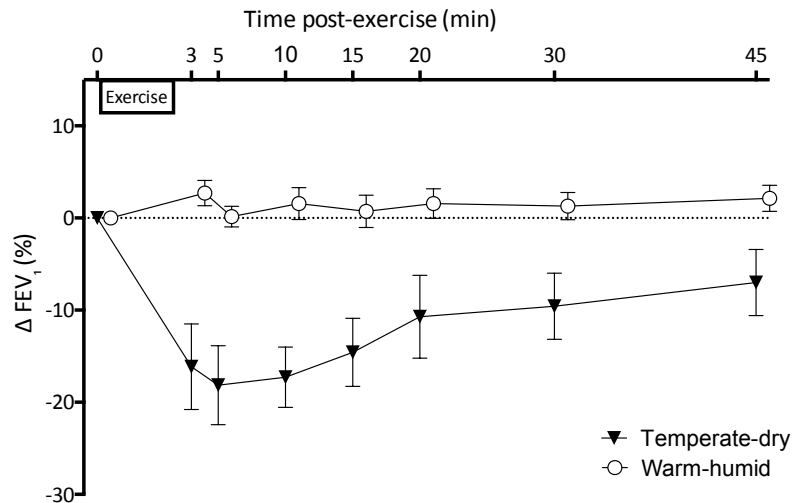
### 7-4.3 Airway response following bronchial provocation with exercise

There were no differences in baseline lung function between conditions (Table 7.2). Inhalation of warm-humid air blocked the bronchoconstriction that was observed following exercise with dry air. The maximum change in FEV<sub>1</sub> post-exercise was reduced from  $-22 \pm 10\%$  (range;  $-12$  to  $-39\%$ ) with the inhalation of dry air to  $-1 \pm 3\%$  (range;  $2$  to  $-6\%$ ) with the inhalation of warm-humid air ( $P < 0.001$ ). The broncho-protection afforded by warm-humid air for maximum % fall in FEV<sub>1</sub> was  $92 \pm 11\%$  (range 71-100%), with all participants afforded complete protection against EIB ( $< 10\%$  fall in FEV<sub>1</sub>). Similarly, the FEV<sub>1</sub>-AUC<sub>0-45</sub> was significantly reduced with the inhalation of warm-humid air compared with dry air during exercise ( $P < 0.05$ ; Figure 7.2). Exercise with the inhalation of dry air caused significant decreases in FVC, PEF, FEV<sub>1</sub>/FVC and FEF<sub>25-75</sub>; these falls in lung volume and expiratory flow rates were all attenuated by inhalation of warm-humid air (Table 7.2).

**Table 7.2.** Lung function results.

Lung function measurement	Warm-humid		Temperate-dry		Humid vs. Dry	
	Baseline	Post-exercise (minimum value)	Baseline	Post-exercise (minimum value)	Baseline	Post-exercise (minimum value)
FEV <sub>1</sub> (l)	$3.69 \pm 0.67$	$3.63 \pm 0.61$	$3.67 \pm 0.66$	$2.84 \pm 0.45^{**}$	$P=0.852$	$P=0.002$
FVC (l)	$5.34 \pm 0.70$	$5.26 \pm 0.70$	$5.24 \pm 0.63$	$4.75 \pm 0.47^{**}$	$P=0.301$	$P=0.005$
PEF (l·s <sup>-1</sup> )	$8.09 \pm 2.10$	$7.71 \pm 1.92^*$	$8.09 \pm 1.92$	$6.68 \pm 1.54^{**}$	$P=0.985$	$P=0.013$
FEV <sub>1</sub> /FVC (%)	$69 \pm 6$	$68 \pm 5.67$	$70 \pm 7$	$59 \pm 6^{**}$	$P=0.245$	$P=0.001$
FEF <sub>25-75</sub> (l·s <sup>-1</sup> )	$2.73 \pm 1.00$	$2.45 \pm 0.64$	$2.69 \pm 0.93$	$2.45 \pm 0.64^{**}$	$P=0.732$	$P=0.002$

Post-exercise (min value), lowest value recorded in the 45 min recovery period following an 8 min exercise bronchial provocation challenge; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; PEF, peak expiratory flow; FEF<sub>25-75</sub> mean forced expiratory flow between 25 and 75% of FVC; \*  $P < 0.05$ , \*\*  $P < 0.01$ , significantly different from baseline.

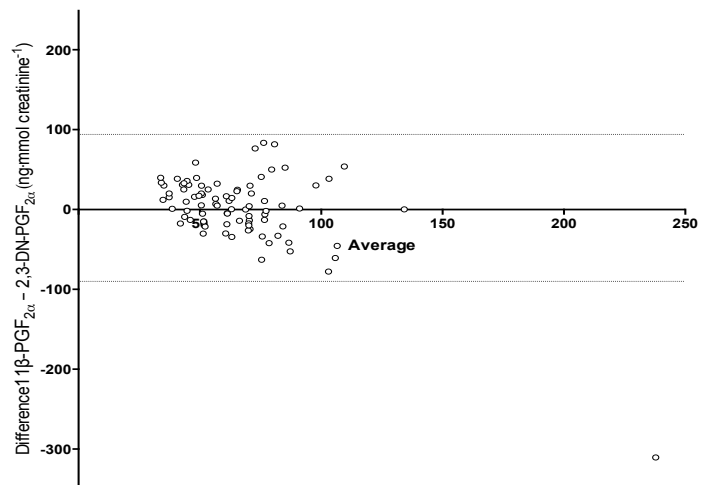


**Figure 7.2.** Mean  $\pm$  SEM percentage change from baseline in forced expiratory volume in 1 s (FEV<sub>1</sub>) after 8 min of exercise while inhaling either temperate-dry or warm-humid air in individuals with exercise-induced bronchoconstriction. FEV<sub>1</sub> AUC<sub>0-45</sub> was significantly reduced with the inhalation of warm-humid during exercise ( $P < 0.05$ ).

#### 7-4.4 Urinary excretion of inflammatory mediator metabolites

##### *Comparison of 11 $\beta$ -PGF<sub>2 $\alpha$</sub> and 2,3DN-PGF<sub>2 $\alpha$</sub>*

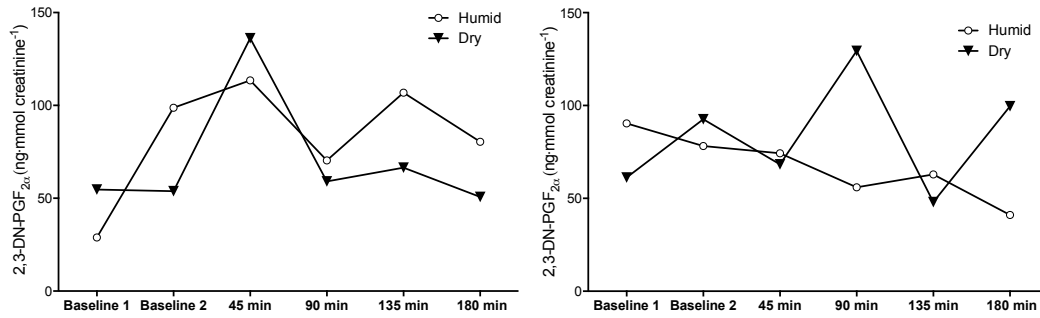
The excretion of PGD<sub>2</sub> was assessed using both ELISA and UPLC-MS/MS *via* measurements of 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  and 2,3DN-PGF<sub>2 $\alpha$</sub> , respectively. As 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  and 2,3DN-PGF<sub>2 $\alpha$</sub>  are cross reactive when assessed using ELISA (Bood et al., 2015), we used the level of agreement between these two metabolites to compare methodologies (i.e., ELISA and UPLC-MS/MS). The comparison between the urinary excretion of 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  measured by ELISA and 2,3DN-PGF<sub>2 $\alpha$</sub>  measured by UPLC-MS/MS indicated a bias of -2 ng· $\mu$ mol creatinine<sup>-1</sup> and a 95% limit of agreement of -90 to 94 ng· $\mu$ mol creatinine<sup>-1</sup> (Figure 7.3).



**Figure 7.3.** Bland-Altman plot showing the agreement between the urinary excretion of  $11\beta$ -PGF<sub>2α</sub> measured by ELISA and 2,3DN-PGF<sub>2α</sub> measured by UPLC-MS/MS

***Peak urinary metabolite excretion post-exercise***

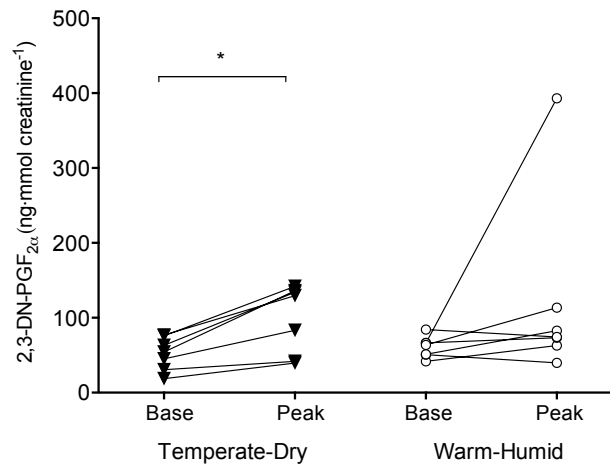
The kinetics of urinary mediator excretion were highly variable between subjects. An example of the between-subject variability in the kinetics of inflammatory mediatory excretion is presented in figure 7.4. Due to this variability, and in line with recent mass spectrometry data published by our collaborators (Bood et al., 2015), analyses were conducted to determine the differences between baseline and peak mediator excretion post-exercise.



**Figure 7.4 (left).** Example of the variable kinetics of urinary mediator excretion between subjects. On the left the peak urinary excretion of 2,3-dinor-PGF<sub>2α</sub> occurs 45 min following exercise with dry air; on the right the peak excretion occurs at 90 min post-exercise.

**Prostaglandin D<sub>2</sub>** – There was a significant increase in the urinary excretion of 2,3-dinor-PGF<sub>2α</sub> (measured using UPLC-MS/MS) following exercise with the inhalation of dry air that was inhibited with the inhalation of the warm-humid air (Figure 7.5). For other metabolites of PGD<sub>2</sub> (i.e., tetranor-PGD<sub>2</sub> measured *via* UPLC-MS/MS and 11β PGF<sub>2α</sub> measured *via* ELISA), a difference between baseline and peak excretion post-exercise could not be identified (Table 7.3).





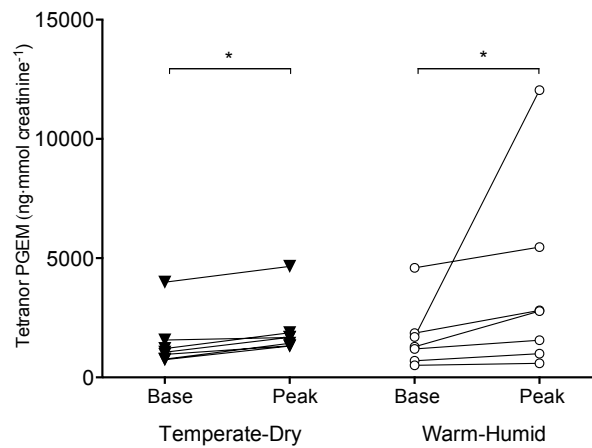
**Figure 7.5.** Urinary excretion of 2,3-DN-PGF<sub>2α</sub> (ng·μmol creatinine<sup>-1</sup>) following 8 min of exercise with the inhalation of temperate-dry and warm-humid air; \* *P*<0.05.

**Table 7.3.** Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) and enzyme linked immunosorbent assays (ELISA)

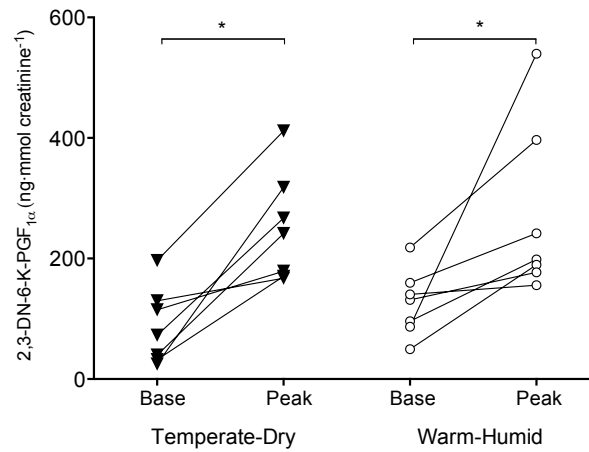
	Temperate-dry		Warm-humid	
	Baseline	Peak	Baseline	Peak
<b>UPLC/MS/MS</b>				
<b>PGD<sub>2</sub></b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>
2,3-dinor-PGF <sub>2α</sub>	54 (31-76)	129 (42-136)*	64 (51-67)	74 (63-114)
Tetranor-PGD <sub>M</sub>	313 (231-377)	299 (232-537)	391(236-457)	308 (212-700)
<b>PGE<sub>2</sub></b>	9 (5-12)	15 (8-20)	10 (8-18)	12 (8-31)
Tetranor-PGEM	1056 (768-1325)	1674 (1325-1871)*	1288 (696-1854)	2782 (992-5462)*
<b>PGI<sub>2</sub></b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>
2-3-dinor-6keto-PGF <sub>1α</sub>	73 (32-130)	241 (170-318)*	131 (87-160)	198 (177-397)*
<b>TXB<sub>2</sub></b>	4 (3-6)	4 (3-10)	4 (4-5)	6 (4-7)
11-dihydro-TXB <sub>2</sub>	3 (2-4)	5 (4 – 9)*	5 (3-7)	6 (3-12)
2-3-dinor-TXB <sub>2</sub>	37 (26-39)	43 (36-90)*	44 (26-71)	65 (38-78)
<b>8-iso-PGF<sub>2α</sub></b>	21 (18-37)	35 (25-42)	26 (21-30)	31 (21-47)
2,3-dinor-8-iso-PGF <sub>2α</sub>	152 (103-186)	232 (166-267)*	142 (115-223)	275 (150 – 369)*
8,12-iPF <sub>2α</sub> -IV	447 (383-625)	754 (526-866)	461 (402-644)	735 (498-1202)
<b>ELISA</b>				
<b>PGD<sub>2</sub></b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>	<b>Not measured</b>
11β PGF <sub>2α</sub>	70 (64-87)	73 (66-84)	67 (57 – 84)	61 (61-83)
<b>LTE<sub>4</sub></b>	31 (19-50)	39 (24-55)	36 (14-114)	37 (19-57)

Data are ng·μmol creatinine<sup>-1</sup> and presented as median and interquartile range (Q1-Q3); \* *P*<0.05 vs. baseline.

**Prostaglandin E<sub>2</sub> and Prostacyclin (PGI<sub>2</sub>)** – Urinary excretion of PGE<sub>2</sub> was present in low levels in both conditions and showed no change following exercise (Table 7.3). However, tetranor-PGEM, a metabolite of PGE<sub>2</sub>, significantly increased in both conditions following exercise (Figure 7.6). Similarly, the PGI<sub>2</sub> metabolite, 2-3-dinor-6keto-PGF<sub>1α</sub> significantly increased following exercise in both conditions (Figure 7.7). For both these metabolites, no significant difference was observed between conditions.

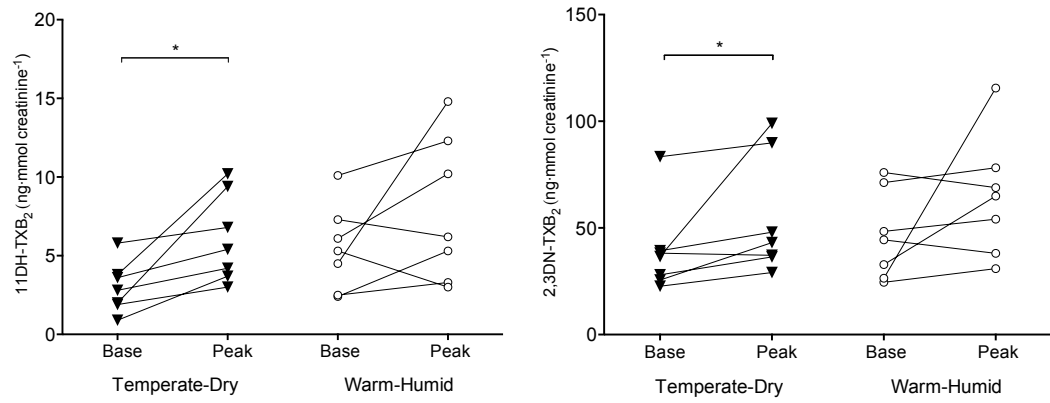


**Figure 7.6.** Urinary excretion of Tetranor PGEM (ng· $\mu$ mol creatinine<sup>-1</sup>) following 8 min of exercise with the inhalation of temperate-dry and warm-humid air; \*  $P < 0.05$ .



**Figure 7.7.** Urinary excretion of 2-3-dinor-6keto-PGF<sub>1α</sub> (ng·μmol creatinine<sup>-1</sup>) following 8 min of exercise with the inhalation of temperate-dry and warm-humid air; \*  $P < 0.05$ .

**Thromboxanes and Isoprostanes** – The TXB<sub>2</sub> metabolites, 11-dihydro-TXB<sub>2</sub> and 2-3-dino-TXB<sub>2</sub>, increased significantly following exercise with the inhalation of dry air (Figure 7.8). There was no difference following exercise with the inhalation of warm-humid air (Figure 7.8). The isoprostanes metabolite, 2,3-dinor-8-iso-PGF<sub>2α</sub>, increased from baseline following exercise in both conditions (Table 7.3).



**Figure 7.8.** Urinary excretion of TXB<sub>2</sub> metabolites 11DH-TXB<sub>2</sub> (left) and 2,3DN-TXB<sub>2</sub> (right) following 8 min of exercise with the inhalation of temperate-dry and warm-humid air; \*  $P < 0.05$ .

## 7-5 Discussion

With this study we demonstrate that UPLC-MS/MS can be used to detect changes in inflammatory mediator release in urine following bronchial provocation with exercise. This is the first report showing that exercise in individuals with mild-to-moderate EIB is associated with significantly increased urinary excretion of PGE<sub>2</sub>, PGI<sub>2</sub> and 8-isoprostanes (irrespective of the presence of bronchoconstriction), whilst urinary concentrations of 2,3-DN-PGF<sub>2α</sub> (a metabolite of the potent bronchoconstrictor PGD<sub>2</sub>) and of 11-dihydro-TXB<sub>2</sub> and 2-3-dino-TXB<sub>2</sub> (metabolites of thromboxane) are solely increased when bronchoconstriction occurs. We also confirm that individuals with mild-to-moderate EIB gain full broncho-protection from inhaling warm-humid air during exercise. Taken together, our findings support that a range of broncho-constricting as well as broncho-protective mediators are released in response to exercise. They also suggest that removal of the osmotic stress at the level of the airways limits the release of broncho-constricting mediators, thereby preventing airway narrowing.

One novel aspect of this study is the use of UPLC-MS/MS to detect a spectrum of lipid mediators possibly involved in EIB. This work extends previous findings based on ELISA analysis alone, which identified PGs and cyst-LTs as the main mediators of bronchoconstriction in individuals with asthma and/or EIB (Brannan et al., 2006; Kippelen, Larsson, Anderson, Brannan, Dahlén, et al., 2010a; Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b; Larsson et al., 2011; Mickleborough et al., 2003). While we were unable to reproduce earlier findings of an increased urinary excretion of 11β PGF<sub>2α</sub> (as measured by ELISA) after bronchial provocation with

exercise in individuals with mild asthma (O'Sullivan et al., 1998) and in elite athletes with EIB (Mickleborough et al., 2003), the observation of an increase in urinary 2,3-DN-PGF<sub>2a</sub> (detected through UPLC-MS/MS in the dry air condition only) nevertheless supports the role of mast cells in the pathophysiology of EIB (Anderson & Daviskas, 2000).

Mast cells are the primary source of PGD<sub>2</sub>, rendering urinary PGD<sub>2</sub> metabolites a convenient tool for assessment of mast cell activation. In the past, induced bronchoconstriction in laboratory settings using exercise (Haverkamp et al., 2005; Mickleborough et al., 2003; O'Sullivan et al., 1998) and its surrogates (Brannan et al., 2006; Kippelen, Larsson, Anderson, Brannan, Dahlén, et al., 2010a; Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b; Larsson et al., 2011) as well as allergen challenges (Balgoma et al., 2013; Daham et al., 2014) has been associated with increased excretion of various metabolites of PGD<sub>2</sub> (11β PGF<sub>2α</sub>, 2,3-DN-PGF<sub>2a</sub> and/or Tetranor-PGD<sub>2</sub>). Variability in the levels of PGD<sub>2</sub> metabolites has however been noticed, which may be attributed, at least in part to: i) differences in the study population (e.g., elite athletes with EIB *versus* untrained individuals with clinical asthma), ii) variance in the mode of action of the different provocation challenges (e.g., Ig-E dependent *versus* osmotic-driven mast cell activation), and iii) anti-body cross-reactivity (when using ELISA). As highlighted in a recent publication from our collaborators (Bood et al., 2015), it is likely that levels of 11β-PGF<sub>2α</sub> measured by ELISA correspond to levels of 2,3DN-PGF<sub>2α</sub> measured by UPLC-MS/MS due to cross-reactivity. That we observed reasonable agreement between these two metabolites supports this idea. Taking this methodological fact into consideration, our PGD<sub>2</sub>

results are therefore broadly aligned with the literature, and support mast cell activation in EIB.

We add to the previous literature by demonstrating that exercise, independent of the occurrence of bronchoconstriction, is associated with the release of the broncho-protective PGE<sub>2</sub> and of the main metabolite of prostacyclin in individuals with mild-to-moderate EIB. A similar result was recently observed following hyperpnoea of dry air in individuals with mild asthma (Bood 2015), suggesting that increased ventilation (rather than exercise *per se*) triggers the release of broncho-protective mediators. Release of lipid mediators with protective and anti-inflammatory properties in the airways, such as PGE<sub>2</sub> (Säfholm et al., 2015) and PGI<sub>2</sub> (Hardy, Bradding, Robinson, & Holgate, 1988), could help to maintain airway patency under conditions of high osmotic, thermal and/or mechanical stress (as occurs within the airways when ventilatory demand increases). On the other hand, an increased production of broncho-constricting mediators, such as PGD<sub>2</sub>, cyst-LTs and thromboxanes, could precipitate airway narrowing.

While we were unable to detect an increased excretion of the metabolites of cyst-LTs post-exercise in our study population, urinary concentrations of the two metabolites of thromboxane (11-dihydro-TXB<sub>2</sub> and 2-3-dino-TXB<sub>2</sub>) significantly increased in the dry air condition. In line with the recently published data based on UPLC-MS/MS urinary mediator analysis following allergen challenge in asthmatics (Daham et al., 2014), this points toward a possible role of thromboxane in bronchoconstriction. Thromboxane is a well-known broncho-constrictor, with the

selective and potent thromboxane A<sub>2</sub> (TxA<sub>2</sub>) receptor antagonist having been shown to inhibit PGD<sub>2</sub>- and allergen-induced bronchoconstriction in individuals with asthma (Beasley et al., 1989). In regards to cyst-LT, we previously noticed that the signal in urine obtained through ELISA may be weaker compared to PGD<sub>2</sub> after induced bronchoconstriction (Kippelen, Larsson, Anderson, Brannan, Delin, et al., 2010b). Therefore, additional testing is required to increase the sample size and the power of our statistical analysis to clarify the involvement of cyst-LT in EIB.

Interestingly, in a seminal paper in 1979, Anderson and colleagues demonstrated that warm-humid air could inhibit the post-exercise fall in FEV<sub>1</sub> and discussed the possibility that this may be due to the inhibition of inflammatory mediator release (Anderson et al., 1979). Here, with a similar methodology, we confirmed that the inhalation of warm-humid air inhibits EIB. Furthermore, our observation that 2,3-DN-PGF<sub>2a</sub> is not increased following exercise with warm-humid air suggests that mast cell activation is inhibited when the osmotic stress to the airways is removed. These data, therefore, constitute the first direct evidence that the attenuation of EIB with the inhalation of warm-humid air during exercise may be caused by the inhibition of mast cell mediator release. Due to our small sample size (n=7), however, these interpretations should be treated with caution and further validation is required.

In conclusion, using mass spectrometry, we were able to identify a spectrum of lipid mediators that are released in response to exercise in individuals with mild-to-moderate EIB. While we identified the presence of the metabolites of the potent



broncho-constricting mediators PGD<sub>2</sub> and thromboxane in urine in conditions of induced bronchoconstriction (when dry air was inhaled during exercise), the broncho-protective mediators PGE<sub>2</sub> and PGI<sub>2</sub> were released following exercise when bronchoconstriction was either induced or inhibited. We also confirmed that the inhalation of warm-humid air during exercise fully inhibits airway narrowing. Collectively, our findings suggest that warm-humid air breathing blocks EIB by decreasing the release of the potent broncho-constricting mediators PGD<sub>2</sub> and thromboxane, while maintaining the production of the broncho-dilating mediators PGE<sub>2</sub> and PGI<sub>2</sub>.

# **CHAPTER 8**

## **General Discussion**

The overall aims of this thesis were; to investigate factors that could affect the severity of EIB; further understand the pathophysiology of the condition; and identify strategies that could improve the management and/or prevent the development of EIB in athletes. To achieve these aims four investigations were conducted. In this chapter, the main findings of these investigations will be discussed in the context of these aims and the existing literature, and recommendations for future research will be provided.

### **8-1 AIM 1: Investigate factors that could affect the severity of EIB**

Following a review of the literature, it was established that there was relatively little information regarding the effect of whole-body dehydration on airway responsiveness and lung function. Given that:

- changes in the volume and composition of ASL during exercise may contribute to the pathophysiology of EIB (Anderson & Daviskas, 2000)
- and that whole-body dehydration may reduce the movement of water towards the airway surface,

we reasoned that whole-body dehydration could increase the severity of airway narrowing following exercise.

Our findings in Chapter 4, however, did not support our hypothesis. Indeed, we found that exercise-induced mild-dehydration (2.3% reduction in body mass) did not affect the airway response to dry air hyperpnoea. We propose that the magnitude of respiratory water loss during dry air hyperpnoea (Daviskas et al.,

1991) may overwhelm the relatively small volume of water available at the airway surface (Anderson, 1984). Thus the airway response to hyperpnoea of dry air would be maximal irrespective of the effect of whole-body dehydration on the initial volume of ASL. To overcome this methodological limitation, we recommend for further work to be based on indirect bronchial provocation testing with step-wise increments (such as the mannitol or hypertonic saline challenge).

Additional results from Chapter 4 did indicate that whole-body dehydration may have a deleterious effect on small airway function. Indeed, exercise-induced whole-body dehydration reduced FVC and increased RV by ~300 ml and ~220 ml, respectively. We propose that exercise-induced whole-body dehydration may interfere with the hydration of the small airways, consequently increasing their collapsibility.

That exercise-induced dehydration can impair small airway function may be an important observation for endurance athletes prone to whole-body dehydration (Rüst et al., 2012; Zouhal et al., 2011). This novel finding therefore warrants further investigation. Firstly, the effect of whole-body dehydration should be confirmed using additional measures of small airway function. At present there is no gold standard test for the assessment of the small airways; instead the use of a range of tests is recommended (Konstantinos Katsoulis, Kostikas, & Kontakiotis, 2013). Small airway function in Chapter 4 was assessed primarily by changes in FVC and RV. The

measurement of FVC and RV both require maximal expiratory manoeuvres and, therefore, changes in FVC and RV may reflect changes in small airway stability at low lung volumes. In contrast, the forced oscillometry technique (FOT) and multiple breath washout technique (MBW) assess small airway function during normal tidal breathing. Therefore, the use of such tests could help to identify whether whole-body dehydration affects small airway function during normal breathing, or whether small airway function is only affected at low lung volumes.

Further support that whole-body dehydration can affect small airway function should also be sought using a dose-response protocol, whereby lung function is measured at varying levels of whole-body dehydration. We propose that levels of up to 8% loss of body mass should be investigated, as this level of dehydration has been reported in field-based studies in athletes competing in endurance events (Rüst et al., 2012; Zouhal et al., 2011).

Following confirmation that whole-body dehydration impairs small airway function, the next step would be to establish the functional relevance of these findings. There is a rationale to suggest that whole-body dehydration may affect breathing mechanics, athletic performance and may cause epithelial injury. In Chapter 4, in addition to a reduction in FVC and an increase in RV, we noted that whole-body dehydration increased FRC. We know that an increase in FRC can increase the work of breathing (Ferguson, 2006). Furthermore, it has been demonstrated that the

work of breathing can significantly affect athletic performance (Harms, Wetter, St Croix, Pegelow, & Dempsey, 2000). Therefore, if the increase in FRC we noted at rest also occurs during exercise, breathing mechanics could be altered, and athletic performance could be impaired. The normal response to exercise is to decrease EELV in order to increase tidal volume and meet the ventilatory demand of exercise (Sharratt, Henke, Aaron, Pegelow, & Dempsey, 1987). If during exercise in a dehydrated state the reduction in EELV infringes upon the increased closing volume, peripheral airway closure could occur. *In vitro* models suggest that the re-opening of closed airways can cause epithelial injury (Bilek et al., 2003). Therefore, impaired small airway stability could cause the cyclic opening and closing of the small airways during exercise and lead to epithelial injury. This is relevant in that repeated epithelial injury has been implicated in the development of EIB in athletes (Anderson & Kippelen, 2005).

With reference to our findings, and with the knowledge that a large number of athletes experience whole-body dehydration during exercise (Zouhal et al., 2011), we propose that the effect of whole-body dehydration on breathing mechanics and epithelial injury should be further investigated.

## **8-2 AIM 2: Further the understanding of the pathophysiology of EIB**

With Chapter 6 and Chapter 7 we have extended the knowledge of the pathophysiology of EIB. Firstly, we confirmed that EIB is associated with mast cell activation. This was demonstrated by an increase in the urinary excretion of metabolites of PGD<sub>2</sub> (a mast cell specific mediator) following bronchial provocation with EVH (Chapter 6) and with exercise (Chapter 7).

In this thesis we discussed the possibility that bronchial provocation with exercise (and EVH) may increase the concentration of mast cell metabolites in urine due to osmotic stimulation of mast cells at the level of the airways. Chapter 7 provided support to this theory. Indeed, the removal of the osmotic stress by administration pre-conditioned air at the mouth level during exercise inhibited bronchoconstriction, as well as mast cell mediator release. This constitutes the first direct evidence of a broncho-protective effect of warm-humid air breathing through inhibition of mast cell activation.

In Chapter 7, the simultaneous analysis (*via* UPLC-MS/MS) of an array of lipid mediators allowed for the first documentation of the release of the broncho-protective mediators PGE<sub>2</sub> and PGI<sub>2</sub> in response to exercise. The precise role of these protective mediators in EIB is yet to be fully established. Given the rise in urinary excretion of PGE<sub>2</sub> and PGI<sub>2</sub> metabolites in conditions of induced bronchoconstriction through exercise (Chapter 7) and EVH broncho-provocation

(Bood et al., 2015), we suggest that occurrence of airway narrowing is dependent upon the balance between broncho-protective and broncho-constrictive mediator release. At this stage, a closer examination of the inter-play between various broncho-active mediators in conditions of induced and inhibited bronchoconstriction is warranted.

Further research could investigate the role of mediator release (incl. the protective mediators) following multiple bouts of exercise. This is based on the fact that many individuals with EIB display a refractory response to a second bout of exercise. Indeed, Schoeffel and colleagues (1980) demonstrated that when individuals with asthma and EIB repeated the same exercise twice within an hour, half of the individuals experienced airway narrowing that was <50% of the magnitude of the initial airway response. The mechanism of this refractoriness is not fully understood and many theories have been proposed to explain its occurrence. These theories include: an increased level of circulating catecholamines; the depletion of mast cell mediators; a decreased release of neuropeptides; the decreased responsiveness of airway smooth muscle; the desensitisation and/or down regulation of receptors on the airway smooth muscle following the initial challenge; and the release of protective mediators following the initial challenge (Larsson et al., 2013). That a release of PGE<sub>2</sub> and PGI<sub>2</sub> was observed after a single exercise test in individuals with mild-to-moderate EIB (Chapter 7) support the latter proposal.



Recently Bood and colleagues (2015) conducted the first comprehensive analysis of mediator release following repeated bouts of EVH in individuals with mild asthma (Bood et al., 2015). In line with our own results (Chapter 7), they noticed increased concentrations of PGI<sub>2</sub> and PGE<sub>2</sub> in urine following the initial hyperpnoea challenge, suggesting these mediators might mediate refractoriness. However, at this stage, decreased responsiveness of airway smooth muscle due to the down regulation of receptors on the airway smooth remains another potential factor contributing to the refractory period (Larsson et al., 2013). Our intervention with warm-humid air naturally inhibited EIB, while maintaining PGE<sub>2</sub> and PGI<sub>2</sub> production. We therefore propose a follow-up investigation using repeated bouts of exercise where EIB is initially inhibited with warm-humid air; this would allow for the contribution of PGE<sub>2</sub> and PGI<sub>2</sub> in the refractory period to be established, when the airway smooth muscle has not been previously contracted.

### **8-3 AIM 3: Identify strategies that could improve the management and/or prevent the development of EIB in athletes**

The primary action of inhaled  $\beta_2$ -agonist medication is to prevent or alleviate bronchoconstriction. In Chapter 5, we demonstrated that the majority of athletes with EIB receive significant broncho-protection following hyperpnoea of dry air with the prophylactic administration of a single, therapeutic dose of terbutaline.

Furthermore, we demonstrated that the level of broncho-protection afforded to athletes (~54%) following exercise is similar to that previously reported in individuals with asthma (66%; range 29% - 91%) (Bonini et al., 2013). These data

suggest that the mode of action of inhaled  $\beta_2$ -agonists for the prevention of EIB in athletes is likely to be similar to that of individuals with asthma.

In addition to its primary action of relaxing the airway smooth muscle, inhaled  $\beta_2$ -agonist medication has multiple effects, mediated through  $\beta_2$ -receptors situated on various cell types (Barnes, 1999). In Chapter 5 and Chapter 6 of this thesis we investigated two additional pathways by which inhaled  $\beta_2$ -agonists may be therapeutically beneficial to athletes with EIB. Firstly, in Chapter 5 we demonstrated for the first time in humans that the inhalation of terbutaline attenuated epithelial perturbation following hyperpnoea of dry air. This finding extended previous work done in animals (Omori et al., 1995; Wang et al., 1992). We propose that terbutaline, *via* ion-mediated water secretion (Davis et al., 1979), reduced dehydration injury of the airway epithelium. This finding may be particularly relevant to athletes who regularly damage their airway epithelium through exercise-hyperpnoea and who put themselves at risk for EIB (Anderson & Kippelen, 2005).

The second pathway whereby inhaled  $\beta_2$ -agonist medication may be therapeutically beneficial to athletes is *via* mast cell stabilisation (such as demonstrated in Chapter 6). This finding is in line with observations of a similar mast cell stabilising effect of supra-therapeutic doses of long acting  $\beta_2$ -agonist medications (Brannan et al., 2006; Szczeklik et al., 1998). Given the wide range of damaging effects of mast cell

mediators (Bradding et al., 2006), the stabilisation of mast cells during exercise could be beneficial to the long term respiratory health of athletes. However, regular usage of inhaled  $\beta_2$ -agonists is known to have side effects, which include the development of tolerance and increased AHR. Hancox and colleagues (2002) demonstrated that following one week of regular usage of the inhaled  $\beta_2$ -agonist salbutamol (200  $\mu\text{g}$  administered four times daily), individuals with asthma displayed a larger fall in  $\text{FEV}_1$  following exercise and a diminished speed of recovery from bronchoconstriction. A similar increased recovery time was noted to a methacholine challenge following one week of daily treatment with the long acting  $\beta_2$ -agonist formoterol (12  $\mu\text{g}$  twice daily) (Haney & Hancox, 2005). Tolerance to  $\beta_2$ -agonists can however be reversed after only 72 h of discontinuation of therapy (Haney & Hancox, 2006). Prophylactic administration of inhaled  $\beta_2$ -agonist could be used, in moderation, to prevent the adverse effects of mast cell mediators released following exercise in athletes with EIB.

In addition to preventing mast cell mediator release and providing acute broncho-protection, the use of  $\beta_2$ -agonist medication to reduce the risk of long-term development of EIB in athletes could be envisaged. However due to the high training load of athletes and the above-mentioned side effects of chronic use of inhaled  $\beta_2$ -agonists, it is not currently recommended for athletes to take  $\beta_2$ -agonist medication prior to every training session. An alternative strategy might be to identify training sessions most likely to induce epithelial injury (such as those performed in cold dry air) and to pre-medicate athletes solely prior to those

sessions (with the minimal required medication). For athletes at high risk of recurrent airway epithelial injury (such as cross country skiers), other strategies (pharmacological and non-pharmacological) should be devised.

## **8-4 Conclusion**

EIB is the most common chronic medical condition in elite athletes and has been the subject of much investigation over the past 50 years. This thesis has contributed to this large body of research, and provides some novel insights into the mechanisms underlying this complex, and often under-recognised, respiratory condition. Furthermore, it has highlighted directions for future research that may lead to the development of new therapeutic strategies for EIB prevention.

In this thesis, we first demonstrated that mild exercise-induced whole-body dehydration does not affect the airway response to dry air hyperpnoea in symptomatic recreational athletes. Interestingly, however, we did establish that whole-body dehydration may affect baseline small airway function. We propose that this effect may be mediated by changes in volume and/or composition of the airway surface liquid, which compromises the stability of the small airways. Further work is required to establish the functional and clinical relevance of these original findings, especially in regards to breathing mechanics and airway epithelial injury during exercise.

With our experimental work, we have also established that a therapeutic dose of the short acting inhaled  $\beta_2$ -agonist terbutaline may be beneficial to athletes with EIB. Firstly, we demonstrated that prophylactic administration of terbutaline offers significant broncho-protection following hyperpnoea of dry air. Secondly, we showed that terbutaline reduces urinary excretion of the pneumo-protein CC16 following dry air hyperpnoea. This suggests that  $\beta_2$ -agonist medication may be used to attenuate exercise-induced epithelial injury in humans. Finally, we demonstrated that a single therapeutic dose of  $\beta_2$ -agonist medication reduces the urinary excretion of  $11\beta$ -PGF<sub>2 $\alpha$</sub>  following hyperpnoea of dry air, indicating an attenuation of mast cell activation. With reference to these findings, a re-evaluation of the usage of inhaled  $\beta_2$ -agonists to optimise EIB treatment in athletes is probably warranted.

In the last section of this thesis, we confirmed that the inhalation of warm-humid air during exercise completely blocks bronchoconstriction in individuals with EIB. We therefore used this model of naturally-inhibited EIB to investigate the key mediators of bronchoconstriction. Our results demonstrated that potent bronchoconstrictive mediators PGD<sub>2</sub> and thromboxane are released in association with bronchoconstriction (i.e., when dry air was inhaled during exercise) and inhibited with the inhalation of warm-humid air. On the other hand, the broncho-protective mediators PGE<sub>2</sub> and PGI<sub>2</sub> are released following exercise irrespective of the occurrence of airway narrowing (i.e., these mediators were released following exercise with the inhalation of both temperate-dry and warm-humid air). These findings directly implicate the osmotic stimulation of mast cells in the pathophysiology of EIB in athletes. Furthermore, the identification of broncho-

protective mediators released with-in the airways during exercise may provide a target for future therapeutic interventions.

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# Appendix

# Ethics approval

Head of School of Sport & Education  
Professor Ian Rivers

**Brunel**  
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Heinz Wolff Building,  
Brunel University, Uxbridge,  
Middlesex, UB8 3PH, UK  
Tel +44 (0)1895 266494  
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www.brunel.ac.uk

Andrew Simpson  
PhD (Sport Sciences) Student  
School of Sport and Education  
Brunel University

9<sup>th</sup> August 2013

Dear Andy

## **RE52-12 Effect of whole-body dehydration on airway responsiveness**

I am writing to confirm the Research Ethics Committee of the School of Sport and Education received your application connected to the above mentioned research study. Your application has been independently reviewed to ensure it complies with the University/School Research Ethics requirements and guidelines.

The Chair, acting under delegated authority, is satisfied with the decision reached by the independent reviewers and is pleased to confirm there is no objection on ethical grounds to grant ethics approval to the proposed study.

Any changes to the protocol contained within your application and any unforeseen ethical issues which arise during the conduct of your study must be notified to the Research Ethics Committee for review.

On behalf of the Research Ethics Committee for the School of Sport and Education, I wish you every success with your study.

Yours sincerely



 Dr Richard J Godfrey  
**Chair of Research Ethics Committee**  
School Of Sport and Education



THE QUEEN'S  
ANNIVERSARY PRIZES  
FOR HIGHER AND FEEDBACK EDUCATION  
2011



## National Research Ethics Service

Central London REC :

Room 4W/12, 4th Floor Wes  
Charing Cross Hospital  
Fulham Palace Road  
London  
W6 8R

Telephone: 020 331 1728  
Facsimile: 020 331 1728

26 October 2010

Dr Pascale Kippelen  
Lecturer  
School of Sport & Education  
Brunel University  
Uxbridge, Middlesex  
UB8 3PH

Dear Dr Kippelen

**Study Title:** Effect of Terbutaline on Hyperpnea-Induced Airway  
Injury in Athletes with Asthma  
**REC reference number:** 10/H0716/30

Thank you for your letter of 23 September 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

### Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

### Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

### Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

This Research Ethics Committee is an advisory committee to London Strategic Health Authority  
*The National Research Ethics Service (NRES) represents the NRES Directorate within  
the National Patient Safety Agency and Research Ethics Committees in England*

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

**It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).**

### Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
Investigator CV	1	06 June 2010
Covering Letter	1	22 July 2010
Advertisement	2	03 June 2010
GP/Consultant Information Sheets	2	22 July 2010
Participant Information Sheet	3	22 July 2010
Response to Request for Further Information	1	07 June 2010
Response to Request for Further Information	1	04 August 2010
Response to Request for Further Information		23 September 2010
Participant Consent Form	3	22 July 2010
Questionnaire	2	04 June 2010
Restrictions Sheet	2	04 June 2010
EucapSys air mixture with adjustable CO2 levels		
Evidence of insurance or indemnity	Ref: NAP	12 August 2010

### Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

### After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "*After ethical review – guidance for researchers*" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

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The National Research Ethics Service (NRES) represents the NRES Directorate within  
the National Patient Safety Agency and Research Ethics Committees in England

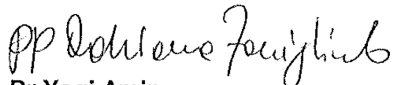
The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email [referencegroup@nres.npsa.nhs.uk](mailto:referencegroup@nres.npsa.nhs.uk).

10/H0716/30

Please quote this number on all correspondence

Yours sincerely

  
Dr Yogi Amin  
Chair

Email: [adriana.fanigliulo@imperial.nhs.uk](mailto:adriana.fanigliulo@imperial.nhs.uk)

*Enclosures:* "After ethical review – guidance for researchers"

*Copy to:* Dr Gary Armstrong, School of Sport & Education, Heinz Wolf Building  
Brunel University, Uxbridge UB8 3PH, Middlesex UB8 3PH

Head of School of Sport & Education  
Professor Susan Capel

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Andrew Simpson  
PhD Sport Sciences Student  
School of Sport and Education  
Brunel University

20<sup>th</sup> November 2012

Dear Andy

**RE10-12 – Effect of repeated exercise and humid air breathing on airway injury and urinary mediator release in individuals with exercise-induced bronchoconstriction.**


I am writing to confirm the Research Ethics Committee of the School of Sport and Education received your application connected to the above mentioned research study. Your application has been independently reviewed to ensure it complies with the University/School Research Ethics requirements and guidelines.

The Chair, acting under delegated authority, is satisfied with the decision reached by the independent reviewers and is pleased to confirm there is no objection on ethical grounds to grant ethics approval to the proposed study.

Any changes to the protocol contained within your application and any unforeseen ethical issues which arise during the conduct of your study must be notified to the Research Ethics Committee.

On behalf of the Research Ethics Committee for the School of Sport and Education, I wish you every success with your study.

Yours sincerely



Dr Richard Godfrey  
**Chair of Research Ethics Committee**  
School of Sport and Education



## Consent form



**Brunel**  
UNIVERSITY  
L O N D O N

School of Sport & Education  
Heinz Wolff Building  
Uxbridge, Middlesex,  
UB8 3PH, UK

Subject Identification Number:

### CONSENT FORM

Title of Study: **Effect of whole-body dehydration on airway responsiveness**

**The participant should complete the whole sheet him**

**Please tick box**

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected, and without affecting my future employment as a member of staff of the University or my progression or assessment as a student of the University.
3. I understand that information that is obtained during this study may be looked at by the chief investigator and the researchers associated to the study and that it will be treated as private and confidential. It will not be released or revealed to any person without my written consent. However, the information may be used for statistical or scientific purposes with my right to privacy retained. I give permission for these individuals to have access to the data collected during the above mentioned study.
5. I understand that this project has been approved by the Research Ethics Committee of the School of Sport & Education, Brunel University.
6. I agree to take part in the above study.

\_\_\_\_\_  
Name of Volunteer

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Name of Person taking consent

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature



## Health questionnaire



**Brunel**  
UNIVERSITY  
L O N D O N

School of Sport & Education  
Heinz Wolff Building  
Uxbridge, Middlesex  
UB8 3PH, UK

Title of study: **Effect of whole-body dehydration on airway responsiveness**

### PRE-PARTICIPATION HEALTH CHECK QUESTIONNAIRE

Health and safety within this investigation is of paramount importance. For this reason we need to be aware of your current health status before you begin any testing procedures. The questions below are designed to identify whether you are able to participate now or should obtain medical advice before undertaking this investigation, Whilst every care will be given to the best of the investigators ability, an individual must know his/her limitations.

**Subject name:**.....

**Date of birth:**.....

**Doctors Surgery Address:**.....

**Emergency Contact Name:**.....

Please answer the question below (circle as appropriate). If you are unsure about any of these questions, please ask.

1. Has your doctor ever diagnosed a heart condition or recommended only medically-supervised exercise? **Yes / No**
2. Do you suffer from chest pains or heart palpitations? **Yes / No**
3. Do you have known high blood pressure? **Yes / No**
4. Do you have low blood pressure or often feel faint or have dizzy spells? **Yes / No**
5. Do you have known hyper-cholesteremia? **Yes / No**
6. Do you suffer from diabetes? **Yes / No**
7. Do you suffer from any medical condition other than asthma, exercise-induced asthma or exercise-induced bronchoconstriction? **Yes / No**
8. Has your doctor ever diagnosed asthma, exercise-induced asthma or exercise-induced bronchoconstriction? **Yes / No**
9. Do you commonly cough, get wheeze, get short of breath, feel tightness in your chest and/or produce a lot of mucus after exercise? **Yes / No**
10. Have you had an asthma exacerbation in the last 4 weeks? **Yes / No**



11. Have you had a chest infection or a cold in the last 4 weeks? **Yes / No**  
12. Do you suffer from seasonal allergy? **Yes / No**  
    If so, have you had allergic symptoms in the last 4 weeks? **Yes / No**  
13. Do you have a history of anaphylactic shock? **Yes / No**  
14. Do you suffer from epilepsy? **Yes / No**  
15. Are you taking any medication? If so, please list below **Yes / No**

16. Are you taking any vitamins or health supplements? If so, please list **Yes / No**

17. Do you have any injuries that cause pain when exercising? **Yes / No**  
18. Are you currently enrolled in any other studies? **Yes / No**  
19. Are you a smoker? **Yes / No**  
20. If you are female, to your knowledge, are you pregnant? **Yes / No**  
21. Do you exercise on a regular basis? **Yes / No**  
22. Describe your exercise routines (mode, frequency, intensity/speed, race times):

If you feel at all unwell because of a temporary illness such as a cold or fever please inform the investigator. Please note if your health status changes so that you would subsequently answer YES to any of the above questions, please notify the investigator immediately.

**I have read and fully understand this questionnaire. I confirm that to the best of my knowledge, the answers are correct and accurate. I know of no reasons why I should not participate in physical activity and this investigation, and I understand I will be taking part at my own risk.**

Participant's name & signature: \_\_\_\_\_ Date: \_\_\_\_\_

Investigator's name & signature: \_\_\_\_\_ Date: \_\_\_\_\_

## Creatinine analysis: Modified Jaffe's method

### CREATININE IN URINE, by hand 96 wells

This is a modification of Jaffe's creatinine protocol. For further details see:  
[http://www.searo.who.int/en/section10/section17/section53/section481\\_1755.htm](http://www.searo.who.int/en/section10/section17/section53/section481_1755.htm)  
(2006-04-27)

**NOTE! The solutions used are toxic. Consider the safety notes, and always protect yourself by wearing lab coat and gloves! Wear goggles when preparing reagent A.**  
Read the risk assessment available in the binder in EIA lab before you start the lab work.

**1. Thaw the urine samples.** Max 22 per 96 well plate

Thaw at RT if you are going to use the samples within some hours. Very diluted samples will take longer time to thaw than the concentrated ones.

Thaw o.n. at +4C if the samples are going to be used the next day.

**2. Fill in a protocol**

Note sample nr and comments for very weak or strong colour.

**3. Solutions: Reagents A, B and C. Standards and acetic acid.**

Prepare as below. Aq dest is deionised water from the light green tap labelled avj.

<b>Reagent A</b>	store at +4C	stable for 3 months
400 ml aq dest in a 500 mL beaker		
add 4,4 g NaOH <b>wear goggles!</b>		VWR 1.06469 (plätchen #6498)
mix to dissolve		
add 9,5 g trisodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ )		VWR 6578
mix to dissolve		
add 9,5 g sodium tetra borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) <b>toxic!</b>		Sigma B9876-500g
mix to dissolve		
Check that pH is >10. Adjust with 1M NaOH drop by drop if necessary.		
Add aq dest up to 500 ml. Mix well.		

<b>Reagent B</b>	store at RT	stable for 6 months
10 g SDS (sodium dodecyl sulfate) pellets, Serva 20765		Fischer 166-207652
Dissolve in aq dest and add aq dest up to 250 ml.		

<b>Reagent C</b>	stored in original bottle at RT in cupboard for solvents	
Picric acid saturated solution 1,3%		Sigma P6744
<b>Note! Picric acid is toxic.</b>		

<b>Creatinine standard set</b>	store at +4C	Sigma C3613-1 set
0.01 mg/mL; 0.03 mg/ml; 0.1 mg/ml		

<b>30% (V/V) Acetic acid</b>	store at RT	Stable for 3 months
Dilute 30 ml glacial acetic acid to 100 ml with aq dest.		

### 1. Working reagent

At analysis, freshly mix equal volumes of the reagents A, B and C.  
Store in a dark bottle at RT for max one week. Label with date and signature  
15 mL is required for each 96-well plate.

Reagent A + B + C (mL)	total volume (mL)	nr of plates
2 + 2 + 2	6	< ½
6 + 6 + 6	18	1
10 + 10 + 10	30	2
15 + 15 + 15	45	3

### 2. Dilute the samples

Turn the samples upside down a couple of times.  
Dilute the samples in the same way with aq dest in Ellerman tubes and mix well (vortex).

Serial dilution	Sample uL	aq dest (ul)	final dilution
1:3	100	200	1:3, save
1:5 from 1:3	100	400	1:15, to plate
1:2 from 1:15	100	100	1:30, to plate
1:2 from 1:30	60	60	1:60, use if necessary

### 3. Add the blank, standards and samples to a 96 well plate, suggested template

Add 40 uL of each blank/standard/sample dilution in duplicates. The blank is aq dest.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	BLANK	1	2	3	4	5	6	7	8	9	10	11
<b>B</b>	BLANK	1	2	3	4	5	6	7	8	9	10	11
<b>C</b>	STD 1	1	2	3	4	5	6	7	8	9	10	11
<b>D</b>	STD 1	1	2	3	4	5	6	7	8	9	10	11
<b>E</b>	STD 3	12	13	14	15	16	17	18	19	20	21	22
<b>F</b>	STD 3	12	13	14	15	16	17	18	19	20	21	22
<b>G</b>	STD 10	12	13	14	15	16	17	18	19	20	21	22
<b>H</b>	STD 10	12	13	14	15	16	17	18	19	20	21	22

For the samples: Add from dilution 15 and 30 for all samples in the first run.

**1. Add 150 uL of working reagent to all wells**

Use a 2.5 mL Combitip. Setting is '3'.

**2. Incubate for exactly 30 minutes**

Try to keep this incubation time as consistent as possible.

Incubate on the Orbital shaker, speed 150.

Use a post-it note with an empty 96-well plate on top as lid.

The colour will turn to more yellow by time. Dark yellow= high creatinine value.

**3. Start the plate reader before the 30 minutes are gone!**

**4. Read the plate at 490 nm = BEFORE.**

Print and save your result. The standard curve should be linear. The duplicates should have low standard deviation values (<10%)

**5. Rerun samples where both dilutions failed or if the CV is not acceptable.**

Use the saved dilution 3 if results are too low.

Dilute the 1:30 sample further to 1:60 and run if the first results are too high.

**6. Add Acid reagent (30% V/V Acetic Acid), work in the ventilated hood.**

Add 5 uL of Acid reagent to all wells. Use a 0.5 mL Combitip. Setting is '0,5'.

**7. Incubate for at least 5 minutes.**

Incubate on the Orbital shaker, speed 150. Use a post-it note with an empty 96-well plate on top as lid. The colour will turn back to the original yellow if the assay works OK.

**8. Read the plate again at 490 nm = AFTER**

Print and save your result. All the wells should be de-stained and no proper standard curve visible. Abs values should all be close to zero.

Repeat the assay if all dilutions for the same sample fails to de-stain. In very rare cases this is due to renal disease.

**9. Transfer the stock urine samples back to minus 20 if not already done.**

**10. Calculations**

If possible, add the proper formulas into the plate reader so the program makes all the necessary calculations for you.

Bring the Excel or text file generated to your computer for further calculations.

Use the BEFORE values and the conversion factor below to express data as mmol/L.

Sometimes, creatinine values are expressed as mg/dL. This goes for the standards used in the method.

A factor of 0,088 is used to transform data to mmol/L

$\text{mmol/l} = \text{mg/dl} \times 0.088$
---

As long as the Abs AFTER values are close to zero, there is no need to use them in the calculations. The BEFORE results should be enough for further analysis.

If you wish to stick to the original protocol, the AFTER values should be subtracted from the BEFORE values before the calculations are done.

### 1. Expected creatinine values in urine are 1-25 mmol/L

In our experience, obtained  $\text{LTE}_4$  and  $9\alpha11\beta$   $\text{PGF}_2$  values in urine are not reliable when the creatinine values are very low;  $< 1$  mmol/L

### 2. QC

Check that the creatinine values obtained are high when sample colour is dark and low when sample colour is light.

### 3. Plastic ware, equipment and chemicals used

1	96 well plate	NUNC maxisorp	439454	VWR
1	Combitips Eppendorf	2.5 mL	613-3521	VWR
1	Combitips Eppendorf	0.5 mL	0030 069.226	VWR
	Ellerman tubes		525-3110	VWR
	Automatic pipettes		0,5-20; 20-200; 200-1000 uL	
	Eppendorf Multipette Plus		613-3669	VWR
	Vortex			
	Plate shaker			
	Plate reader for 96 well plates		Possibility to read at 490 nm	
	aq dest			deionised water
	NaOH, pellets		6498	VWR
	trisodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ )		6578	VWR
	sodium tetra borate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ )		B9876-500g	Sigma
	SDS, pellets preferred! Serva 20765		166-207652	Fischer Scientific
	Picric acid		P6744	Sigma
	Creatinine standard set		C3613-1set	Sigma
	Acetic acid	Glacial acetic acid		