Impact of Ozone-Pollution and Heat on Athletic Performance and Pulmonary Responses

Elisa Couto Gomes

A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University for the award of Doctor of Philosophy

November 2009

Declaration

It is hereby declared that this thesis and the research work upon which it is based were conducted by the author, Elisa Couto Gomes.

Elisa Couto Gomes

You can achieve anything you want in life. Just make Perseverance your best friend, Frustration your arch-enemy, Hope and Focus your dear siblings and Passion your driving force.

Acknowledgements

I would first like to thank my supervisors Professor Vicki Stone and Dr. Geraint Florida-James for their dedication and support throughout my PhD. Their professionalism and profound knowledge have been inspiring and of essence for the completion of this work.

Thanks to my fellow PhD colleagues that were great fun in and out of uni. Our nights out, coffee breaks, football and basketball games (especially all the ones my team won) were always fun – apart from my injuries - and will be dearly remembered! Thanks to all the technicians for their assistance and help - especially during some "panic" moments that happened during my data collection!

A big thanks to my great friends. Martinha, for all our conversations, trips and conferences, moments of unstoppable laughter (oops!), moments of happiness, help and support! Simone, my dear sister, for our wonderful friendship, for being there whenever needed, for living "close" by, for the great moments, travels and experiences we shared! Gabi, for the sambas and football chats, for the lovely company and conversations! Heli, my flat-mate, gym-buddy, uni colleague and lovely friend for all our amazing times together and great films/series ("hey-hey") that we watched! Thank you all for being part of my life!

To my dad, the best dad in the world!!! For all our life and science talks! For the support, guidance, help and love throughout these tough PhD years. To my wonderful and caring mum, also the best mum in the world!!! For all her unconditional love, motivation, affection and encouragement that were essential for my perseverance!!!! To my big brother Renato! For the inspirational talks, for the light-hearted conversations, for believing in me and for our great and strong friendship!!! Even so far away, you were all present every day in my heart, thoughts and skype calls!!! I love you all and miss you too!!!

And finally I have to greatly thank the participants of my studies, who I know endured lots of not-very-pleasant procedures during the experiments, yet kept coming back for the next trials. Without them, this thesis would not have been possible!

ABSTRACT

Epidemiological studies have reported that ozone-pollution has a negative impact on human health. This pollutant is associated with high temperatures and is expected to continue to rise with the predicted global warming. People and athletes that exercise outdoors are of particular concern because, the more intense and the more prolonged the activity, the higher the ozone dose delivered to the lungs and potentially the higher the risk for performance impairment, lung function decrement, onset of lung inflammation, lung injury and oxidative stress.

The main aim of the studies contained in this thesis was to evaluate the impact of ozone-pollution (0.1 ppm), heat and humidity on well trained runners taking part in an 8 km time trial run. Different end points were investigated for an analysis of the impact on performance outcome, lung function, lung inflammation and oxidative stress. In addition, a second aim was to investigate whether a 2-week supplementation period of vitamin C (500 $mg \cdot day^{-1}$) and vitamin E (100 $IU \cdot day^{-1}$) would provide any beneficial effects to the participants.

The participants' lung function was measured by spirometry. Lung inflammation and oxidative stress status were assessed by a variety of markers both in the upper respiratory airways, by nasal lavage, and in the plasma. The markers assessed included: neutrophil count, clara cell protein (CC16), interleukin-8 (IL-8), uric acid, GSH/Protein and trolox equivalent antioxidant capacity. The results showed that the athletes performance was significantly decreased in the hot and humid condition (mean ± SD: 32min 35sec ± 2min 25sec) and in the hot, humid and ozone-polluted condition (33min 09sec ± 2min 44sec) when compared with the ozone alone condition (30min 27sec ± 2min 23sec) and the control condition (30min 15sec ± 1min 58seg). Ozone alone had little effect on the performance variable. The participants' lung function was not affected by the adverse environmental conditions. Evidence of early lung epithelial injury, however, was observed by an increase in CC16 in the upper respiratory airways immediately after the exercise trial in the hot, humid and ozone-polluted environment;

though this was not observed for any other marker of inflammation at this time point. In this same adverse environmental condition, an increase in the GSH/Protein concentration in the upper respiratory airways was found immediately after the exercise.

It was observed that the 2-week supplementation protocol improved the runners' time to complete the 8 km time-trial run in the hot, humid and ozone-polluted environment by 2.6%. In addition, the supplementation was shown to be effective in decreasing the lung inflammation induced by the combination of ozone pollution, heat and intense exercise. This was observed by a smaller increase in the concentration of CC16 in both the upper respiratory airways (0.67 ± 0.5 mg·l^{-1}) and plasma (39.4 ± 17.4 ng·ml^{-1}) in the vitamin treatment compared to the placebo. In addition, in the vitamin treatment the, cortisol concentration (29.2 ± 14.8 ng·ml^{-1}) after the run, the IL-8 concentration (75.8 ± 43.2 pg·ml^{-1}) and neutrophil percentage (22.6 ± 17.2 %) in the airways 6 h after the run were also reduced compared to the values in the placebo treatment (49.9 ± 13.4 ng·ml^{-1}; 126.6 ± 103.2 pg·ml^{-1}; 25.2 ± 22.6 % respectively).

Taken into consideration together, these results provide evidence that heat and humidity combined with ozone have a detrimental effect on athletes' performance in an 8 km time trial, it cannot be discounted that this was simply due to the heat and humidity as there was no differences in the two heat performances. The hot, humid and ozone environment elicited an early epithelial damage characterized by increase in CC16 concentration in the airways. Moreover, an increase in the antioxidant concentration in the upper respiratory airways in that same trial, as indicated by the nasal lavage GSH/protein, suggests a protective mechanism against the oxidative stress stimulated by the high intensity exercise in association with ozone, heat and humidity. Heat and humidity alone had a similar detrimental effect in performance. While, ozone alone had little effect on the variables. In addition, it can be suggested that 2 weeks of a low dosage of vitamin C and E supplementation might present some benefits for the performance outcome and immune system of trained individuals when taking part in a running competition in an ozone-polluted, hot and humid environment. These benefits will, however, depend on the regulation of the antioxidants uptake and metabolism of each subject.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND SYMBOLS

α	Alpha
ABTS	2,2-azinobis 3-ethylbenzothiazoline-6sulfonic acid
BAL	Bronchoalveolar lavage
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSA	Bovine serum albumin
CC16	Clara cell protein
CC10	Clara cell protein
CRP	C-Reactive Protein
DHA	Dehydroascorbic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoasorbant assay
eNOS	Endothelial nitric oxide synthase
ESR	Electron spin resonance
FEF_{25-75}	Forced expiratory flow during the middle half of the forced vital capacity
FEV_1	Forced expiratory volume in 1 second
f_R	Respiratory frequency
FVC	Forced vital capacity
GSH	Reduced glutathione
GSSG	Oxidazed glutathione
GSSG-to-GSH	Ratio between oxidized glutathione and reduced glutathione
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin

iNOS	Inducible nitric oxide synthase
IU	International unit
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
Mn-SOD	Magnesium superoxide dismutase
NF-kB	Nuclear factor kappa B
NL	Nasal lavage
NO	Nitric Oxide
NO_x	Nitrogen oxides
O_3	Ozone
OPT	*o*-phthaldialdehyde
PBS	Phosphate buffered saline
PM_{10}	Particulate matter measuring 10μm or less
ppm	Parts per million
RDA	Recommended daily allowance
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
SEM	Standard error of the mean
SO_2	Sulfur dioxide
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant capacity
TNF-α	Tumor necrosis factor alpha
VE	Ventilatory exchange
VO_2 max	Maximum volume of oxygen
WHO	World Health Organization
μg	Microgram
μl	Microlitre
μm	Micrometre

Chapter 1: General Introduction

Since the industrial revolution, there has been a rapid global urbanization process. As a result, large urbanized areas suffer from, amongst other problems, a high concentration of pollutants such as sulfur dioxide (SO_2), particulate matter (PM_{10}), nitrogen oxides (NO_x) and ozone (O_3) (Baldasano *et al.*, 2003). The analyses of recent epidemiological studies, conducted in various urban centres, have provided coherent evidence that elevated levels of air pollution are associated with an increased risk of respiratory disease and mortality, via impacts on the respiratory and cardiovascular systems (Brunekreef & Holgate, 2002; Katsouyanni, 2003; Bell *et al*., 2006). This has been shown to be the case in both developed and developing countries (Brunekreef & Holgate, 2002; Stedman, 2004; Bates, 2005; Bell *et al*., 2006). These adverse effects on health caused by pollution also lead to economic burden in the countries affected (Bates, 2005). The World Health Organisation (WHO) and the government of various countries have proposed guidelines for maximum concentrations of the different pollutants. Nevertheless, this does not mean that the countries are able to consistently maintain the pollution levels within the guidelines. In addition, some countries such as China and Mexico, still have a series of anti-pollution measures to implement in order to promote some improvement in their air quality (An *et al*., 2006; Bell *et al*., 2006).

One pollutant that is of concern to the general population and also to athletes is ozone. This pollutant is a strong oxidant gas and studies have shown that ozone alone has a negative impact on human health, being associated with, amongst other health problems, an increase in pulmonary diseases, reduced pulmonary function, increase in hospital admissions, and an increase in mortality (Xu *et al*., 1998; Knowlton *et al*., 2004). Ozone pollution is derived from the interaction of nitrogen oxides, volatile organic compounds and solar radiation. The World Health Organization guideline (WHO, 2006) recommends a limit value equivalent to 0.05 ppm. This value is exceeded however, in a number of large cities, especially in developing countries (Baldasano *et al*., 2003), but also in developed ones (Fiala *et al*., 2003). This oxidant gas, which contributes to the urban air pollution, should be distinguished from the stratospheric ozone, which protects the Earth against the potentially harmful ultraviolet light.

Ozone is mainly a summer time pollutant because the high temperatures and increased

sun light enhances its formation. In fact, temperature can be used as a marker for ozone-pollution production (Bloomer *et al.,* 2009) and interaction between ozone and temperature during hot weather can potentially be very harmful to the population. During summer and heat wave conditions, the population is affected by high levels of ozone and also by high temperatures, both of which have been shown to impact on mortality rates (Bloomer *et al.,* 2009). Additionally, some epidemiological studies have reported that the ozone effect increases in size and significance when interacting with higher temperatures and humidity (Thurston & Ito, 2001; Stedman, 2004). This is quite worrying to the general population and to athletes, taking into consideration that the temperature across the globe has been increasing. According to the assessment report on climate change of the Intergovernmental Panel (IPCC, 2007), there is an estimate of an increase of around 0.3ºC/decade for some regions of the globe, while other regions might have an even larger increase due to other factors besides global greenhouse gas emissions. Therefore, due to the prediction of global warming, a simultaneous rise in the ozone levels is also expected. According to Bloomer *et al.* (2009) over the last couple of years, there has been a slight decrease in ozone levels in some regions despite a slight rise in temperature (0.002 ppm/ºC). This reduction was observed together with a reduction in the ozone precursor NO_x in those regions. Assuming that the concentration of NO_x is going to keep falling, then the ozone concentration will possibly continue to decrease (Bloomer *et al.,* 2009). Nevertheless, the ozone levels have also been shown to have risen in certain areas with controlled pollution policies. This occurs because ozone is a gas and is easily shifted, for example via the wind, to different areas in spite of where it is generated. Taking into consideration that the emission of NO_x is increasing in some regions of the world, ozone is most likely to increase more than expected if temperature also rises (Bates, 2005; Bloomer *et al.,* 2009). Knowlton and colleagues (2004) estimate that by the year 2050 there will be an increase of 4.5% in deaths related to the increase of ozone due to the changes in climate alone. If the increase in its precursors is added to this estimation, this value has a 4.4% median increase. In addition, areas less densely populated would also be affected because of the translocation of this oxidant gas.

This issue is of high relevance to the general population due to the adverse health effects of ozone. The active and athletic population is of special concern because of the amount of time they spend training outdoors eliciting high ventilation rates that result in

higher ozone delivery to the lungs. Another consequence of the urbanization process is that most of the major sports events (e.g. Summer Olympic Games, Football World Cup) take place within or near large cities that, besides having a relatively high pollution level, can experience high temperatures during the summer. The environmental conditions of the last Olympic Games venues (Barcelona 1992; Atlanta 1996; Sydney 2000; Athens 2004; Beijing 2008), with exception of Sydney where the 2000 Olympic Games was held during the spring season, proved to be hot, humid and polluted for the athletes (Peiser & Reilly, 2004; Streets *et al.*, 2007). Other pollutants such as PM_{10} and NOx also have strong oxidant properties (Kelly, 2004), however it is the association of ozone-pollution with heat which makes it unique. In addition, due to the similar properties of these pollutants, the effect of ozone-pollution can be extended to the other pollutants.

The impact of ozone-pollution, heat and humidity on athletic performance has indeed led to interest of researchers and governments. Part of the study conducted within this thesis was funded by the British Olympic Medical Institute, which was concerned about how these adverse environmental factors would affect the performance and lung function of British athletes travelling to Beijing 2008. The impact of the ozone toxicity to the exercising individuals and how it leads to lung inflammation and oxidative stress is also an area of interest which is analysed in this thesis. This area of study is interdisciplinary bringing together exercise physiology, exercise immunology and toxicology. Even though research in this field is methodologically challenging, more investigation is necessary in order to investigate how an environmentally relevant concentration of ozone, together with a higher temperature affects exercising individuals and what could minimize the adverse effects of such exposure. The data presented within this thesis will hopefully help this process.

The main aim of the studies in this thesis was to evaluate the impact of ozone-pollution, heat and humidity on athletic performance, lung function, lung inflammation and oxidative stress of well trained runners taking part in a simulated competition in such adverse environment. In addition, analyzing if the ingestion of antioxidant vitamins would affect the outcome of the results was also of interest. In order to investigate these questions two main studies were conducted. To allow for a better comprehension this thesis was divided into 8 chapters. Below is an overview of the chapters:

Chapter 1: Provides a general introduction to the research developed.

Chapter 2: Contains the main literature review relevant for the studies described in the thesis.

Chapter 3: Details the general materials and methods used in the experimental part of the studies. Any specific methodology is described in the relevant chapter.

Chapter 4: Presents part of the data from the first study. This chapter focuses mainly on the effect of the different environmental factors – ozone-pollution, heat and humidity – on the performance, lung function and respiratory symptoms of runners.

Chapter 5: Presents data from two separate studies. One study is a continuation of the study presented in *Chapter* 4 and focuses on the effect of ozone-pollution, heat and humidity on aspects of lung inflammation and oxidative stress. The second study in this chapter investigates the reliability of the nasal lavage method. This method is used to assess the inflammatory and oxidative stress markers of the upper respiratory tract of the individuals taking part in the studies presented within this thesis.

Chapter 6: Provides data that is part of the third study of this thesis. This study was elaborated based on the results obtained in the previous chapters. This study investigates the effect of vitamin supplementation on athletic performance in an ozone-polluted, hot and humid environment. This chapter presents the results related to performance, antioxidant status, oxidative stress and lung function of the runners.

Chapter 7: Presents further data from the third study. This chapter contains the results relative to lung inflammation and immunoendocrine response of the runners.

Chapter 8: Contains the general discussion and the conclusions of the studies presented. In addition, it covers the limitation of the research conducted in this thesis and discusses further research questions and areas for future study.

Chapter 2: Literature Review

This chapter provides a general overview of the relevant literature for the studies described within this thesis. Each of the subsequent chapters provides a more detailed reference to the relevant literature. It must be noted that the literature provided in this thesis is mainly focused on studies with humans. However, *in vitro* and animals studies have been cited whenever there is relevant or additional information. This chapter is divided into four main sections: Section 2.1 analyses the effect that ozone pollution has on lung function and inflammation in the lungs; Section 2.2 describes the role of Clara Cell Protein as a marker of inflammation and how ozone and exercise affects its concentration in the blood and lungs; Section 2.3 focuses on free radical and reactive oxygen species production. It provides information on how exercise influences oxidative stress in the human body and it also refers to the effect of air pollution on this process. Section 2.4 describes the role of antioxidants in reducing the oxidative stress levels that may arise during exercise. This last section focuses on three different antioxidants: vitamin C, vitamin E and glutathione. It also examines the conflicting literature on antioxidant supplementation for people partaking in exercise bouts both in a non-polluted and a polluted environment.

2.1 OZONE EXPOSURE

Several studies evaluating the effects of ozone exposure (0.08-0.40 ppm) on healthy subjects have shown airway inflammation and also deterioration of lung function not only on low and moderate exercising individuals, but also on athletes performing high intensity exercise (Horstman *et al.*, 1990; Adams and Schelegle 1983; Devlin *et al.*, 1996). Some studies have also shown an increase in respiratory symptoms – amongst them are cough, throat tickle, headaches and breathlessness, all of which are associated with exposures to large concentrations of ozone (Schelegle and Adams 1986).

When ozone (O_3), or indeed any gas, is inhaled, it first comes into contact with the respiratory tract lining fluid (RTLF). In fact, ozone is not able to infiltrate further than the RTLF and the membranes of cells from the lung-air interface, yet it is still known to cause damage beyond the lungs (Pryor *et al.*, 1995). The RTLF covers the respiratory epithelium cells and contains a great variety of antioxidants to help protect the lungs. These antioxidants include urate, GSH, ascorbic acid, and in a smaller concentration α-

tocopherol (Van der Vliet *et al.,* 1999). Thus, once O_3 comes into contact with the RTLF, it induces a direct oxidative stress, the intensity of which varies depending on the O_3 inhaled dose and also on the antioxidants present in the lining fluid. It also induces an indirect oxidative stress because, even though O_3 does not react directly with the epithelial cells, these cells do react in response to the oxidation products produced in the RTLF, consequently releasing a variety of pro-inflammatory mediators and more reactive oxygen species. As a result, if the oxidative stress is sufficient there is an activation of an inflammatory response which may lead to an increase in airway neutrophilia and tissue injury (Pryor, 1994; Pryor *et al.*, 1995).

The airway inflammation is detected by an increase in inflammatory cells, such as neutrophils and macrophages, as well as inflammatory mediators, such as interleukin-6 (IL-6) and interleukin-8 (IL-8). The depletion of antioxidants found in the airways, characterizes the oxidative process that triggers an inflammatory response due to epithelial damage (Mudway *et al.*, 1999a; Bloomberg, 2000). Both the inflammatory mediators and the antioxidants can be measured using different techniques, each with its advantages and disadvantages. Bronchial biopsies and brochoalveolar lavage (BAL), though frequently used, are quite invasive procedures and, therefore, require local anesthesia and need to be performed in a medical environment (Jörres *et al.,* 2000). Another technique is the sputum induction procedure which is less invasive and less technically difficult than bronchoscopy (Ratto *et al.*, 2006). Similarly, the nasal lavage technique is not only a relatively noninvasive procedure but can be repeated at multiple time points (Mudway *et al.*, 1999a). When reviewing studies, it is important to take into consideration that these different techniques could be sampling different airway compartments (Ratto *et al.,* 2006), thus, explaining some differences in the outcome of studies.

Lung function is usually assessed using spirometry, which is an important and efficient way to evaluate disorders of the respiratory tract (Booker, 2005; Wagner *et al.,* 2006). A variety of lung function measurements can be assessed by spirometry. Nevertheless, some parameters have been shown to be more important when evaluating an individual's lung function (Booker, 2005). Such parameters include forced vital capacity, forced expiratory volume in 1 second, forced mid-expiratory flow and peak expiratory flow (Gong *et al.,* 1986; Folinsbee *et al.,* 1984; Blomberg *et al.*, 1999).

Forced Vital Capacity (FVC) and Forced Expiratory Volume in 1 second (FEV_1): The FVC is the total volume of air expired, in litres, after full inspiration. It measures the respiratory stroke volume. The FEV_1, on the other hand, is the volume of air delivered in the first second of the FVC manoeuvre and is affected by the airway resistance. In people with normal lung function both the FVC and the FEV_1 should be above 80% of the predicted (Booker, 2005). Any reduction indicates a decrease in the individual's ventilatory capacity, which is often associated with different lung diseases – e.g. fibrosis, asthma, bronchitis. Moreover, a significant increase in both these measurements or in either one indicates that the airflow obstruction is reversible (Pellegrino *et al.,* 2005). Another important and much used index when assessing airflow obstruction is the ratio FEV_1/FVC. The normal ratio for FEV_1/FVC is 80% (West, 2003). A decrease in this percentage may be a sign of chronic obstruction pulmonary disease (Celli *et al.,* 2004).

Forced Expiratory Flow ($FEF_{25-75\%}$): Forced expiratory flow is the volume of expired air measured during the middle half (25-75%) of an FVC manoeuvre. It is the volume in litres divided by the time in seconds. Similarly to FEV_1, $FEF_{25-75\%}$ is affected by changes in the airway resistance and, therefore, these two measures are often closely correlated (West, 2003).

Peak Expiratory Flow (PEF): The PEF can be defined as the maximum flow (rate) during a forced expiration after full inspiration (West, 2003). Taking into account that PEF is effort-dependent, factors such as the subjects' motivation and their respiratory muscle strength will affect the PEF outcome. Furthermore, PEF is measured within the first 100 ms of the forced expiration and assesses the calibre of the large airways. Thus, if just this lung function variable is being measured, the subject need not make a prolonged forced expiration (Jain, 1998). The standard error of this measure is considered large, therefore, differences of up to 100 $l \cdot min^{-1}$ observed between different testing days does not necessarily represent an abnormal value. Equally, if individuals present normal PEF values, it does not necessarily mean that they may not present a small degree of airway obstruction (Gregg & Nunn, 1973). In addition, due to the variation in the neural mechanisms responsible for bronchoconstriction (Barnes, 1992),

the PEF varies throughout the day, achieving its peak in the afternoon around 4pm. Therefore, some intraindividual variation can also be explained by this circadian rhythm if the time of measurement is not consistent (Jain, 1998).

When using the spirometer it is important to take into consideration some factors in order to optimize the results obtained. Firstly, a daily calibration check is recommended in order to determine the onset of any technical problem. Secondly, when an individual is re-evaluated the professional conducting the test should be the same and the time of the day should not exceed a two-hour difference from the previous tests. Moreover, it is important to explain the whole procedure for the subject and ensure that they understand what has to be done (Pellegrino *et al.*, 2005).

Decreases in pulmonary function and increases in airway neutrophilia have been observed to be related to the total O_3 effective dose (ED). This index is defined as the product of O_3 concentration, average expired ventilation, and duration of exposure (Gibbons *et al*., 1984; Gong *et al*., 1986; Mudway & Kelly, 2004) and has been shown to be a reliable surrogate for the actual dose (Rigas *et al*., 2000). It is important to take the ED into account when analyzing the pertaining scientific literature since athletes can achieve a higher ventilatory exchange ratio during exercise and, consequently, inhale more air and more ozone per minute than non-athletes. Hence, being exposed to the same ozone concentration but having higher minute ventilation, as seen in athletes, might result in more pronounced side effects.

2.1.1 Ozone and Airway Inflammation

Airway inflammation and any other inflamed tissue can be characterized by an increase in inflammatory cells, such as neutrophils and macrophages, as well as inflammatory mediators, IL-6, IL-8, and prostaglandins. Neutrophils are granulocyte cells and are part of the innate immune response, that is, these cells can recognise and act against a foreign particle, including bacteria and viruses, without prior exposure. The mechanism of action of a neutrophil consists of phagocytosis and digestion of the foreign particle or antigen. An increase in neutrophil numbers and percentage is a good indicator of the beginning of an inflammatory response because these cells account for 50-60% of the

total white blood cells (leukocytes) in the circulation and are the first cell type to migrate to sites of injury and inflammation. When there is an inflamed tissue there is an increase on the expression of adhesion molecules of the selectin family (E- and P-selectin molecules) on the local endothelium, this is mediated by cytokines and other inflammatory mediators. Neutrophils present in the blood recognise the site of inflammation because of these adhesion molecules, which binds to molecules (mucin-like cell-adhesion molecules, CAM) on the neutrophil surface. This step is referred to as rolling and is the first step to the attachment of neutrophils on the endothelium. In order for them to adhere firmly and be able to migrate through the endothelial to the inflamed site, the neutrophils are activated by various chemoattractants derived from epithelial cells exposed to a foreign body, IL-8 being an important one. Once the adhesion processes is successful the neutrophils can initiate their transendothelium migration (Figure 2.1). Upon arrival to the inflamed tissue neutrophils release a number of chemoattractants to amplify the inflammatory response by recruiting other cells (Pyne, 1994).

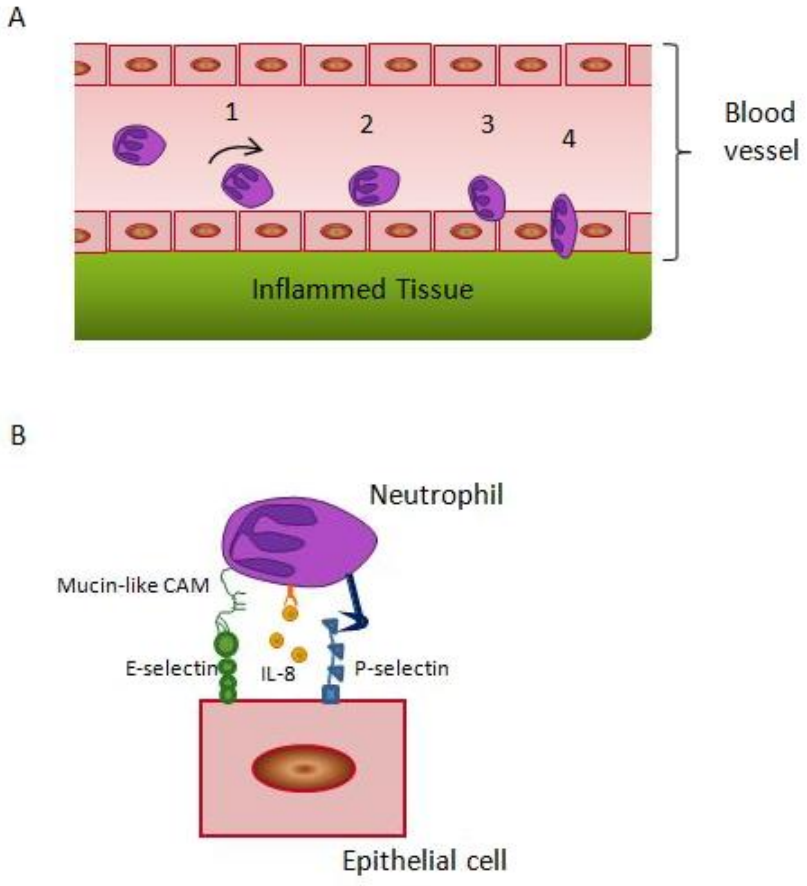


Figure 2.1. Neutrophil transendothelium migration. A- Four sequential steps in the migration process: 1- Rolling 2- Activation 3- Adhesion 4- Transendothelium migration. B- Cell-adhesion molecules and chemokines envolved in the migration process (adapted from Kuby, 1997).

The cytokine IL-8 is a mediator of the immune function and helps to regulate the immune response. It is secreted by a variety of cells, including neutrophils, macrophages and endothelial cells, and is a chemotactic for cells such as neutrophils and T cells. In addition, it has been linked to a wide variety of pathologic conditions characterized by an increase in neutrophil count, thus an increase in IL-8 levels is linked to an increase in neutrophils (Holz *et al.,* 1999). IL-6 is another important mediator in the development of an inflammatory process. It is produced mainly by T-cells and macrophages, and together with IL-1 and TNF-α stimulate both local and systemic changes seen in an inflammatory response. This cytokine has been thoroughly studied in immunological responses to exercise (Steensberg *et al.,* 2000; Febbraio & Pedersen, 2002).

Athletes usually present at rest a clinically normal level of blood circulating neutrophil number, but there have been some studies that have reported lower neutrophil counts in trained distance cyclists (Blannin *et al.,* 1996) and elite distance runners (Hack *et al.,* 1994). The elite runners presented a lower neutrophil count at rest during a period of intense training (102 $km \cdot wk^{-1}$) compared with non-athletes. This baseline difference was not apparent when the training was of moderate intensity (89 $km \cdot wk^{-1}$) (Hack *et al.,* 1994). During and after exercise however, the number of circulating neutrophils is known to substantially increase, the magnitude of which depends mainly on the exercise mode, duration and intensity (Blannin *et al.,* 1996; Mackinnon, 1999). This increase is seen in both trained and untrained individuals, and occurs mainly because of the detachment of these cells from the vascular endothelium. After endurance exercise the neutrophil count remains elevated for several hours (Steensberg *et al.,* 2002).

Contrary to the blood neutrophils, the basal neutrophil concentration in the airways of athletes and individuals who go through a chronic endurance training period, are usually higher than compared to the general population (Bonsignore *et al.,* 2001; Morice *et al.,* 2004; Belda *et al.,* 2008). This elevated number has been attributed to, amongst other factors, a prolonged period of high ventilation which can lead to epithelial damage in long distance runners and cyclists (Bonsignore *et al.,* 2001; Morice *et al.,* 2004; Denguezli *et al.,* 2008).

Inhalation of ozone has been shown to stimulate airway inflammation due to its

oxidative nature. Devlin *et al.* (1996) analyzed the concentration of a broad range of inflammatory mediators in BAL fluid 1 h after ozone exposure. In this study, volunteers performed intermittent heavy treadmill exercise (66 $l \cdot min^{-1}$) for 2 h in a chamber where the ozone concentration was of 0.4 ppm. An increase was observed in mediators of inflammation such as neutrophils, IL-6 and lactate dehydrogenase (LDH), which is an indicator of cell damage. Similarly Holz and colleagues (1999) observed a significant increase in neutrophil count and percentage in induced sputum 1 h after participants completed 3 h of light intermittent exercise - 14 $l \cdot min^{-1} \cdot m^{-2}$ of body surface area - exposed to 0.25 ppm of O_3. Nevertheless when the participants performed the same exercise bout exposed to a lower O_3 concentration (0.12 ppm) no changes in neutrophils were observed. Furthermore, sputum IL-8 concentration was reported to be elevated only after the 0.25 ppm exposure.

Contrasting some of the previous findings, Blomberg *et al.* (1999) were unable to find either mucosal and airway neutrophilia or LDH increase at 1.5 h after a 2 h exposure to 0.2 ppm O_3 in subjects performing intermittent moderate cycling exercise producing an average minute ventilation of 20 $l \cdot min^{-1} \cdot m^{-2}$ of body surface area. This difference might be explained by the lower exercise and O_3 levels in the latter study, consequently the inhaled O_3 dose was also lower. Nevertheless, Blomberg *et al.* (1999) were able to detect in tissue obtained from bronchial mucosal biopsies increases in the expression of vascular endothelium P-selectin and ICAM-1 after the ozone exposure. These molecules mediate adhesion and rolling of leukocytes on the vessel walls. Hence, it was suggested that although there was an increase in the expression of vascular adhesion molecules in the vascular endothelium, this had not yet resulted in an increase in neutrophil numbers at the analyzed sites. Stenfors *et al.* (2002) using the same study design as the previous study, demonstrated a significant increase in BAL neutrophil number and percentage 6 h after the exercise trial. In addition, vascular endothelium P-selectin and ICAM-1 were also elevated. This reinforces the role of these adhesion molecules in the inflammatory response, recruiting inflammatory cells into the airways of healthy individuals.

The airway inflammation response to chronic ozone exposure has also been investigated, though more in-depth studies are also required. Christian *et al.* (1998) showed an attenuation of the inflammatory response in BAL after four consecutive

days' exposure to ozone. Nevertheless, it seems that although neutrophil recruitment and IL-6 concentration in the respiratory tract is attenuated with multiday short-term exposures, airway epithelial injury may continue to occur. The data from Jörres *et al.* (2000) support the previous finding and additionally they reported that after four consecutive exposures an increase in airway mucosa inflammation as well as the neutrophil count was observed in bronchial mucosal biopsies. These data, thus, demonstrate that airway inflammation persists despite attenuation of some inflammatory markers in BAL. It is important to point out that this persistent injury could lead to airway remodeling, which has been observed in several animal studies, but needs further investigation in humans (Christian *et al.,* 1998).

Using a different technique, sputum induction, to assess airway inflammation, Ratto *et al.* (2006) found an increase in the percentage of neutrophils after 4 h of 0.2 ppm O_3 exposure during 4 consecutive days. This finding is in contrast to the previous mentioned studies using BAL where it was observed that attenuation of inflammatory response occurred. Nevertheless, it was consistent with the increased recruitment of neutrophils to proximal airway tissue demonstrated in endobronchial biopsies samples taken by Jörres *et al.* (2000). Once more, it shows that different techniques sample different airway compartments producing differing results. In addition, the exposure and exercise protocol is also essential for the outcome of the inflammatory process. An important additional factor that appears to affect results in these studies is individual responsiveness due to differences in ozone sensitivity of individuals (Holz *et al.,* 1999; Mudway *et al.*, 2001).

All studies mentioned above were conducted in a thermoneutral environment. Unfortunately, they also used non-athletes and did not measure the performance response to O_3 exposure. Therefore, it is difficult to establish the inflammatory responses that athletes would have had, and how it would affect their performance. It is essential to keep in mind that endurance sport athletes currently present airway neutrophilia, but they are not activated at rest or after exercise. In addition, this increase in the inflammatory cells is not necessarily associated with respiratory symptoms or functional impairment (Bonsignore *et al.*, 2003).

2.1.2 Ozone and Lung Function

Research evaluating the effect of ozone on lung function has been carried out in laboratory settings using either a mouth-breathing face mask (Adams and Schelegle 1983, Gibbons and Adams 1984; Schelegle and Adams 1986; Ratto *et al.*, 2006) or an environmental chamber (Christian *et al.*, 1998; Blomberg *et al.*, 1999; Horstman *et al.*, 1990; McDonnell *et al.*, 1991). Other studies have been performed in outdoor settings (Brunekreef *et al.*, 1994; Korrick *et al.*, 1998) and show, in general, greater O_3 associated changes in pulmonary function than in controlled exposure settings, which could be attributed to the synergistic effect of other pollutants.

Horstman *et al.* (1990) reported impaired lung function and an increase in non-specific airway reactivity in subjects exposed to an ozone concentration as low as 0.08 ppm O_3. In this study, 22 individuals performed 5 h of moderate exercise (mean VE approximately 39 $l \cdot min^{-1}$) while exposed during 6.6 h to different concentrations of ozone – 0.00, 0.08, 0.10 and 0.12 ppm. The authors observed that the FEV_1 was significantly reduced after only 3 h at 0.12 ppm, 4.6 h at 0.10, and 5.6 h at 0.08 ppm. This indicates that O_3 at levels, often found in ambient air, decrease pulmonary responses in individuals engaged in activity representative of a typical day of moderate to heavy work or play. These findings were in accordance with a subsequent study by McDonnell *et al.* (1991) using a similar methodology.

Impairment in lung function has also been reported with shorter ozone exposure durations. Blomberg *et al.* (1999) reported a depressed response of FEV_1, FVC and FEF_{25-75}, following a 2 h exposure of 0.2 ppm ozone. In this study, subjects performed intermittent cycling exercise at an average minute ventilation of approximately 20 $l \cdot min^{-1} \cdot m^{-2}$ of body surface area. In contrast to the previous studies, this one used a higher ozone concentration but a shorter duration and a lower minute ventilation. Other studies utilized different ozone concentrations, with lower exposure duration but with exercise eliciting a much higher ventilatory exchange ratio. Schelegle and Adams (1986), using ten endurance athletes, evaluated the influence of different ozone concentrations – 0.12, 0.18, and 0.24 ppm - while performing a 1 h competitive cycling simulation protocol. One, five and seven subjects did not complete the 0.12, 0.18 and

0.24 ppm O_3 protocol respectively. In addition, a significant increase in the number of reported respiratory symptoms and decreases in FEV_1 and FVC were observed following the 0.18 ppm O_3 exposure.

In relation to studies carried out in the field, Korrick *et al.* (1998) evaluated the effects of ambient O_3 on the pulmonary functions of healthy adults hiking on Mt Washington. The study population was composed by 530 healthy hikers of different ages and physical fitness tested throughout a period of 74 days. The average of each hourly O_3 concentration during each hike ranged from 0.021 to 0.074 ppm. In addition, the average temperature and exercise time were of 19 ºC and 8 h respectively. After adjusting to potential confounding variables – age, gender, other pollutants etc – a significant decrement in FEV_1 and FVC after the exercise was observed. These results are closely related to those of Brunekreef *et al.* (1994), although the former presented a larger decrease in pulmonary function probably due to the amount of time the subjects were exercising in comparison to the latter. Brunekreef *et al.* (1994) conducted a field study on a group of amateur cyclists investigating the effect of outdoor ozone exposure during the Netherlands' summer season. The ozone levels varied between 0.044 and 0.098 ppm, the cycling had an average duration of 75 min and the mean temperature was 17.9 ºC. The authors reported that even though extreme lung function impairments were not present, there was a significant relationship between ozone exposure and lung function decrements. These field studies are very important because they are conducted in a real ambient environment and give us some idea of the implication of athletes competing in a polluted site. On the other hand, it is difficult to point out the true effect of O_3 since there are other influencing variables, some of which may not yet have been even associated with lung function impairments.

Besides ozone, the parameters of heat and humidity per se have been shown to have a detrimental effect on exercise performance (Galloway & Maughan 1997; Gozalez-Alonso *et al.*, 1999; Parkin *et al.*, 1999; Nybo & Nielsen, 2001). However, there is very little research that evaluates the combined effect of pollution with heat and humidity. There are two important studies that have investigated the impact of both heat and ozone on lung function and exercise performance (Gibbons & Adams, 1984; Gong *et al.*, 1986). Unfortunately the humidity was kept low in these studies. The authors

arrived at the conclusion that the combined effects of an ozone polluted environment, heat, and exercise produced a greater negative impact on performance and respiratory discomfort than ozone and exercise alone (Gibbons & Adams, 1984; Gong *et al.,* 1986).

2.2 CLARA CELL PROTEIN

Clara cells are nonciliated, secretory columnar cells found exclusively in the pulmonary airways - predominantly in the respiratory bronchioles and in the terminal bronchioles. The function of these cells is mainly the protection of the respiratory tract (Hermans & Bernard, 1999). They present a high content of xenobiotic metabolizing enzymes which protect our system against inhaled particles (Robin *et al.,* 2002) such as pollutants. The main protein secreted by the Clara cells is a small protein of 16 kDa, that has been given various names throughout the literature, such as Clara cell protein, CC16, CC10 and Uteroglobin. However, hereafter it will be referred to as Clara cell protein or CC16. The exact function of this protein is not clear thus far, but it is believed it plays an important role in decreasing the inflammation of the respiratory tract and protecting it against the harmful effects of oxidative stress (Broeckaert & Bernard, 2000).

CC16 is also produced by the nasal mucosal epithelial cells (Lindahl *et al.,* 1999; Benson *et al.* 2004) making it easy to quantify this protein by the nasal lavage method (Johansson *et al.,* 2005). Small amounts of Clara cell proteins have also been found in other organs such as the male and female urogenital tract, endometrium, fetal lung and kidney, and in the amniotic fluid. Nonetheless, the level of CC16 expression in these organs is on average 20 times lower than in the pulmonary airways (Broeckaerte *et al.,* 2000a). CC16 can also be found in the blood, where it is derived almost exclusively from the airways (Hermans & Bernard, 1999). In normal healthy individuals, the serum level of CC16 ranges on average from 10 to 15 $ug \cdot l^{-1}$ (Helleday *et al.,* 2006). Yet, as a result of pulmonary inflammation and increases in the permeability of the lung epithelial barrier the concentration of this protein in the blood has been shown to increase (Hermans *et al.,* 1999). The mechanisms involved with the transudation of Clara cell proteins from the lung to the blood are explained in detail in the following section.

2.2.1 Mechanisms of Changes in Serum Levels of CC16

The lung–blood barrier offers some resistance to the bi-directional movement of large proteins such as albumin. Nevertheless, the high concentration of CC16 in the respiratory tract secretions and its small size permits its diffusion into the blood (Broeckaert *et al.,* 2000a). The CC16 concentration in the blood can easily be detected by conventional enzyme immunoassays (Gioldassi *et al.,* 2003).

The bi-directional exchange of proteins between lung and blood is regulated by several factors, among which are the size of the proteins, the epithelium permeability and the driving force of the transepithelial concentration gradient. The concentration gradient will allow the movement of proteins from an area of high concentration to an area of low concentration. In the case of CC16 it will be from the lung to the blood; but albumin, for example, moves in the opposite direction. The large difference between the concentration gradients can be related to the difference in the compartment sizes in which the proteins are diluted. The concentration gradient is also influenced by the removal of the protein from the compartment into which it is leaking - proteins that enter the lung interstitium are rapidly cleared by lymphatic drainage (Broeckaert *et al.,* 2000a).

The changes that occur in serum concentrations of CC16 may result from three different mechanisms. The first mechanism would be a result from the increase in the permeability of the lung epithelial barrier, which has as a consequence a higher diffusion of CC16 to the blood. This can happen following exposure to ozone, which causes epithelial lung injury (Christian *et al.,* 1998), more specifically, damage to the tight junctions of the cells (Broeckaert *et al.,* 2000a). A second possibility is the decrease or increase in the production or secretion of CC16 from the Clara cells present in the respiratory tract. A reduction in the number of Clara cells has been shown to occur following chronic exposure to lung toxicants such as silica particles (Bernard *et al.,* 1994). Finally, the levels of serum CC16 can also be altered if there is a reduction of the clearance of the protein by the kidney. Serum CC16 has a half-life of approximately 2-3 h due to the rapid clearance through the kidney (Hermans & Bernard, 1999). Hence, the variation in CC16 serum levels can only be used as a specific biomarker of the

airway epithelium integrity if the subjects in the study do not present renal function problems. For a fuller explanation see Figure 2.2.

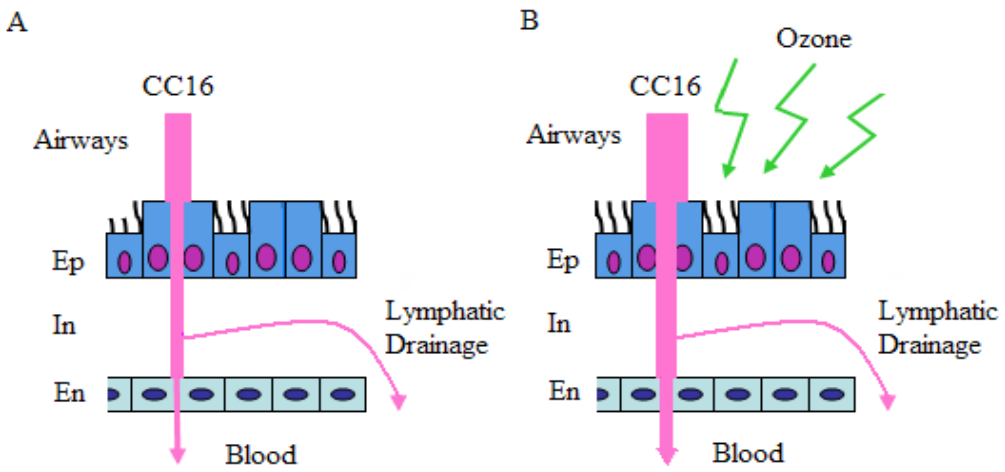


Figure 2.2. Movement of CC16 from the airways to the blood across the different barriers under normal conditions (A) and after exposure to ozone (B). The thickness of the arrows is used to illustrate the relative permeability of the different barriers and increase in the CC16 flux after ozone exposure. Abbreviations: Ep – epithelium; In - interstitium; En – endothelium (adapted from Broeckaert *et al.* 2000b).

An important point which has to be taken into account when carrying out experiments or comparing studies is the diurnal variation in the serum concentration of CC16. In addition, there is also a large inter-individual variation in this protein. Halleday *et al.* (2006) demonstrated diurnal variations on the serum levels of CC16 presenting a significant drop in the concentration between 11:30 am and 10:00 pm. Even though there is a large inter-individual variation, this study showed that the time-dependent diurnal change was similar in all individuals regardless of the baseline concentrations.

2.2.2 Lung Injury

Both acute and chronic exposure to toxicants has been shown to elicit changes in serum CC16 levels. This supports the theory that this protein is a sensitive and suitable biomarker of lung toxicity and lesions involving the respiratory epithelium, which can be assessed in a simple and noninvasive way (Bernard *et al.*, 1994, Hermans & Bernard, 1999).

Acute smoke inhalation has been shown to significantly increase the serum levels of CC16 (Bernard *et al.*, 1997). This study was conducted on firefighters; who in

addition to inhaling the smoke also performed physically demanding tasks. Serum CC16 concentration was measured immediately after exposure and was three fold higher when compared to control subjects. The authors attributed this change in the serum CC16 concentration to a transient increase in the lung epithelial permeability. Moreover, this increase occurred in the absence of any functional sign of lung impairment. Ten days after exposure the CC16 concentration had returned to baseline levels.

A chronic toxic effect on Clara cells has been shown in workers inhaling silica-rich dust for an average of 15 months (Bernard *et al.*, 1994). This study compared two groups of workers, one composed of workers exposed to silica and the other a control group, matched for age, body mass index and proportion of smokers. The mineworkers presented a significant reduction of serum CC16, even though they did not present any lung function impairment or abnormalities in their chest X-ray. The decrease was reported for both the nonsmokers and the smokers. An additional and significant effect of tobacco smoking was also found. The authors associated this decrease with a reduction in the release of CC16 from the Clara cells probably due to their damage from the toxic action of silica.

A similar chronic toxic effect on these cells has been shown in tobacco smokers, who presented a significant decrease in serum CC16 when compared to nonsmokers (Robin *et al.*, 2002). The authors suggested that the toxic metabolites of tobacco smoke not only increases the permeability of the lung epithelium, reflected by an increase in serum concentration of the small surfactant-associated protein B, but it also causes the progressive destruction of the Clara cells.

2.2.3 Ozone, Exercise and CC16

Few studies have investigated the effect that exercise or ozone or the combination of both has on Clara cells and CC16. The majority of these studies however, investigating exercise and Clara cells, have utilized an ozone polluted environment, mainly because of the established relationship between CC16 and lung inflammation. Therefore the relationship between exercise type, intensity, duration and CC16 is still poorly

understood.

A study conducted by Nanson *et al.* (2001) was carried out to detect the effect of different exercise intensities on serum CC16 levels. For this, 14 healthy subjects went through three different trials; no ozone was used in this study. One trial consisted of a no exercise session, another of 30 min moderate exercise simulating firefighting tasks, and a third session composed of a 60 min high-intensity exercise. Blood samples were collected 1 h post-exercise. The results showed a significant increase in serum CC16 for both exercise sessions despite differences in the intensity and duration of the exercise. The authors suggested that this result could be due to an increase in the pulmonary CC16 concentration resulting in a larger diffusion gradient from the lung to the serum. Alternately, the alveolar-capillary permeability could have increased slightly permitting the passage of small molecules. It is important to point-out that the subjects that took part in this study did not participate in regular physical activity. Carbonelle *et al.* (2002) also found an increase in serum CC16 levels of swimmers immediately after a 45 min training session.

The effect of exercise associated with ozone was investigated in the study of Broeckaert *et al.* (2000b) where 24 non-smoking cyclists, performed 2 h of cycling at moderate intensity during episodes of photochemical smog. The average concentration of ozone was 0.076 ppm. Immediately before and after each ride the participants provided blood samples and also performed lung function tests. Significant correlations were found between O_3 concentration and the serum levels of CC16 of the cyclists both pre and post-exercise. By contrast when comparing pre and post rides no decrements on lung function performance were found - these parameters are usually impaired by O_3 exposure. Thus, this study shows that short-term exposures to ambient-levels of O_3 induces an early increase in serum CC16 which takes place before other manifestations of lung toxicity. However there was no control group to verify if this increase was due to the exercise, the ozone or a combination of both. The authors suggested that the increase seen in the serum CC16 was due to an increase in the pulmonary epithelium permeability and not to an increase in the production of this protein. As this study was conducted in the field, it is difficult to attribute these results directly to the O_3 exposure, as there may have been other pollutants that could also have influenced the epithelial

leakage. In addition, the levels of serum CC16 pre-ride were also correlated with the levels of ambient O_3, indicating that the cyclists initiated the exercise with an increased permeability of the lung epithelium.

In another study (Blomberg *et al.*, 2003), this one conducted in the laboratory, 22 subjects performed 2 h of moderate intermittent exercise exposed to two different environment conditions: 0.2 ppm of O_3 and filtered air. The participants' lung function was assessed and peripheral blood samples were obtained 2 h pre, immediately pre, immediately post, 2 and 4 h post-exercise. Significant decreases in the lung function parameters, FEV_1 and FVC, were observed immediately post O_3 exposure. However, at 2 and 4 h post-exercise this decrease was not observed anymore. Moreover, a significant increase in serum levels of CC16 was observed around 2 and 4 h post O_3 exposure. No relationship was noted between CC16 and lung function at any time point. Serum CC16 concentrations were shown to have returned to baseline 18 h post-exposure. Other epithelial permeability markers, albumin and total protein concentration, that were also assessed did not show a significant increase. The data from this study supports the theory that serum CC16 is a more sensitive marker of altered lung epithelial permeability than traditional markers.

Even though the latter findings are in agreement with the enhanced serum levels of CC16 mentioned in the previous study of Broeckaert *et al*., (2000b), it is important to note some differences between these studies. Firstly, the concentration of ozone varied a lot between the studies. In addition, while in the first study (Broeckaert *et al.,* 2000b) the participants were trained cyclists, the second (Blomberg *et al.*, 2003) only mentioned that the subjects were healthy individuals. Neither study detailed the participants' aerobic capacity nor the exercise intensity elicited during the trials. An additional confounding factor is the degree of pre-exposure between the two studies: the first study took place during the summer and the other during the winter. This could be one of the causes for which the former study (Broeckaert *et al.,* 2000b) showed an increase in serum CC16 levels immediately post-exposure. Nonetheless, it cannot be excluded that other air pollutants present in the photochemical smog could have influenced the CC16 leakage immediately post-exercise.

Contrary to the previous studies, Lagerkvist *et al.* (2004) did not find any significant changes in serum levels of CC16 in children (10-11 years of age). They performed 2 h of outdoor exercise, where the maximal O_3 value reached 0.059 ppm. Blood samples and lung function performance were obtained pre and post-exercise. Yet, no decrease in lung function or changes in CC16 were observed. In relation to CC16 concentration, the authors reported that children who regularly visited chlorinated indoor swimming pools presented significantly lower levels of serum CC16 both before and after the outdoor exercise when compared to the non-swimming children. In this study, it is important to observe that the maximum level of O_3 reported is lower than in the previous studies discussed above. Furthermore, the authors mentioned that the children performed light exercise; though, they did not report the type of exercise nor how the exercise intensity was controlled for. More investigation is needed to establish the effect that ozone and exercise have on the airway permeability as there are still contradictions in the literature using CC16 as a marker of lung injury.

2.3 OXIDATIVE STRESS

2.3.1 Free Radicals and Reactive Species

Free radicals can be broadly defined as molecules or ions containing an unpaired electron and capable of existing independently (Halliwell & Gutteridge, 2007). As a result of the unpaired electron, free radicals easily react with other molecules to gain another electron and therefore become stabilized (Radák, 2000). Reactive species are molecules that are capable of oxidizing other molecules. Many of these molecular species are oxygen centred (O_3, H_2O_2, etc) and thus denominated reactive oxygen species (ROS). Some ROS are also free radicals such as superoxide anion (O_2^-) and nitric oxide (NO) because they have an unpaired electron (Radák, 2000). Reactive species can also be formed by a wide range of other atoms such as nitrogen and chlorine (Halliwell & Gutteridge, 2007). Both free radicals and ROS, due to their molecular instability, promote oxidation reactions with cellular proteins, lipids or DNA. However, the body has an elaborate antioxidant defence system that neutralizes the reactive species and free radicals in order to achieve a balance (homeostasis). But, if there is an increase in the oxidant concentrations, overwhelming the available antioxidants, or if the antioxidants are depleted due to disease or poor diet, then oxidative stress and

impaired cellular function may occur (Powers *et al.,* 2004). In fact, oxidative stress has been proved to be the cause and consequence of many diseases (Halliwell & Gutteridge, 2007). Different antioxidants and their mechanisms of action are described in section 2.4.

On the other hand, free radicals and ROS are essential to our well-being. For example, ROS are produced by immune cells – neutrophils and macrophages - during the process of respiratory burst in order to eliminate invading microorganisms (Kuby, 1997). Moreover, other processes can be highlighted as examples such as the stimulation of genes - which encode transcription factors, differentiation, and development; cell-cell adhesion; cell signalling; involvement in vasoregulation and fibroblast proliferation (Sen, 2001).

2.3.2 Measurements of Oxidative Stress in Humans

The molecular detection of free radical species is technically challenging because of their high reactivity and low steady-state concentration. Thus, to analyze acute oxidative stress in response to exercise, different stress markers in the blood, urine, expired air, nasal lavage fluid, and muscle tissue from humans have been examined (Leaf *et al.,* 1997; Sienra-Monge *et al.*, 2004; Child *et al.,* 1999; Hellsten *et al.,* 1996).

The by-products of lipid peroxidation are the markers most commonly measured (e.g. F_2-isoprostane and malondialdehyde). Lipid peroxidation is a process of chemical reactions that occurs when free radicals attack polyunsaturated fatty acids, such as those found in cell membranes (Urso & Clarkson, 2003). In addition to lipid peroxidation, in order to examine the oxidative stress, other processes have also been examined. These include changes in the status of antioxidants compounds, such as glutathione, uric acid, analysis of protein and DNA oxidation products, and antioxidant enzyme activities (Watson *et al.,* 2005). Nonetheless, these are all measures of oxidative stress and not necessarily reflection of ongoing ROS and free radical production. A direct measure, used in some studies (Ashton *et al.,* 1999), is the electron spin resonance (ESR) technique. This technique enables the detection of species that have an unpaired electron, generally meaning that it is a free radical.

2.3.3 Exercise-Induced Oxidative Stress: Experimental Evidence

Under basal conditions the skeletal muscle produces superoxide anions and NO at a low rate. However, during contractile activity, this rate is drastically increased. In fact, physical exercise is associated with an increase in oxygen uptake both by the whole body and especially by the contracting muscle group. Sen (1995) reported an increase of 10-15 fold in the rate of whole body oxygen consumption and an increase of more than 100 fold in the oxygen flux in active muscles during exercise. How the increase in O_2 uptake results in increased ROS production is explained later in this review.

Studies that measured the oxidative stress induced by physical exercise have been reported. Sastre *et al.* (1992) investigated the influence that exhaustive exercise had on the levels of oxidative stress. For this, they analysed the ratio between cellular oxidized glutathione and reduced glutathione (GSSG-to-GSH). Although the GSH levels did not change significantly, they reported an increase in the GSSG-to-GSH ratio due to an increase in the cellular oxidized glutathione levels. Hence, it evidenced an occurrence of oxidative stress post-exercise.

On the other hand, Niess *et al.* (1996) measured plasma levels of malondialdehyde (MDA) at rest before and after an exhaustive bout of exercise. They found no significant increase in MDA following a treadmill test to exhaustion, neither at 15 min post-exercise nor at 24 h post-exercise. Yet they did demonstrate the occurrence of DNA damage in white blood cells following the exercise protocol. More recently, Watson *et al.* (2005) measured exercise-induced oxidative stress by the concentration of F_2-isoprostane in the blood during two different exercise protocols performed in sequence. Initially, the subjects ran for 30 minutes at 60% of their VO_2 max and performed an incremental running test to exhaustion immediately after that. As a result, they reported a decrease in the concentration of F_2-isoprostanes both during and 1 h after the exercise. Nevertheless, it is important to highlight that these studies present some limitations concerning the analytical techniques employed, as all of them were indirect methods, and the reactive intermediates do present different properties contributing to the inconsistencies reported in the literature (Bailey *et al.*, 2003). Furthermore, analyses of ROS generation are mainly collected immediately after

exercise, whereas the true rate of ROS production has been shown to decrease rapidly within the first 1-2 min after muscle contraction ceases (O'Neill *et al.*, 1996). Therefore, the majority of studies probably underestimate the real time of reactive species production during exercise.

Using the direct technique of ESR and also changes in MDA concentrations, Ashton *et al.* (1999) examined free radical production of subjects that cycled to volitional fatigue. They reported an increase in free radical production by both methods. In accordance, Bailey *et al.* (2004) analysed an incremental knee extensor exercise preformed at 25, 70 and 100% of single-leg maximal work rate. Besides ESR, other parameters, including measurements of plasma catecholamines, biomarkers of tissue damage and uric acid were also used to assess oxidative stress. The findings provided direct evidence for an exercise intensity-dependent increase in free radical production. It also provided additional insight into potential sources and mechanisms associated with exercise-induced free radical generation that is addressed further on.

It is important to keep in mind that the exercise models used in the studies also deserve critical evaluation since they recruit different muscle groups and different modes of contraction (Bailey *et al.*, 2003). Coupled with the fact that they use different population groups, it is comprehensible that these studies present different rates of oxidative stress as the result of exercise performance.

2.3.4 Mechanisms of Increased Free Radical Production with Exercise

The causes of increased free radical and ROS production during exercise have not been totally clarified. Although various mechanisms have been identified, there is still a lack of understanding concerning how each of them contributes to the total amount of oxidative stress produced. In addition, the mechanisms may act synergistically, and different types of exercise probably elicit different pathways of free radical production (Vollaard *et al.*, 2005). The mechanisms are described below.

1. *Electron leak at the mitochondrial electron transport chain.* This theory has led

to the interpretation that a substantial increase free radical generation is to be expected during exercise due to a "leak" of electrons in the respiratory chain in the mitochondrial inner-membrane. This would occur due to the inadequate coupling of the electron transfer between the complexes (Figure 2.3) (Vollaard *et al.*, 2005). Despite this theoretical appeal, there is little direct evidence that mitochondrial superoxide anion production is increased during exercise. To further contradict this theory, there are studies with isometric exercise where the oxygen pressure (PO_2) in the mitochondria was proven to be low but still demonstrated an increase in the oxidative stress (Alessio *et al.*, 2000). Bailey *et al.* (2004), mentioned earlier, also demonstrated that free radical outflow of a contracting muscle was associated with decreases in intracellular PO_2 rather than with conditions of increased oxygen flux. Although there is a lack of direct evidence for this mechanism it is unlikely that an increase in mitochondrial oxygen flux is the only, or main, cause for increases in radical production during exercise (Vollaard *et al.*, 2005).

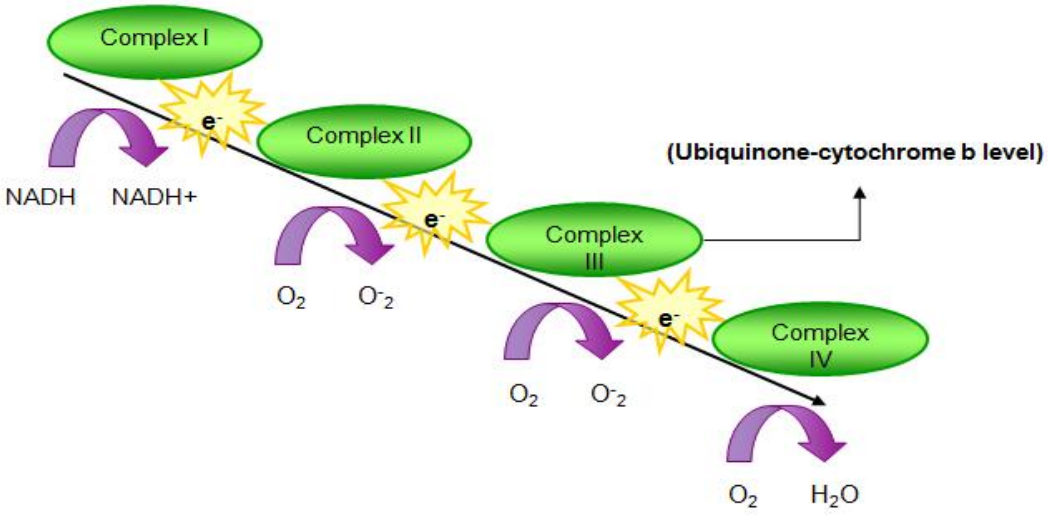


Figure 2.3. The mitochondrial respiratory chain. Electrons are transferred from complexes I, II and III to IV. However, inadequate coupling of electron transfer can cause leakage, generating superoxide anions at different complex levels (adapted from Vollaard et *al.*, 2005).

2. *Ischemia-reperfusion and activation of endothelial Xanthine oxidase.* During exercise, blood flow is shunted from many organs and tissues and redirected to the working muscles. The ischemic conditions trigger the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase. When the exercise ceases and the tissues are reoxygenized, xanthine oxidase produces superoxide (O^-_2) and H_2O_2 as by-products of the degradation of hypoxanthine into xanthine, and

subsequently into uric acid (Halliwell & Gutteridge, 2007) – Figure 2.4.

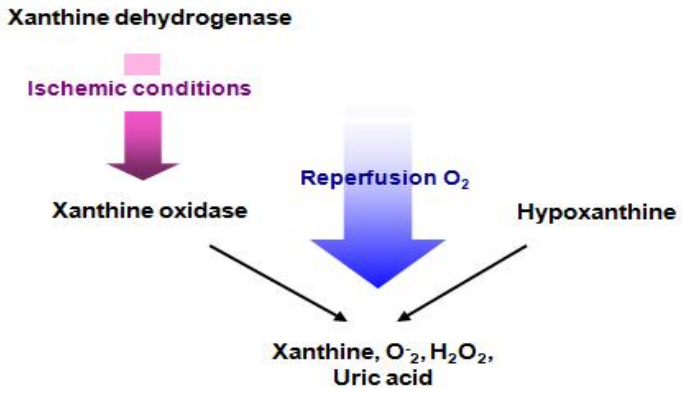


Figure 2.4. A suggested mechanism for the production of free radicals upon reoxygenation of ischemic or hypoxic tissues (adapted from Halliwell & Gutteridge 2007).

3. *Neutrophils and the inflammatory response*. When neutrophils are activated, they release ROS. Despite the fact that this inflammatory response is critical to the removal of damaged proteins and infections, ROS and other oxidants released from neutrophils can also cause secondary damage, such as lipid peroxidation. It has been shown that exercise can elicit muscle injury accompanied by the activation of neutrophils (Ji, 1999). Boyum *et al.* (2002) showed not only an increase in neutrophil number following cycling exercise (65 min 75% VO_{2max}), but also an increase in their respiratory burst activity measured as chemiluminescence. An increase in neutrophil counts has also been described in short duration (less than 20 min) resistance exercise (Ramel *et al.*, 2004). This reinforces the fact that there is an increase in plasma neutrophils even when oxygen consumption is only moderately increased.

4. *Auto-oxidation of catecholamines*. Adrenaline, noradrenaline and dopamine are often referred to collectively as catecholamines. With exercise, there is an increase of these substances (Shinkai *et al.*, 1996; Suzuki *et al.*, 1999) The oxidation of the catecholamines can produce superoxide anion, H_2O_2, and other non-oxygen-derived species in a complicated series of reactions. This can lead to a depletion of cellular glutathione concentrations in the blood (Halliwell & Gutteridge, 2007).

5. Other factors also regulate exercise-induced oxidative stress, including level of fitness, type and intensity of exercise, and dietary antioxidant ingestion.

Despite the need for a better comprehension of how these mechanisms of ROS generation interact, the mechanism by which they affect the exercising muscles is well established. At rest, the low concentration of ROS in the muscles is critical for force generation. During contractile activity there is a rise in ROS production. On one hand, production of reactive species during non exhaustive exercise has been shown to be important for the adaptation of the muscle fibres (Gomez-Cabrera *et al.,* 2008). Nevertheless, during strenuous exercise, the production of ROS can be higher than the buffering capacity of the muscles' antioxidants. As ROS accumulates in the contracting muscles the oxidation of proteins and lipids might cause, amongst other things, inhibition in force production contributing to the development of acute fatigue (Reid, 2001). In addition, this exaggerated increase in ROS levels in response to exercise can also lead to oxidative DNA modification, damage to cell membrane and other cellular compounds (Niess & Simon, 2007).

In order to attenuate the muscle oxidative stress generation and thus improve the muscular performance several researchers have analysed the efficacy of dietary antioxidant supplementation, these are analysed in the next section. Interestingly it has been well documented that training causes an enhancement in the antioxidant enzyme activity in various tissues (Kanter, 1998; Wilson & Johnson, 2000; Gomez-Cabrera *et al.,* 2007). This is an adaptation process that happens because the free radicals produced during muscle contraction act as signaling molecules stimulating the gene expression and hence increased production of antioxidant enzymes and modulates other oxidative stress protection pathways, such as enhancing the activity of DNA repair enzymes in skeletal muscles (Radak *et al.,* 2003; Gomez-Cabrera *et al.,* 2008). This strengthens the body's antioxidant network system which, hence, minimizes the oxidative stress process (Gomez-Cabrera *et al.,* 2008). This stimuli associated with enhanced antioxidant protection occurs not only in the muscles but systemically, so vital organs such as liver and brain also go through this beneficial adjustment (Radak *et al.,* 2008). This adaptation process resultant from regular exercise stimulus can be explained by the hormesis theory. This theory postulates that chemicals and toxic substances may have a low-dose stimulation high-dose inhibitory effect. That means they can provide positive

responses when present in small amount (Radak *et al.*, 2005). In this regard the type and duration of the training are the key for a significant up-regulation of the endogenous antioxidants with long-duration high-intensity endurance training having been shown to be more effective (Powers *et al.*, 1999). Niess *et al.* (1996) reported that trained individuals presented less DNA damage after an exhaustive bout of exercise compared to untrained men. Other evidence for this training adaptation was reported by Miyazaki *et al.* (2001), where the free radical production was reduced after 12 weeks of endurance training. More specifically, they reported a decrease in the neutrophil superoxide anion production and attenuation in the lipid peroxidation process. The contrary is also true as sedentarism not only reduces various physiological functions but also decreases the body's oxidative stress protection mechanisms. Consequently there may be an increase in the occurrence of oxidative stress associated with diseases such as cancer, atherosclerosis, cardiovascular and neurodegenerative diseases (Radak *et al.*, 2008).

2.3.5 Oxidative stress and air pollutants

Air pollution is a major public health issue in urban areas all over the globe. Air pollutants such as ozone, particulate matter and nitrogen dioxide have been shown to be associated with an increase and exacerbation of respiratory and cardiovascular diseases (Katsouyanni, 2003; Bell *et al*., 2006). Little is known, however, about their effects as oxidant compounds in the lungs or about the role and the effectiveness of respiratory tract lining fluid (RTLF) antioxidants in scavenging and protecting against their harmful effects. Data have shown that pollutants such as diesel exhaust particles can induce lung inflammation through stimulation of the oxidative stress process (Baulig *et al.*, 2002). Nevertheless, as has also been confirmed, endogenous antioxidants can attenuate this process, particularly if the inhaled pollutant dose is low (Behndig *et al.*, 2006).

A great variety of antioxidants can be found in human respiratory tract lining fluids (RTLF) and these can easily be accessed by nasal lavage procedure (Mudway *et al.*, 1999). The distribution and concentration of these antioxidants throughout the airways is not equally distributed, with high levels of GSH in the alveolar epithelial regions and uric acid predominating in the upper airways (Penden *et al.*, 1990; Cantin *et al.*, 1987).

Numerous studies have investigated the effects by which the oxidant gas ozone affects the human lungs. Nevertheless, the mechanisms responsible for such adverse effects,

such as an increase in the airway inflammation and impairment in lung function, are only partly understood. It is known that when O_3, or indeed any gas, is inhaled, it first comes into contact with the RTLF. In fact, ozone is not able to infiltrate further than the RTLF and the membranes of cells from the lung-air interface, yet it is still known to cause damage beyond the lungs (Pryor *et al.*, 1995). Once O_3 enters in contact with the RTLF, it induces a direct oxidative stress, the intensity of which varies depending on the O_3 inhaled dose and also on the antioxidants present in the lining fluid. It also induces an indirect oxidative stress because, even though O_3 does not react directly with the epithelial cells, these cells do react in response to the oxidation products produced in the RTLF. As a consequence they release a variety of pro-inflammatory mediators and more ROS (Pryor 1994; Pryor *et al.*, 1995). As a result, if the oxidative stress is sufficient there is an activation of an inflammatory response, characterized by an intense arrival and activation of neutrophils. These neutrophils produce further ROS through the respiratory burst process. Hence, the overproduction of ROS might result in oxidative stress in the airway tissues (Corradi *et al.*, 2002). It has been hypothesized that the antioxidants present in the epithelial lining fluid of the lungs would neutralize the excess production of free radicals and ROS. Thus, it would reduce the ozone induced lung injury (Samet *et al.*, 2001). This will be discussed in the following sections.

2.4 ANTIOXIDANTS

Due to the potential role that ROS and free radicals have in lipid, protein and DNA damage, it is not surprising that there are a variety of antioxidant defense mechanisms present in the body. In general, antioxidants are often reducing agents that have the capacity to react with free radicals and ROS minimizing their reactivity and thus decreasing oxidative stress. They can be both synthesized *in vivo* and absorbed through the diet. A great variety of antioxidant defense mechanisms exist endogenously, both intracellular and extracellular, working synergistically to minimize the generation of free radicals and also to remove ROS (Powers *et al.*, 2004).

Antioxidants can be divided into two groups: enzymatic and non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Each of these enzymes is responsible for the reduction of a different ROS and they are located in different cellular compartments. For example, GPX,

located in both the cytosol and the mitochondria of cells, is responsible for the removal of organic hydroperoxides and hydrogen peroxides (H_2O_2); while SOD, present in the cytosol of cells catalyses the reaction of superoxide radicals into oxygen and hydrogen peroxides (Powers *et al.*, 1999). In the muscles, the activity of these enzymatic antioxidants varies depending on the muscle fibre type, with the greatest activity present in type I fibres that are the slow twitch fibres with higher oxidative capacity. The non-enzymatic antioxidant group includes glutathione, vitamin C, vitamin E, carotenoids, uric acid and others. A more detailed explanation on the mechanisms of action of the specific antioxidants glutathione, vitamin C and vitamin E, will be given in the subsequent sections. Similarly to the enzymatic antioxidant, these are present in different cellular compartments and elicit distinct antioxidant properties which maximize their effectiveness (Powers *et al.*, 2004).

Production of ROS during muscle contraction and specifically exhaustive exercise is well established (Sen & Roy, 2001). As a consequence the excess in ROS release can inhibit locomotory and bactericidal activity of neutrophils, reduce the proliferation of T-lymphocytes and B-lymphocytes, and inhibit Natural Killer cells. In addition it can lead to a decrease in muscle force and hence premature fatigue (Reid, 2001). It has been hypothesized that the increase in the production and release rate of ROS during exercise would be neutralized by an increase in antioxidant concentration in the organism. Studies that have analyzed the efficacy of antioxidant supplementation have used changes in oxidative stress, inflammation and exercise performance as outcome measures. There has been, however, a variety of different results making it hard to draw definite conclusions about this issue. Unfortunately, there are few studies that have investigated the effect of antioxidant supplementation on exercise performance, and the ones to date report little evidence of a positive effect on performance. It is important to point out that on one hand, even though exercise can increase the production of reactive species in the body and thus oxidative stress, on the other hand, when practiced systematically it can be considered an antioxidant as explained previously.

2.4.1 Glutathione

Reduced glutathione (GSH) is a water-soluble low-molecular-weight tripeptide formed from the amino acids glutamate, cysteine and glycine. These three amino acids can be

obtained from food intake. All types of cells are capable of synthesizing GSH. This synthesis process occurs through two sequential reactions and necessitates the action of two enzymes: for the first reaction, γ-glutamylcysteine; and GSH sythetases for the consecutive reaction (Figure 2.5). Cysteine is the key substrate for the production of GSH limiting the production-rate. In addition, the levels of GSH act as a negative control for the first reaction, this helps maintain the adequate production (Anderson, 1998).

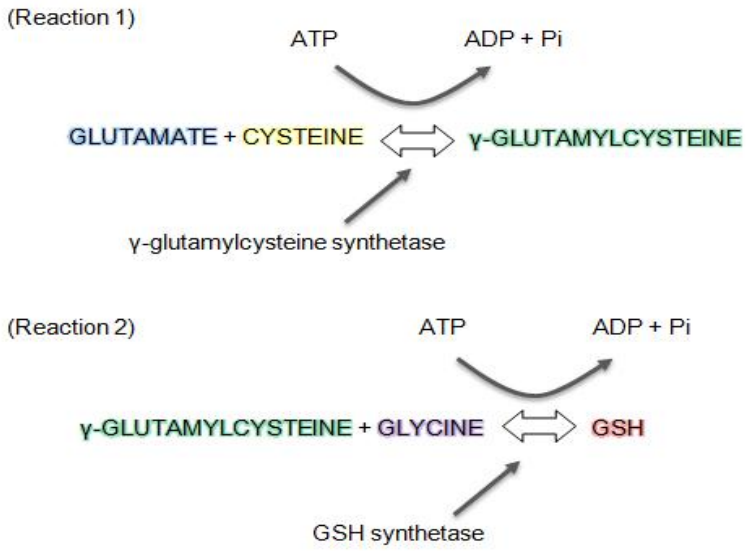


Figure 2.5. GSH synthesis illustrated by two reactions. Enzymes that catalyze the reactions are γ-glutamylcysteine synthetase and GSH sythetase (Adapted from Wu *et al.*, 2004).

GSH can also be obtained directly from food, but only small amounts of the intact tripeptide are absorbed by the gut; most is broken down into amino acids, which are then used in the synthesis cycle. Thus, a healthy diet with a balanced protein intake is of the essence for an adequate GSH homeostasis in the body (Valencia *et al.*, 2001; Wu *et al.*, 2004).

GSH exerts various essential functions in the body. Amongst these functions is the detoxifying role that GSH plays when it reacts with xenobiotics and their metabolites. GSH also stores the amino acid cysteine which, due to its instability outside the cells, easily oxidizes to cystine producing ROS (Lu, 1999). In addition, GSH is essential for the production of an adequate immune response: it is necessary for the proliferation of lymphocytes, for the production of cytokines and is crucial for the activation of T-lymphocytes and neutrophils (Wu *et al.*, 2004). Another major function of GSH, which

is of particular interest here, is its antioxidant role. It efficiently scavenges ROS and free radicals preventing an increase in the oxidative stress process. In these reactions, the reduced GSH is oxidized to form glutathione disulfide (GSSG), this reaction is catalysed by the enzyme glutathione peroxidise. Note that GSSG is formed by two GSH molecules linked via a disulfide bond due to oxidation of the thiol (SH) groups. Once oxidised, GSSG can be reduced back to its original GSH form by the enzyme GSSG reductase and nicotinamide adenine dinucleotide phosphate (NADPH). Nevertheless, when there is a high level of oxidative stress, NADPH becomes depleted and there is an intracellular accumulation of GSSG. This excess GSSG can either be exported out of the cell or it can form a mixed disulfide. Despite what happens to the oxidized glutathione, depletion of cellular GSH can be observed when an intense oxidative stress process occurs (Lu, 1999).

As mentioned previously GSH is produced in the cells' cytoplasm, with the liver being the most active organ for its production and delivery. The intracellular concentration of GSH ranges from 0.5-10 $mmol \cdot l^{-1}$, and about 10 to 20% of this GSH is present in cell's mitochondria. Nonetheless, this organelle is not capable of producing this antioxidant and has to obtain it from the cytoplasm through transporters in the inner membrane (Halliwell & Gutteridge, 2007). The intracellular GSH concentration is quite high, especially when compared to the relatively low plasma concentration which varies from 2-20 $umol \cdot l^{-1}$. Most of the blood GSH is present in the erythrocytes, which explains the low plasma concentration. In addition, the plasma GSH is derived mainly from the liver production, this organ is also responsible for most of the GSH that is taken up by the kidneys and lungs (Lu, 1999). In fact, the respiratory tract lining fluid has a high concentration of reduced GSH, around 300-400 $\mu mol \cdot l^{-1}$. It has been shown that, where smokers are concerned, these values are greatly increased to protect these individuals against the exacerbated oxidative stress levels (Dalle-Donne *et al.,* 2006). Inflammatory cells present in the respiratory tract, such as macrophages, lymphocytes and fibroblasts are also responsible for the GSH levels in the lung seeing as they release this antioxidant (Cantin *et al.,* 1987).

The change in plasma GSH concentration has been linked to a variety of pathophysiological conditions including cancers, diabetes mellitus, HIV, cardiovascular

diseases and lung inflammatory diseases. Indeed, it has been shown that the GSH levels are decreased in the epithelial lining fluid of asthmatics or during an asthma exacerbation episode, whereas in chronic smokers the concentration of this antioxidant is increased (Dalle-Donne *et al.,* 2006).

Measuring the plasma level of GSH or its oxidized form (GSSG) is a widely accepted method of detecting oxidative stress and can be reported as GSH concentration, GSSG or GSH/GSSG ratio. It is not only a good indicator of systemic oxidative status, but also a useful indicator of disease risk (Rossi *et al.,* 2002). It has been used in various studies to indicate the free radical production during exercise (Gohil *et al.,* 1988; Sastre *et al.,* 1992; Sen *et al.,* 1994; Medved *et al.,* 2003). However, these studies do present some divergent results. This could possibly be explained by the difference in subject fitness, exercise protocol and method of determining the glutathione concentration.

Gohil *et al.* (1988) were the first to show that throughout a submaximal exercise, 90 min of cycling at 65% VO_2 peak, there was a significant increase in blood GSSG and a decrease in the reduced GSH form. In the study of Sastre *et al.* (1992), trained athletes performed a bout of running to exhaustion, according to the Bruce protocol. Blood GSH and GSSG concentration were measured pre-exercise, immediately post-exercise, 30 and 60 min within the recovery period. There were no differences in blood GSH concentration when pre and immediately post values were compared. However, after 30 and 60 min of exercise cessation, these values had significantly dropped below pre-exercise levels. With the oxidized glutathione, the pattern was slightly different: GSSG levels had a significant increase after the exhaustive exercise, with the concentration returning to pre-exercise levels after 60 min of recovery. Similar GSH and GSSG patterns were encountered by Medved *et al.* (2003) when healthy subjects performed a high intensity intermittent exercise to exhaustion. Furthermore, the study of Sen *et al.* (1994) concurs partially with the previous studies. They reported a significant increase in blood GSSG concentration following 3 different exercise bouts: a maximal test and two submaximal 30 min exercise bouts. In contrast to the previous studies, however, there were no changes in the reduced GSH concentration post-exercise. Nevertheless, in the latter study, blood samples were taken only pre-exercise, immediately post-exercise and 24 h post-exercise. It could be that the change in reduced GSH was not observed

due to time of sampling. The increase in blood GSSG concentration reflects an increase in the oxidative stress process during the exercise protocols. As for the decrease in blood GSH concentration during the recovery period, this can be attributed to a decrease in the GSH synthesis which has been shown to occur with adrenergic stimulation (Sastre *et al.*, 1992).

2.4.2 Vitamin C

Vitamin C is a water-soluble vitamin and refers to both ascorbic acid and dehydroascorbic acid (DHA). Ascorbic acid is the main form of the vitamin found *in vivo*. This vitamin, also referred to as ascorbate, has clearly been shown to play an essential role in connective tissue biosynthesis and its deficiency results in scurvy, a disease which leads to the deterioration of collagen production and results in fragile blood vessels and impaired lesion healing. This disease is reversible once the individual restarts the ingestion of ascorbate. Vitamin C is also a strong reducing agent, due to its facility in donating electrons, with important antioxidant properties (Institute of Medicine, 2000). It can inactivate a variety of reactive species minimizing damage to body tissues.

Ascorbate is found in relatively high levels in different tissues throughout the body. It occurs in high concentration in activated neutrophils and macrophages, present predominantly in the cells' cytosol. This vitamin is implicated in a variety of anti-infective functions, including promotion of T-lymphocyte proliferation, prevention of corticosteroid-induced suppression of neutrophil activity, production of interferon, and inhibition of virus replication. Vitamin C also occurs in high concentration in the adrenal glands and is necessary for the production of several hormones that are secreted in relation to stress, such as adrenalin, noradrenalin, and cortisol as previously described (Jukendruop & Gleeson, 2004). Furthermore, it is also present in the brain, eye tissue and respiratory tract lining fluid providing a high antioxidant defense. This antioxidant is also present, in low concentration, in plasma and in saliva (Institute of Medicine, 2000).

When ingested, vitamin C is absorbed in the intestines either by active transport, if the availability of the vitamin is low, or by simple diffusion if present it high concentrations. If ingested in excess, it is degraded in the intestines and can cause

diarrhea and intestinal discomfort. Besides the absorption and degrading mechanisms occurring in the intestines, the kidneys are also responsible for conserving or eliminating unmetabolized ascorbic acid to help maintain the body's ascorbic acid balance (Institute of Medicine, 2000). The average limit uptake of vitamin C by the body is 200 mg daily. This amount is enough to saturate cells and body fluids and it can be achieved with no difficulty by ingesting five serving of fruits and vegetables per day (Halliwell & Gutteridge, 2007). It has been shown that, 2 h after supplementation there is a significant increase in the ascorbic acid concentration in both the plasma and in the nasal lavage fluid. The levels in the nasal lavage represent the ascorbic acid concentration present in the respiratory tract lining fluids. The correlation between the increases of this antioxidant in both these compartments suggests a permeable and efficient communication between them when ascorbic acid is concerned (Schock *et al.*, 2004). During oxidation reactions, only small amounts of ascorbate are lost because, once it is oxidized, it can be reduced back to ascorbic acid by reductants such as glutathione, nicotinamide adenine dinucleotide (NADH) and NADPH. Similarly, vitamin C is also known to regenerate other antioxidants, such as vitamin E and glutathione, back to their reducing state, thus maintaining a balanced network of antioxidants (Rietjens *et al.*, 2002).

It has been hypothesized that, by increasing the plasma ascorbate levels, there would be a greater availability of this antioxidant to react with ROS and free radicals and consequently minimize the oxidative stress process that may occur with exercise (Mudway *et al.*, 1999b). Enhancing intrinsic antioxidant defense, by increasing vitamin C intake, is therefore theorized to benefit athletes engaged in heavy training and competition (Peters, 1997). This issue, however, has generated some conflicting data. Nieman *et al.* (1997) found that 8 days of vitamin C supplementation (1000 mg) had no influence on immune parameters after a 2.5 h run (75-80% VO_{2max}). Here blood samples were analyzed for cortisol, cathecolamines, and a variety of immune measures such as leukocyte subsets, NK-cell activity, neutrophil phagocytosis and activated oxidative burst. Similar results were encountered for a 1500 $mg \cdot day^{-1}$ of vitamin C intake on a group of ultramarathon runners (Nieman *et al.*, 2002). No significant correlation was found between plasma ascorbic acid levels, immune or oxidative stress measures after the race. Importantly, Nieman *et al.* (2002) controlled for carbohydrate ingestion during the ultramarathon. This is an essential process in field studies since it is

known that carbohydrate ingestion, throughout an exercise bout, affects stress hormones and inflammation markers response after exercise. In these studies, plasma ascorbic acid was significantly higher post-race, whether in supplemented or non-supplemented athletes. This could represent an up-regulation of several major antioxidant enzymes, in spite of its additional intake, and an enhancement of overall antioxidant status during periods of greater oxidative stress, minimizing excessive cellular damage (Dekkers *et al.*, 1996). This natural increase in plasma vitamin concentration could possibly abolish the effect of additional vitamin supplementation, though this needs to be further explored. No impact on the athletes' performance was reported.

Adrenal hormones (cortisol, adrenaline and noradrenaline) possess potent anti-inflammatory and immunosuppressive properties. During intensive physical exercise, there is an increase in the circulating concentration of these hormones (Shinkai *et al.*, 1996, Suzuki *et al.*, 1999). This has an impact on the magnitude of the post–exercise immunodepression (Pedersen & Ullum, 1994). Whether vitamin C affects the response of adrenal hormones to exercise is an issue which has raised contradictory discussions. Palmer *et al.* (2003) measured the influence of vitamin C supplementation in relation to placebo on oxidative stress markers (F_2-isoprostane and lipid hydroperoxides) in runners during and following an ultramarathon race. The athletes in the supplemented group ingested 1500 mg·day^{-1} during a 7 day period prior to the race. Vitamin C did not show any protective effect on the exercise-induced oxidative stress. Additionally, the authors reported that vitamin C had no influence in cortisol increases post-exercise.

The afore mentioned results are in contrast to those of Nieman *et al.* (2000) and Peters *et al.* (2001) as they support the hypothesis that vitamin C supplementation attenuates an exercise induced increase in cortisol levels. In both these studies (Nieman *et al.*, 2000; Peters *et al.*, 2001), the athletes undertook an ultramarathon race in the same way as in the study of Palmer *et al.* (2003). The supplementation protocols were different, though; consisting of 1500 mg·day^{-1} during a 1-week period for Nieman *et al.* (2000) and 1000 mg·day^{-1} during a 1-week period for Peters *et al.* (2001). The cortisol response was blunted after the long duration exercise, but there were no differences in the inflammatory markers between placebo and the vitamin C supplemented group (Peters *et al.*, 2001). In addition, while the pre-exercise levels of plasma vitamin C was significantly increased in the supplemented group, post-race there were no alteration

in these values, contrary to the placebo group that despite presenting lower levels of vitamin C pre-exercise presented an increased value after the race. This result suggests that there was mobilization of ascorbic acid into the plasma in the placebo group. Conflicting findings between the studies can possibly be explained when one takes into consideration that subjects were not randomized, that there were different numbers of subjects in the treatment groups in the last studies (Peters *et al.,* 2001; Palmer *et al.,* 2003), and that carbohydrate intake was not controlled for. Although these studies have inherent limitations, such as not having used a crossover design, they do have the advantage of being field studies.

A more recent vitamin C supplementation study has shown that acute intake of vitamin C during a prolonged exercise bout (2.5 h of cycling at 60% VO_{2max}) did not alter the plasma levels of IL-6 or cortisol post-exercise in moderately trained males (Davison & Gleeson, 2005). The lack of changes in this study could be explained by the supplementation protocol used. As vitamin C was given to the participants only during the exercise period, there might have been a lack of uptake of this antioxidant by the leukocytes and muscle tissues. Conversely, in a subsequent study, the same authors (Davison & Gleeson, 2006) used a different supplementation protocol and obtained slightly different results. The supplementation consisted of daily vitamin C (1000 $mg \cdot day^{-1}$) or placebo intake during a 2 week period. The study was carried out with trained cyclists using a randomized crossover design with a washout period of at least 14 days. The athletes performed the same exercise bout as in the previous study. Again the authors showed that the antioxidant had no effect on plasma IL-6 or on malondialdehyde (marker of lipid peroxidation) levels post-exercise and it did not affect the decrease in neutrophil functional capacity. Nonetheless, the supplementation resulted in slightly lower leukocytosis, neutrophilia and cortisol responses. This suggests that vitamin C might have a direct effect on cortisol synthesis or release. It is important to mention that the subjects in this study only presented a modest oxidative level post-exercise so it might have been necessary to undergo a more strenuous exercise protocol to observe greater benefit of the antioxidant supplementation. It can also be suggested that healthy subjects who consume a balanced diet present sufficient ascorbic acid in their organism to exert the necessary antioxidant defense so additional supplementation would not promote extra benefits. However, it could be speculated that the supplementation might be beneficial if the athletes were to experience an increased

oxidative stress burden such as with the addition of ozone-pollution.

Even though there has been many studies analyzing the effect of vitamin C on different aspects of the immune system and oxidative stress, there is still controversy in the results. The different doses and supplementation periods together with the exercise protocol and fitness level of the subjects analyzed are the main variables which may explain the discrepant results. There has been little recent interest in how this antioxidant impacts on the well being and performance of athletes and whether or not it has an effect on oxidative stress and immune function. In the 1970s, when the first studies analyzing the effect of vitamin C on exercise were conducted, researchers did focus on the effect of this vitamin on exercise performance. These studies however, did not find any strong evidence of an improved aerobic or anaerobic performance for people taking vitamin C supplementation (for review see Urso & Clarkson, 2003). In the study of Howard *et al*. (1975), for instance, athletes were supplemented for a 2-week period with both placebo and 1000 mg of vitamin C. The authors investigated the impact of the placebo and the antioxidant on the performance of a cycling incremental test to exhaustion. They reported that for a given submaximal workload, when subjects ingested vitamin C, they presented a lower heart rate. This could mean a more economic heart function during exercise and, thus, a better work capacity. Nevertheless, this was not translated into a better total work performance with the vitamin C ingestion. The mechanism through which vitamin C would exert a positive effect on performance remains unclear. It seems, though, that healthy subjects ingesting sufficient vitamin through their diets would not gain any further benefits, be it enhancing their immune system, antioxidant capacity or performance, with such vitamin supplementation.

Vitamin C is not only associated with immune and oxidative stress parameters, but there is also a link between vitamin C and aspects of pulmonary function. In fact, a number of epidemiological studies have shown evidence that consuming a diet rich in fruits and vegetables results in a reduced risk of COPD and have a beneficial effect against asthma in adults (Romieu *et al.,* 2006; Varraso *et al.,* 2007a; Varraso *et al.,* 2007b). Even though studies do report a positive effect of such a diet, there is some inconsistency, especially in regard to vitamin C, that seems to have a greater effect on lung function than on respiratory symptoms (Romieu *et al.,* 2006). Studies analysing the effect of

vitamin C supplementation on asthmatic subjects have provided different results. Fogarty *et al.* (2003) studied the effect of a 16 week supplementation period of vitamin C (1000 mg·day^{-1}) on a cohort of over 200 patients with asthma. The authors reported no correlation between the antioxidant and asthma in these patients. Yet, contrary to those findings, a recent study by Tecklenburg *et al.* (2007) reported a positive effect of vitamin C supplementation on exercise-induced bronchoconstriction in asthmatic individuals. The authors investigated the impact that a diet supplemented with vitamin C had not only on pulmonary function, but also on various biological markers of airway inflammation in asthmatic subjects after an exercise bout to exhaustion. This study had a double-blind, randomized crossover design and 8 subjects took part in it. All subjects had mild to moderate asthma and also exercise induced bronchoconstriction (EIB), characterized by a 10% decrease in FEV_1 after an exercise bout (Rundell & Jenkinson, 2002). The supplementation consisted of 3 x 500 mg·day^{-1} of vitamin C during a 2-week period. There was also a 1 week washout period before they were assigned to the alternate treatment/placebo period. The authors reported an improvement in the post-exercise pulmonary function and reduced severity of EIB when subjects ingested vitamin C. They also reported a decrease in the severity of respiratory symptoms post-exercise and a decrease in the airway inflammation markers. It is important to point out that the airway inflammation markers assessed in this study were obtained from urine. It is difficult to directly compare these 2 studies, because of the added exercise variable. Nevertheless, other studies investigating the effect of vitamin C on lung function seem to concur with the later study (Smit *et al.*, 1999). Thus, it seems reasonable to suggest that vitamin C does have a positive impact on lung function and provides some protection against airway inflammation and airway narrowing in asthmatic subjects after exercising.

2.4.3 Vitamin E

Vitamin E, also referred to as α-tocopherol, is a lipid-soluble vitamin. There are a variety of vitamin E molecules that differ in structure. The various forms of this vitamin differ significantly in their metabolic functions and bioavailability. In humans, over 90% of vitamin E encountered in the body is α-tocopherol. However, not all the α-tocopherol forms are maintained in the plasma. The α-tocopherol, that is encountered naturally in food together with synthetic forms of the α-tocopherol (the isomeres RRR-, RSR-, RRS-, and RSS- α-tocopherol), can be maintained in the human plasma and

tissues. When ingesting α-tocopherol supplements, the RRR- α-tocopherol form is preferable (Lodge *et al.,* 2000; Institute of Medicine, 2000).

This vitamin has been shown to have beneficial effects in some diseases, it has also been associated with a decreased risk in cardiovascular disease, and it can help slow the progress of degenerative diseases such as atherosclerosis. In addition, α-tocopherol deficiency, in patients that present impairment in intestinal fat absorption, has been associated with neuronal degeneration (Cerqueira *et al.,* 2007). Nevertheless, a recent review study (Bjelakovic *et al.,* 2007), which thoroughly analyzes over 47 antioxidant supplementation research papers on all-cause mortality, has unexpectedly shown the negative effect of some supplements. Vitamin E, for example was one of the antioxidants that was associated with an increase in mortality. Vitamin C, on the other hand, did not have such an effect, but there was also no evidence that it might increase longevity. It is difficult to determine the specific biochemical and physiological mechanisms that may have led to this result. More investigation is, therefore, necessary to answer questions that such research generates. In addition, these results concerning synthetic antioxidants should not be transferred to effects that fruit and vegetables have on human health.

Similarly to vitamin C, vitamin E has important antioxidant properties. Due to its capacity for scavenging ROS and free radicals, particularly peroxyl radical ($ROO^{\bullet}$), it exerts the important function of protecting cellular membranes and plasma lipoproteins against lipid peroxidation. This is possible because vitamin E has a great affinity for reducing peroxyl radicals, preventing their interaction with the membrane phospholipids or lipoproteins (Institute of Medicine, 2000). Indeed, vitamin E can be considered one of the major inhibitors of lipid peroxidation *in vivo* (Halliwell & Gutteridge, 2007). Once oxidized, it can be regenerated back to its reduced state by vitamin C, as mentioned previously. Nevertheless, it has been shown that increased levels of α-tocopherol radicals that are not converted back to the reduced form can act as pro-oxidants, initiating oxidative stress processes by themselves. In addition, an environment that lacks other antioxidants or that has a high level of oxidative stress will contribute for vitamin E to act as a pro-oxidant (Rietjens *et al.,* 2002).

Once ingested, vitamin E is absorbed from the intestinal lumen via chylomicrons, however it has been shown that depending on the availability of fatty acids, vitamin E can also be incorporated and transported by high-density lipoprotein (HDL) (Rigotti, 2007). These structures are taken up by the liver, which is the main storage site of tocopherols. In the liver, specifically the α-tocopherol form will be incorporated selectively into the very low-density lipoproteins (VLDL) secreted into the blood. Tissues acquire α-tocopherol and other tocopherol forms from the lipoproteins present in the circulation. Indeed plasma α-tocopherol levels are highly correlated to plasma lipid levels as lipoproteins are the major transporters of vitamin E (Institute of Medicine, 2000; Rigotti, 2007). Moreover, the amount of supplemented vitamin E that is actually absorbed is dependent not only on the fat content of the meal that the supplement is taken with, but also the type of food that is ingested. For example, in a randomized crossover study subjects ingested 200 IU of vitamin E supplementation with different types of food. The labeled plasma α-tocopherol concentration was higher when ingested with meals that had a greater fat percentage. Surprisingly, when subjects ingested toast with butter they presented a higher labeled plasma α-tocopherol concentration compared to when they ingested cereal with full fat milk, even though these two meals were matched for total fat content (Lodge *et al.*, 2004). A potential explanation for this is that foods have different effects on gastric emptying, therefore also influencing absorption.

As mentioned at the start of this section, vitamin E has been found to protect cellular membranes from lipid peroxidation. Hence, it is logical to assume that this vitamin could protect the muscle cells against exercise-induced damage. Early studies analyzing the effects of vitamin E supplementation and exercise investigated its effect on performance. Most of the studies, however, reported no benefit of vitamin E whether for muscle strength or for endurance performance (Urso & Clarkson, 2003). Furthermore, it has been hypothesized that vitamin E supplementation could have a protective effect against the contraction-induced muscle damage oxidative stress that may occur after an intense exercise bout. This rationale is based on the knowledge that this vitamin can stabilize muscle membranes by interacting with its phospholipids which would, thus, provide some protection against the increase in oxidative stress or muscle damage observed after certain types of exercise (Urso & Clarkson, 2003). Yet results of the

various studies have been quite contradictory (Jackson *et al.*, 2004).

In a study by Nieman *et al.* (2004), athletes were divided into 2 groups: one taking a daily supplementation of 800 IU α-tocopherol, the other taking placebo during a 2 month period prior to an Ironman competition (Triathlon World Championship in Kona, Hawaii). Plasma α-tocopherol levels were significantly higher before the race, and this difference was maintained post-race. There was no significant difference in the performance time between the 2 groups. Nevertheless, unexpectedly, the plasma levels of IL-6, IL-8 and F_2-isoprostanes increased significantly after the race in the supplemented compared with the placebo group. This surprising result may have occurred due to the dualist behavior that vitamin E can have. As explained previously, when there is an antioxidant balance in the body, vitamin E would have an effective antioxidant action. If, however, there is high quantity of this vitamin especially under conditions of high oxidative stress and low levels of other antioxidants, the increase in α-tocopherol radicals would result in a pro-oxidant effect (Rietjens *et al*., 2002). In fact, in this study, the amount of vitamin E given to the athletes does greatly exceed the recommended daily allowances (RDA) of 30 IU (Institute of Medicine, 2000). Moreover, the plasma α-tocopherol levels were positively correlated with the IL-6 concentration post-race, but that was not correlated to the athletes' performance.

It has also been suggested that a high vitamin E intake would reduce the incidence of asthma, by decreasing the oxidative stress and consequently the epithelial cell injury that exacerbates the asthma process. The study of Pearson *et al.* (2004), however, has shown no such association. In this study, 72 individuals with mild to moderate asthma were randomly assigned to either a placebo or a vitamin E supplemented group. The supplementation had a 6 week duration period with the participants having a daily ingestion of 500 mg of vitamin E. Although, there was an increase in the plasma levels of vitamin E in the supplemented group, no effect on bronchial reactivity or other asthma aspects were observed. The authors suggested that, to see any improvements in such a group, the ingestion of a combination of antioxidants and not just an isolated one might be necessary.

2.4.4 Supplementation with antioxidant combinations

In the last section, the potential pro-oxidant toxicity of vitamin E has been briefly reviewed. This pro-oxidant toxicity also occurs with other antioxidants, such as vitamin C, carotenoids and flavonoids (Rietjens *et al.,* 2002). With respect to vitamin C, in addition to its scavenging properties, it has been shown to play an important role in reducing the vitamin E radical back to its original state. Through this process, however, vitamin C is oxidized to a vitamin C radical (semiascorbyl). One way to recycle semiascorbyl back to its original vitamin C form is via interaction with other antioxidants, such as glutathione (Powers *et al.,* 2004). Moreover, when vitamin C is found in high concentrations together with the presence of transitional metals (Fe^{3+} or Cu^{2+}) it reduces these metals, transforming them into potent catalysts in the production of free radicals (Powers *et al.* 2004). All together, these facts clearly indicate the need for a balanced antioxidant network in order to maximize the benefits and reduce potential toxic effects of the antioxidants. Taking this into consideration, many studies have investigated the effects of a combined antioxidant supplementation protocol on different post-exercise variables.

In Petersen *et al.* (2001), subjects were divided into a supplemented (500 mg vitamin C + 600 IU of vitamin E daily for 2 weeks) and a placebo group. The exercise protocol consisted of a 5% downhill treadmill run for 1.5 h, the workload corresponding to 75% of VO_{2max}. No group differences were measured for exercise-induced changes in cytokine and lymphocyte subsets. The authors concluded that exercise induced inflammatory responses are not induced by free radicals. However, in this study, the authors did not report any oxidative stress measurements. The study of Mastaloudis *et al.* (2006) investigated the effect of antioxidant supplementation (vitamins E and C) on exercise-induced muscle damage and recovery rate from a muscle-damaging exercise bout. There were 2 groups: supplemented and placebo. The former received a 6-week supplementation period (450 IU vitamin E + 1000 mg vitamin C) before taking part in an ultramarathon competition. No statistically significant differences between the supplemented and placebo group were reported in the time to complete the race. In addition, the antioxidant supplementation protocol elicited no difference on exercise-induced muscle damage or recovery.

In an attempt to specifically investigate the antioxidant supplementation effect on

oxidative stress caused by an eccentric exercise bout, Goldfarb *et al*. (2005) had participants randomly assigned to either a placebo or a supplemented group (400 IU vitamin E + 1000 mg vitamin C + 90 ug of selenium daily for 2 weeks). The exercise protocol consisted of a total of 4 sets of 12 repetition of eccentric arm exercise, and blood samples were collected up to 48 h post-exercise to analyze protein, lipid and glutathione oxidation after the eccentric exercise. The antioxidant supplemented group did present a smaller increase in the blood protein oxidation and MDA following the exercise bout. Nevertheless, no effect could be observed for the blood glutathione levels. It is possible to conclude that a combined antioxidant therapy was effective in minimizing the oxidative stress elicited by a bout of eccentric arm exercise in non-trained subjects (Goldfarb *et al.,* 2005). Yet it is with caution that these results should be extended to other population performing different types of exercise.

Davison *et al.* (2007) showed that a 4-week period of combined antioxidant supplementation (1000 mg vitamin C + 400 IU vitamin E daily) might blunt the cortisol responses to a 2.5 h of cycling at an intensity of about 60% of VO_{2max}. This result was similar to their previous study, which was mentioned earlier (Davison and Gleeson 2006). Nevertheless, this study used an antioxidant combination instead of just vitamin C supplementation. The supplementation protocol was also different between studies. Contrary to the previous study, however, no leukocytosis or neutrophilia was observed after exercise in the supplemented group. Both studies reported similarly that the reduction in cortisol levels post-exercise occurred in spite of any changes in plasma oxidative stress markers, IL-6 concentration and neutrophil function when compared to the non-supplemented group. The authors concluded that even though further investigation is required in this field, it seems that daily supplementation of a high antioxidant dose does not provide any practical benefit.

There are also studies that have restricted the participants' dietary antioxidants intake. Watson *et al*. (2005) investigated the effect of a dietary antioxidant restriction on exercise performance, antioxidant defenses and oxidative stress of athletes. The exercise protocol consisted firstly of a 30 min run at a submaximal speed corresponding to 60% of their VO_{2max}, followed immediately by a running incremental test to exhaustion. The results showed that at rest there was no difference in plasma F_2-isoprostane levels when

participants restricted their antioxidant intake. This indicates that the body's antioxidant reserve was still capable of protecting against resting production of reactive species. However, in response to the exercise bout, the F_2-isoprostane levels in the restricted diet group increased significantly when compared to the group which maintained normal antioxidant intake even though there was no difference in the exercise time to exhaustion. As for the plasma antioxidant concentrations, they did not differ between the groups. The authors concluded that for athletes participating in acute high intensity exercise with up to 40 min duration, there would be no reason to recommend antioxidant supplements because the amount obtained from their diet would suffice to protect them. All in all, these studies present mixed results on the effectiveness of antioxidant supplementation in decreasing exercise-induced oxidative stress. It is important however, to keep in mind that the different training status of the participants, the exercise performed, and the supplementation protocol all have a great influence on the outcome of the studies.

2.4.5 Air pollution, exercise and antioxidant supplementation

Air pollutants, and especially ozone, can cause oxidative stress and trigger inflammatory response in the lungs, as has been well documented and discussed in previous sections. Thus, it has been proposed that an increase in the antioxidant intake which consequently increases the availability of antioxidant defenses in the respiratory-tract lining fluid might provide protection against the adverse effects of ozone pollution.

Antioxidant supplementation has been proposed to be beneficial for people exercising in an ozone-polluted environment. The rationale behind such hypothesis is that increasing the availability of antioxidants in the respiratory-tract lining fluid will provide additional sacrificial substrates for ozone which will in turn decrease oxidation reactions occurring within this fluid and the underlining epithelial cells. As a result, its toxicity would be decreased limiting the inflammation response of the cells from the epithelium tract (Kelly, 2004). Indeed, some studies have shown a small protective effect on lung function when subjects are supplemented with a mix of antioxidants (Grievink *et al.,* 1998, Grievink *et al.,* 1999; Romieu *et al.,* 1998; Samet *et al.,* 2001). Nevertheless, the results have not been shown to prevent the increase in inflammatory markers (Samet *et al.,* 2001; Mudway *et al.,* 2006). Moreover, there is limited information about the effects of antioxidant supplementation and performance in a polluted environment and

pollution combined with heat and humidity.

2.4.6 Antioxidant combination, asthma and ozone

It has been suggested that the ingestion of combined antioxidants might indeed attenuate the severity of asthma. A study that analyzed the effect of antioxidants supplementation by asthmatics together with their exposure to ozone has shown a protective effect (Trenga *et al.,* 2001). The supplementation consisted of a 4-week period of daily ingestion of vitamin C (500 mg) and vitamin E (400 IU). The participants were exposed to 0.12 ppm of O_3 during 45 min while taking part in intermittent exercise. The degree of ozone induced bronchial hyperresponsiveness was measured through SO_2 challenge after the exposure protocol. The group that received supplementation showed a decrease in the bronchial hyperresponsiveness. It is important to note that the protective effect of the vitamin mixture was more pronounced among participants that had more severe asthma and that showed a higher sensitivity to SO_2. Still in respect to asthmatic individuals, there have been other studies which support a beneficial effect of vitamin supplementation. Sienra-Monge *et al.* (2004) observed that asthmatic children, with low intake of vitamin E, supplemented with a combination of vitamin E and C presented a decrease in nasal lavage inflammatory mediators (IL-6 and IL-8). These children lived in Mexico City and, therefore, were constantly exposed to air pollution.

Even though there is still controversy in the literature, it has been better established that asthmatics or non-asthmatic individuals exercising in a polluted environment will have more beneficial results from the antioxidant supplementation (Grievink *et al.,* 1998, Grievink *et al.,* 1999; Samet *et al.,* 2001; Trenga *et al*., 2001) than exercising in a non-polluted environment (Peters *et al*., 2001; Nieman *et al*., 2002; Palmer *et al*., 2003). A possible explanation for this fact is that, as mentioned previously, exercise training can be considered an antioxidant. So if an athlete already has an antioxidant system that balances the oxidative process that occurs during exercise, there will not be any positive effects of extra antioxidants intake. Still, if the same athlete were to exercise in a polluted environment, they would have, in addition to the reactive species produced during exercise, more reactive species production stimulated by inhaling the polluted air. In this case, it would be beneficial to increase the availability of antioxidants through supplementation. In addition, the antioxidant supplementation is more efficient

in protecting against oxidative stress if it consists of a mixture of antioxidants. Finally, more investigation is required to analyze how an antioxidant supplementation might benefit athletic performance in a polluted environment.

2.5 SUMMARY

The literature review suggests that an individuals' health is deteriorated with exposure to air pollution. Taking into consideration that the levels of ozone-pollution rises with increase in temperature, it is possible that over the next decades we will see an increase in this pollutant due to the prediction of global warming. This oxidant gas has been shown to decrease lung function and lead to lung inflammation and oxidative stress. Athletes and active individuals who exercise outdoors would be quite a susceptible population to this pollutant because of the increased dose inhaled when performing exercise. Some people have been shown to be more sensitive to ozone, resulting in exacerbated symptoms. It has been suggested that adding heat to the ozone exposure further impairs lung function, performance and increases respiratory symptoms, but how the combined stressors would affect lung inflammation and oxidative stress has not yet been investigated.

The mechanism responsible for the adverse effect of ozone in the lungs, which might possibly also lead to a systemic outcome, would be its oxidative reaction with molecules and cells present in the airways. The presence of a network of antioxidant in the respiratory tract lining fluid helps to balance out the oxidation reactions of ozone and thus, diminishing the oxidative stress. This fact leads to the hypothesis that supplementing an individual with antioxidants might enhance the concentrations of sacrificial molecules in the lungs which would provide a further protection for the adverse effects of ozone pollution. Nevertheless, there is little evidence to fully back up this theory. One of the reasons for that is because most of the studies in the literature have investigated the effects of vitamin supplementation on performance and inflammation of exercising individuals without the addition of ozone-pollution; and, because of the diversity in the methodology used those studies yielded rather inconsistent results.

All in all, there is a lack of literature investigating the effects of ozone pollution and heat on athletes' health and performance. In addition, there is also limited evidence on methods to counteract the adverse effects. As stated in *Chapter* 1 the main aim of this thesis was to specifically address that question and be able to provide data that can add to the current body of knowledge in this area that is still quite novel in the Sport Science realm. Furthermore, it is hoped that this thesis provide some practical insight in improving and maintaining the immune health of athletes and the general population exposed to air pollution. The specific aims of the data chapters comprised in this thesis were as follows:

Chapter 4: To investigate how four different environmental conditions (Control; Control + ozone; Heat and humidity; Heat, humidity and ozone), set to resemble that of large urbanized centres, affect lung function, performance and respiratory symptoms of well-trained male runners taking part in an 8 km time-trial run.

Chapter 5: To examine how four different environmental conditions (Control; Control + ozone; Heat and humidity; Heat, humidity and ozone) affects lung inflammation, lung injury and oxidative stress after well-trained runners performed an 8 km time-trial run. The aim of the second study presented in this chapter was to assess the reliability of the nasal lavage method to assess the upper respiratory tract.

Chapter 6: To examine if a 2-week supplementation period with the antioxidants vitamin C and E would have an impact on performance, oxidative stress and respiratory symptoms of well-trained runners when taking part in an 8 km time-trial run in a hot, humid and ozone-polluted environment.

Chapter 7: To investigate the impact of a 2-week supplementation period with vitamin C and E on the immunoendocrine response, lung injury and lung inflammation of well-trained runners taking part in an 8 km time-trial run in a hot, humid and ozone-polluted

environment.

Chapter 3: General Materials and Methods

3.1 INTRODUCTION

This chapter is divided into five main sections that describe the general methodology used in the studies. Specific methods and participants' characterization is elucidated in detail in the appropriate chapter. Section 3.2 describes the criteria for recruitment of the participants. Section 3.3 details the exercise protocols and the variables accessed during the trials. Section 3.4 outlines the biochemical assays used for analyses of the participants' plasma and nasal lavage fluid. The statistical analysis is described in section 3.5. All chemicals and reagents used were purchased from Sigma-Aldrich, Dorset - UK, unless stated otherwise.

3.2 RECRUITMENT CRITERIA FOR PARTICIPANTS

The same criteria for recruiting participants was used for the two main studies comprised within this thesis. This included selecting participants that were healthy, well trained male runners of a decent competitive standard; this was assured by recruiting participants that achieved a minimum of 60 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$ on a $\dot{V}O_{2\,max}$ test. This test was undertaken during the familiarization trial (see below). The participants were asked to complete a general medical history questionnaire, a specific medical questionnaire and a physical activity questionnaire where they described their weekly training levels (see Appendices I, II and III for these). For the reliability study described in *Chapter 5*, the volunteers had to be non-smoking, healthy and physically active individuals. All participants received a document with information about the study, the benefits and possible risks and discomforts they might experience when taking part in the experimental protocols. In addition, they were fully aware about their right to withdraw from the study at any time without need to justify their reasons to the investigator. All participants signed an informed consent form. Ethical approval was obtained from Edinburgh Napier University, Faculty of Health, Life and Social Sciences ethics committee and the study was conducted in accordance with the Declaration of Helsinki.

3.2.1 Preliminary Measurements

All participants, prior to the data collection, were required to go through a familiarization trial where they performed a $\dot{V}O_{2\,max}$ test (Simpson *et al.*, 2006) on a

treadmill (Woodway, ergo ELG 55, Germany), practiced the lung function test and also the nasal lavage procedure. The $\dot{V}O_{2\,max}$ test was initiated with the participant running at a speed of 10 km·h^{-1} and 0% gradient. The speed of the treadmill was increased by 3 km·h^{-1} every 3 min until achieving a maximum speed of 16 km·h^{-1}. After running 3min, at this speed, the gradient of the treadmill increased 2.5% every minute until the runner reached volitional fatigue. During this test, the participant's oxygen uptake ($\dot{V}O_2$) was measured using online gas analysis (CPX MedGraphics, Oldham, UK). The runner had previously been fitted with a face-mask which was connected to the gas analyser by a sample line. In addition to the $\dot{V}O_2$ being measured, the carbon dioxide production ($\dot{V}CO_2$), the respiratory exchange ratio (RER) and the ventilation rate (V_E) were also measured. The criteria used to establish that the runners had reached their $\dot{V}O_{2\,max}$ was the following: heart rate was approximately 220 beats·min^{-1} minus the runner's age; RER value was greater than 1.10; and VO_2 had reached a plateau (Withers *et al*., 2000). During the familiarisation trial the participants practiced the lung function test and became accustomed to the nasal lavage procedure. Prior to the preliminary measurements, all equipments were carefully calibrated

3.3 EXERCISE PROTOCOL

All participants were asked to refrain from any intense physical activity for 24 h prior to the trial. On the day of the test they were required to drink 500 ml of water 2 hours before arriving at the lab to standardize their hydration state and guarantee that they would initiate the exercise hydrated. In addition, the participants completed a daily health questionnaire (Appendix IV) to ensure they did not present any cold or flu symptoms and that they were in good health to partake in the exercise. They also signed a blood donation form (Appendix V). The exercise consisted of an 8 km time trial run on a treadmill (Woodway, PPS 55 Med, Germany), which the participants were required to complete as fast as they could. As an incentive for the athletes to perform maximally, a cash prize was offered for the participant who completed the trials in the shortest amount of time. The exercise trials were performed in an environmental chamber (Weis-Gallenkamp, UK) where the temperature and humidity could be programmed and controlled. The ozone was generated with a silent generator (Ozone Tech Systems ACT5000) and the concentration in the chamber was continuously monitored by a second device (ATNI Satellite R, Germany) to ensure that the concentration was at the

required level.

Once the exercise protocol commenced, subjective ratings of perceived exertion – RPE – on a scale of 6-20 (Borg, 1998) and heart rate (Polar Electro, Finland) were recorded at the end of each kilometre ran. In addition, the athletes' running speed was recorded every 500 metres – the participants had control of the speed they were running at, however, they did not have access to the value of the speed nor to the total time they took to complete each trial. In addition, a 30 second air sample was collected, at the 7.5 km, in a Douglas Bag (Douglas Bags 100 L, Cranlea, England) to analyze the athletes' minute ventilation and to calculate the O_3 effective dose. For safety reasons, core temperature was measured throughout the trials using a rectal probe (Grant, Cambridge, England).

3.3.1 Assessment of Respiratory Symptoms

The severity of respiratory symptoms was assessed by a written questionnaire (Schelegle & Adams, 1986) after the athletes completed the exercise protocol. The symptoms comprised shortness of breath, cough, excess sputum, throat tickle, raspy throat, wheezing, congestion, pain on deep inspiration, headache, nausea and eye irritation. In addition, the participants also answered if they thought they would be able to compete maximally in a competition if it took part in such an environment (see appendix VI).

3.3.2 Lung Function Tests

All participants received the same verbal instructions each time they performed the lung function test. In a standing position the participants would inhale filling their lungs maximally and would then blow into the mouthpiece of the spirometer (Compact II: Type C, Vitalograph Ltd., UK) as hard and as long as they could until all air was expired. Forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), forced expiratory flow in the middle half of an expiration (FEF_{25-75}) and peak expiratory flow (PEF) were measured. The participants performed the test three times and the best values were recorded.

3.3.3 Blood Sample Collection and Plasma Separation

Samples of whole blood were collected in 6ml vacuum tubes containing EDTA or Sodium heparin as anticoagulant (Becton-Dickinson, Oxford, UK). Whole blood was collected from anticubital vein by venepuncture. The tubes were centrifuged for 10 min at 1000g at room temperature (Mistral 2000R, Sanyo, Leicester, UK). The plasma was removed, aliquoted into eppendorfs and immediately stored at -80°C until further analysis.

3.3.4 Nasal Lavage Procedure

The nasal lavage (NL) procedure was collected according to methods described by (Keman *et al*., 1998). This method consists of having the participants seated with their head tilted backwards during all nasal lavage collection. All participants were instructed in the same way each time they went through this procedure. They were asked to elevate the palate to close the nasopharynx, 4 ml of sterile pre-warmed (37°C) saline was then inserted into one nostril with a 10ml sterile pipette. After 10 seconds, the participant would put his head forward and expire the lavage into a 15ml centrifuge tube via a polyamide gauze-filtered funnel (100 meshes) to separate the mucus. This procedure was repeated with the other nostril. The tube was immediately placed on ice and the total volume collected was recorded. The tube was centrifuged for 10 min at 600g (Mistral 2000R, Sanyo, Leicester, UK) at a temperature of 4°C. The supernatant was aliquoted into eppendorfs (400µl) and immediately stored at -80°C until further analysis.

The remaining cell pallet (200µl) was re-suspended with 100µl of saline. In an eppendorf tube, 25µl of the nasal lavage suspension was mixed with 10µl of trypan blue. From this mixture 20µl was placed into a Neubauer haemocytometer (Assistant, Germany) and the cell count was performed immediately with the microscope set with the x10 lens. On each side of the haemocytometer, four 1 mm^2 squares were counted and then averaged. The amount found was multiplied by 1 x 10^4 to determine the number of cells per ml. From the remaining re-suspended volume, 150µl was used for the cytospin (Cytospin3, Shandon, England), 1000 rpm for 5 min. After this procedure,

the slide was left to dry and then stained with Romanowsky stain (Raymond A. Lamb, London, UK). The cells were counted and differentiated according to their morphological appearance. This procedure was conducted in a blinded manner to eliminate the possibility of examiner bias. The nasal lavage sampling technique and the processing of the samples were performed in a consistent and tightly regulated manner to ensure reliability of the results.

3.4 BIOCHEMICAL ASSAYS

3.4.1 Inflammatory Markers

3.4.1.1 IL-6 and IL-8

Nasal lavage pro-inflammatory cytokines, IL-6 and IL-8, were measured using commercially available enzyme linked immunoabsorbent assay (ELISA) kits (R&D, Abigdon, UK) in accordance with the manufacturer's instructions. The nasal lavage supernatant samples were thawed to room temperature before initiating the experiment. In brief, 96-microwell plates were coated with the specific capture antibody (100µl in PBS), sealed and left overnight at room temperature. The following morning, after the plates were washed twice using the washing buffer, they were coated with the blocking buffer (0.5% BSA, in PBS). The plates were then placed on a microplate shaker for 2 hours. Following this period, the plates were washed again; standards and samples were added to the microwells in duplicate. For IL-6 the standard concentrations varied from 1000 to 15.12 $pg \cdot ml^{-1}$, while for IL-8 they varied from 400 to 6.25 $pg \cdot ml^{-1}$. Once more, the plates were placed on a microplate shaker for 2 hours after which they were washed three times. Then, the detection antibody was added to the microwells (100µl in standard diluent), and the plates were left for another 2 h-period on the microplate shaker. Following this incubation, the plates were washed (x4) and streptavidin-HRP (in standard diluent) was added to each microwell. The plates were then incubated in the dark for 20 minutes, following that, they were washed four times again. Finally, 100 µl of substrate solution was added and another 20 min incubation period in the dark was necessary for the colourimetric reaction. The reaction was terminated by the addition of 50 µl of 1M sulphuric acid to each well. Within 30 minutes, the plates were read on a plate reader (Dynex Magellan Bioscience MRX Revelation, West Sussex, UK) at a wave length of 450 nm. The standard curve was obtained on a log-log fitted line plot

against absorbance. The cytokine concentrations of each sample were obtained from the standard curve.

3.4.1.2 Clara Cell Protein

Nasal lavage and plasma CC16 were measured using an ELISA kit (DiaMed EuroGen, Turnhout, Belgium) in accordance with the manufacturer's instructions. The nasal lavage and plasma samples were thawed to room temperature before initiating the experiment. Due to the high concentration of this protein in the nasal lavage samples, these were diluted 1in100, while plasma samples were diluted 1in 20 with the Sample Diluent. Standards (5 $ng \cdot ml^{-1}$ – 0.1 $ng \cdot ml^{-1}$) and diluted samples (125µl) were pipetted in duplicate in the 96 microwell plate provided by the manufacturer. The plates were then incubated in a moist atmosphere for 2 h at 37°C. After this, the plates were washed 5 times, before adding 125µl of Biotin-anti-CC16 Conjugate. The plates were incubated again, in a moist atmosphere, for 1 h at 37°C. The washing process was repeated and 125µl of Streptavidin-HRP Conjugate was added. Once more the plates were incubated in a moist atmosphere, at 37°C, this time for a 20 min period, after which the washing process was repeated. Chromogen Solution (100µl) was then added before incubating it at room temperature for 20 min. Following this, 50µl of Stopping solution was added to each microwell and the absorbance read with a 450 nm wavelength (Dynex MRX II, West Sussex, UK). The standard curve was obtained on a log/linear fitted line plot against absorbance. The CC16 concentrations of each sample were obtained from the standard curve and the average value of the duplicate used.

3.4.2 Oxidative stress markers

3.4.2.1 GSH/Protein

The method used to assess the nasal lavage and plasma GSH was adapted from Hissin and Hilf (1976) and is described as follows: after samples had been thawed, 250µl of each sample was placed into an eppendorf and 500µl of the Extraction buffer (9mM tetra sodium EDTA in 14% perchloric acid, PBS was added just prior to use, pH 7.4) was added. Samples were left for 15min on ice and then centrifuged at 5000rpm for 5min at 4ºC (Mistral 2000R, Sanyo, Leicester, UK). The supernatant was transferred to

flow falcon tubes and 500µl of Neutralisation buffer (1M potassium hydroxide and 1M potassium bicarbonate in distilled water) was added before centrifugation at 5000rpm for 5min at 4ºC. The supernatant was separated and 10µl of each sample was added in duplicate in a 96 microwell plate. GSH standards were made up using GSH buffer (1M sodium dehydrogen orthophosphate and 0.005M tetra sodium EDTA in distilled water, pH 8). For the plasma assay, the standard curve varied from GSH 50µM to 3.125µM; whereas, for the nasal lavage assay, the standard curve varied from GSH 12.5µM to 0.7813µM. GSH buffer (180µl) was added into each well as was 10µl of OPT (o-phthaldehyde in methanol). The plate was read using a fluorescence microplate reader (FLUOstar Optima, BMG Labtech) at 350 nm excitation and 420 nm emission wave length. The GSH concentrations of each sample were obtained from the standard curve and the average value of the duplicate used.

Total protein was measured using the BioRad protein assay which quantifies colourimetrically the protein concentrations using the dye Coomassie Brilliant Blue G-250. BioRad stock reagent was diluted 1 in 5 with distilled water and filter through a paper filter. BSA, diluted in distilled water, was used for the standard curve which varied from 2 to 0.03 $mg \cdot ml^{-1}$ for the plasma samples and 1 to 0.03 $mg \cdot ml^{-1}$ for the nasal lavage samples. The plasma samples were diluted 1 in 100 with distilled water. Standards and samples were added in duplicate into 96-well plates. The BioRad, 200µl, was then added to the wells. The absorbance values were measured using a plate reader (Dynex Magellan Bioscience MRX Revelation) at a wavelength of 595 nm. The total protein concentrations of each sample were obtained from the standard curve and the average value of the duplicate used.

3.4.2.2. Trolox

The trolox equivalent antioxidant capacity of plasma and nasal lavage samples were measured by a colourimetric assay (Cayman Chemical Company, Boldon, UK) that compares the capacity of the antioxidants in the sample to prevent 2,2-azinobis 3-ethylbenzothiazoline-6sulfonic acid (ABTS) oxidation with that of a water-soluble tocopherol analogue, Trolox. The result is quantified as milimolar trolox equivalents. Firstly, 10µl the Trolox standards and samples were pipetted in duplicate into the microwells of a 96 well plate. Before initiating the experiment, the plasma samples were

diluted 1 in 20 with the Assay buffer. The standard curve varied from 0.33 to 0.044 mM Trolox. Metmyoglobin (10µl) and chromogen (150µl) were added into each well. The reaction was then initiated by the addition of hydrogen peroxide working solution (40µl) and the covered plates were left to incubate, on a shaker, for 5 min at room temperature. The absorbance was then read at 750 nm (Dynex MRX II, West Sussex, UK). The total antioxidant capacity of each sample were obtained from the standard curve and the average value of the duplicate used.

3.5 STATISTICAL ANALYSIS

All statistical analysis was conducted using Minitab15 Statistical Software. Specific statistical tests used to analyse the data within each chapter is provided in the respective methods section. All data were checked for normality before statistical analysis and if necessary normalised. The majority of the results are represented as mean values and standard deviation (SD), unless stated otherwise. Statistical significance was accepted at $P < 0.05$.

Chapter 4: Investigating Performance and Lung Function in a Hot, Humid and Ozone Polluted Environment

4.1 INTRODUCTION

This chapter will describe and discuss some of the results obtained in the first study of this thesis. In this study, trained individuals took part in a time-trial run in different environmental conditions. This chapter presents the results related to performance, lung function and respiratory symptoms, while *Chapter 5* analyses the results related to oxidative stress and lung inflammation of these individuals.

Ozone and Lung Function

Several studies evaluating the effects of ozone exposure on healthy subjects have shown airway inflammation and also deterioration of lung function not only at low and moderate exercise levels, but also in athletes performing high intensity exercise (Adams & Schelegle, 1983; Devlin *et al*., 1996; Horstman *et al*., 1990; McDonnell *et al*., 1991). As detailed in *Chapter 2*, the methods used in these studies vary considerably, whether in relation to the different settings - mouth-breathing face mask, chamber and field studies – or to the large range in ozone concentration (0.07-0.4 ppm), most of which are not environmentally relevant. The diversity in the exercise protocols employed and fitness level of the participants are other factors, which together contribute to the different outcomes reported. Most studies investigating the effect of ozone on lung function have shown impairment in this function after exposure, but this is not a unanimous conclusion. For example, Holz *et al*. (1999) reported no change in lung function of neither healthy nor asthmatic individuals following exposure to 0.12 ppm of ozone. The exposure consisted of a 3 h duration period with an intermittent cycling exercise divided by 15 min of rest and 15 min of a low intensity cycling exercise eliciting a minute ventilation of 14 l/(min·m^2). A similar result was reported by Krishna *et al*. (1997) when individuals were exposed to the same ozone concentration (0.12 ppm), but to a 2 h period. A small but significant impairment in lung function was reported by Brunekreef *et al*. (1994) in a field study conducted with amateur cyclists. As described in *Chapter 2*, the study of Brunekreef and colleagues (1994) took place throughout the summer, the mean temperature was a temperate 17.9 degrees and the ozone levels varied between 0.04 and 0.1 ppm. The cyclists exercised for an average duration of 75 min on each occasion and the authors reported a significant relationship between ozone exposure and lung function decrements, which means that the higher the ozone values the greater the lung function decrements observed. Horstman *et al.* (1990) and McDonnell *et al.* (1991) reported a significant impairment in lung function after

participants were exposed to ozone during a long period of time (6.6 h) performing light intermittent exercise. They analyzed different concentrations of ozone: 0.00, 0.08, 0.10 and 0.12 ppm. Significant reductions in FEV_1 were observed after 3 h in the exposure period when the ozone concentration was at its highest (0.12 ppm); after 4.6 h of exposure at 0.10 ppm of ozone, and after 5.6 h of exposure when the concentration was at the lowest value 0.08 ppm.

Impairment in lung function has also been reported with higher ozone concentration and shorter exposure durations. Blomberg *et al.* (1999) reported a depressed response of FEV_1, FVC and FEF_{25-75}, following a 2 h exposure of 0.2 ppm ozone. In this study, subjects performed intermittent cycling exercise at low intensity 20 l/(min·m^2). As for the use of a high intensity exercise protocol, Schelegle and Adams (1986), using a 1 h competitive cycling simulation protocol, evaluated the response of ten endurance athletes exposed to different ozone concentrations – 0.12, 0.18, and 0.24 ppm - while performing. The authors reported a significant relationship between impairment of performance and ozone concentration: one, five and seven subjects did not complete the 0.12, 0.18 and 0.24 ppm O_3 protocol respectively. In addition, a significant increase in the number of reported respiratory symptoms and decreases in FEV_1 and FVC were observed following the 0.18 ppm O_3 exposure. As described, most studies have used a high ozone dose or a high exercise protocol, or the combination of both. Nevertheless, when analyzing the pertinent scientific literature, it is essential to take into not only the ozone concentration, but also the amount that is inspired and the exposure duration. This would represent the total effective dose of ozone (ED). This index has been related to both decreases in pulmonary function and increases in airway neutrophilia. ED is defined as the product of O_3 concentration, average inspired ventilation, and duration of exposure (Gibbons & Adams, 1984; Gong *et al*., 1986; Mudway & Kelly, 2004). For a summary of the studies mentioned above see Table 4.1.

The decrease in lung function occurring immediately after exposure, as has been reported in various studies, has been explained by different mechanisms. One of them is by an airway narrowing process mediated by parasympathetic stimulation (Becket *et al*., 1985; Blomberg *et al*., 1999; Holz *et al*., 1999). Another possible explanation is by the exacerbation of respiratory symptoms, such as pain on deep inspiration, which would lead to a decrease in maximal inspiration volume, affecting the lung function as a

consequence (Passannante *et al*., 1998). This would occur via neural stimulation of sensory fibers present in the lungs (Hazucha *et al*., 1989). In more reactive individuals, the ozone could activate "irritant" receptors leading to changes in respiratory muscle force or mechanic properties of the lungs due to contraction of alveolar smooth muscles (Hazucha *et al*., 1989). Endurance exercise alone has also been shown to alter the lung function by means of affecting the airway smooth muscles due to hyperventilation (Scichilone *et al*., 2005). It has been reported that the lung function has returned to baseline values as quick as 2 h after the termination of the exercise protocol (Blomberg *et al*., 2003), showing that the changes which occur are temporary. In addition, multiple exposures to ozone have been shown to elicit an adapted response that would depend both on intensity of the exposures and on individual sensitivity to ozone (Jörres *et al*., 2000; Frank *et al*., 2001). Yet, the exact mechanism leading to this adaptation is still unclear.

Table 4.1. Summary of studies evaluating the effect of ozone exposure on lung function.

Study	Subjects	Exercise and ozone levels	Results
Adams & Schelegle, 1983	Male endurance runners	1 h training or competition simulation 0; 0.2; 0.35 ppm	↓ Lung function at 0.2 and 0.35 ppm ↑ Respiratory symptoms with higher O_3 concentration, impairment in performance
Schelegle & Adams, 1986	Male endurance cyclists	1 h competitive cycling simulation protocol 0; 0.12; 0.18; and 0.24ppm	↓ Lung function following 0.18 and 0.24ppm ↑ Respiratory symptoms with higher O_3 concentration, impairment in performance
Horstman *et al.*, 1990; McDonnell *et al.*, 1991	Healthy male individuals	6.6 h light intermittent exercise 0.08; 0.10 and 0.12ppm	↓ Lung function after 3h at 0.12 ppm ↓ Lung function after 4.6h at 0.10 ppm ↓ Lung function after 5.6 h at 0.08 ppm ↑ Respiratory symptoms with higher O_3
Brunekreef *et al*., 1994	Field study, amateur male cyclists	Training: 75 min Mean 17.9 ºC O_3: 0.04 – 0.1 ppm	Small ↓ Lung function Relationship between O_3 exposure and degree of lung function impairement
Krishna *et al*., 1997	*Healthy individuals	2 h intermittent cycling 0.12 ppm	No effect on lung function
Blomberg *et al.*, 1999	*Healthy individuals	2 h intermittent cycling 0.2 ppm	↓ Lung function following

Holz *et al*., 1999	*Mild asthmatics + nonasthmatics	3 h intermittent cycling 0.12 ppm	No effect on lung function

*Non-smokers, male and female, the study does not report their physical fitness level.

Heat, Ozone-Pollution and Performance

As mentioned at the outset, large urbanized areas have a polluted environment and can also reach high temperatures and humidity levels. It has been well documented that heat (González-Alonso *et al*., 1999; Parkin *et al*., 1999; Nybo & Nielsen, 2001) and heat and humidity (Galloway & Maughan, 1997; Nielsen *et al*., 1997) can have a significant detrimental effect on exercise performance. This is true for exercises that depend on muscle force production (Nybo & Nielsen, 2001), submaximal, maximal and supramaximal $VO_{2\ max}$ intensity (Galloway & Maughan, 1997; González-Alonso *et al*., 1999; 2008; Parkin *et al*., 1999) as well as events with different durations, i.e. marathon races (Ely *et al*., 2007), middle distance cycling and running events (Peiser & Reilly, 2004; González-Alonso *et al*., 2008). Performance impairment in a hot environment is caused by a multifactorial system. An increased demand for skin blood flow, in an attempt to dissipate the extra heat and the metabolic demands of the contracting muscles to increase local blood flow, especially when the exercise recruits large muscle groups, such as running, will result in a compromised skin circulation leading to an increase in body temperature. The high level temperatures of the body and the central nervous structures, specifically the brain, combined with cardiovascular strain from the exercise limits the individual's ability to continue the exercise. However, a variety of factors play a part in influencing the fatigue: body temperature before the exercise; dehydration; thermoregulatory capacity; fitness level and exercise duration are just a couple of examples. The thermoregulatory capacity of the individual will be affected in part by the humidity of the environment. Indeed, it has also been shown that when heat is combined with high levels of humidity it results in a higher stress to the body due to the difficulty in dissipated the heat through the evaporation process impairing performance to a higher degree (Nielsen *et al*., 1997).

As ozone-pollution occurs with high temperatures it is relevant to study these two variables together. The addition of humidity is also important because in lots of regions the summer time is hot and humid, which adds another stressor to exercising

individuals. Nevertheless, there is very little research evaluating the combined effect of pollution with heat and humidity. Two important studies have investigated the impact of both heat and ozone, but not the addition of high humidity levels, on lung function and exercise performance (Gibbons & Adams, 1984; Gong *et al.*, 1986). One of these studies was conducted by Gibbons and Adams (1984). The authors came to the conclusion that the combined effects of an ozone polluted environment with heat produces a greater negative impact on performance than ozone and exercise alone. In their study, 10 female participants underwent 6 randomized protocols at different temperatures, 24 and 35 °C, and at different ozone concentrations: filtered air, 0.15, and 0.30 ppm. The exercise regime consisted of cycling for 1 h, eliciting a minute ventilation of approximately 55 $l \cdot min^{-1}$. All participants were able to complete the trials in the filtered air condition irrespective of the ambient temperature. Nonetheless, three women stopped cycling prematurely when exposed to ozone (0.30 ppm) in the hot environment (35°C); one could not complete the trial in the moderate heat (24 °C) with the same ozone concentration; and one stopped the trial prematurely in the moderate heat with the lower ozone concentration (0.15 ppm). In addition, at all ozone concentrations tested, the volunteers reported a higher respiratory discomfort.

A subsequent study by Gong *et al.* (1986) also reached similar conclusion. In this study competitive cyclists exercised for 1 h at 70% of their VO_2 max followed by an incremental test to exhaustion on a cycle ergometer. In addition to ozone (0.12 and 0.20 ppm), the subjects were exposed to a high temperature of 31 ºC, while the humidity was low (37%). The authors reported a decrease in FVC and FEV_1 at 0.12 that intensified at 0.20 ppm O_3. Impairment in the maximal exercise performance was also observed for the higher O_3 concentration. Albeit the lack of explanatory mechanisms underlining this outcome, the stress in certain physiological variables and subjective limitations is clearly possible when ambient heat is associated with photochemical ozone during exercise performance.

At present there is still a need to investigate the effect of ozone-pollution, at an environmentally relevant burden, on lung function and performance of exercising individuals. The addition of heat and humidity is of essence, seeing as during the summer months there is an increase in the number of people that practice outdoor

sports. In addition, important sports events also take place during the summer and therefore athletes are exposed to these potentially harmful environmental stressors. The current study investigates how different environmental conditions, set to resemble that of large urbanized centres, affect lung function, performance and respiratory symptoms of well trained male runners taking part in a time-trial run. Aspects of the immune system and oxidative stress were also investigated, but these will be explored in *Chapter 5*. Even though there are various studies that have separately investigated similar aspects of this study, the novelty of the current research is that these variables are investigated together for the first time. In addition, this study has a crossover randomized design that allows for the analysis of different environmental conditions with each participant acting as their own control. Part of this study was done in association with the British Olympic Medical Institute and, therefore, the choice of using well-trained individuals as the participants was made so as to allow the extrapolation of these results to the competitive population. It was hypothesized that performance, lung function and respiratory symptoms would be affected by the environment that contained just ozone pollution and that the addition of heat and humidity would have a further impact on these variables.

4.2 METHODS

Detailed descriptions of the methods are elucidated in *Chapter 3*. Any methodology that was used specifically in this study will be fully described in this section, while the methods previously described will be summarized. The specific methods and participants used in this study are identical to those used in *Chapter 5*.

4.2.1 Participants

Ten male well trained runners took part in this study (mean ± SD = 24 ± 6 years; 72.9 ± 7.1 kg; 180.8 ± 8.5 cm; 64.4 ± 4.4 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$). The participants were all competitive runners who had to achieve a criterion $\dot{V}O_{2\,max}$ minimum value of 60 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$. The adequacy of each subject's $\dot{V}O_{2\,max}$ was tested during the familiarization trial (see section 3.2.1 for further details).

4.2.2 Experimental Procedures

After the familiarization trial, the athletes completed four trials with the same exercise protocol, each in a different environmental condition, and separated by at least 7 to a maximum of 10 days. When the participants arrived in the lab they completed a health questionnaire to assess if they were in good health to take part in the exercise trial (appendix IV). If they presented signs of cold or flu they did not take part in the exercise and were asked to return 2 weeks after they had recovered. Before the exercise the participants performed the lung function test and values of FVC, FEV_1, FEF_{25-75} and PEF were measured. The exercise trials took place in an environmental chamber (details are given in the section 3.2). The exercise consisted of an 8 km time trial run on a treadmill (Woodway, PPS 55 Med, Germany), which the participants were required to complete as fast as they could. The runners controlled their own speed, but they did not have access to the value of the speed. The environmental conditions were Control: filtered air at 20 ºC + 50% relative humidity (rh); Control + O_3: 0.1 ppm O_3 at 20 ºC and 50% rh; Heat: filtered air at 31 ºC and 70% rh; Heat + O_3: 0.1ppm O_3 at 31 ºC and 70% rh. Immediately after the run the participants completed a respiratory symptom questionnaire (appendix VI), had blood samples taken, performed the lung function test again and then went through the nasal lavage procedure (see Figure 4.1). The trials were single blinded and performed with a crossover randomized design with each subject acting as their own control. The tests were carried out at the same time of the day for each athlete, to avoid any diurnal variation of the analyzed variables. In addition, the runners were asked to refrain from any physical activity 24 h before the test.

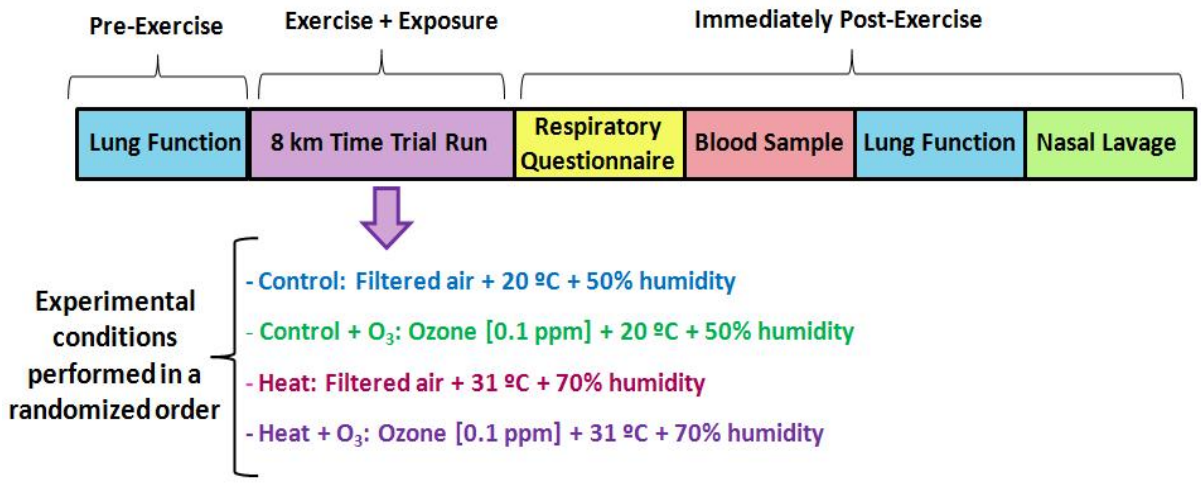


Figure 4.1. Single-blinded randomized crossover design.

Once the exercise protocol commenced, subjective ratings of perceived exertion (RPE) (Borg, 1998), heart rate (Polar Electro, Finland) and the athletes' running speed were continuously recorded. In addition, a 30 seconds air sample was collected, at 7.5 km, in a Douglas Bag (Douglas Bags 100 L, Cranlea, England) to analyze the athlete's minute ventilation in order to calculate the ED of ozone. The athletes had access to water *ad libitum* during the trial.

Statistical Analysis

All data were checked for normality and homogeneity of variance before statistical analysis. Results are represented as mean values ± standard deviation (SD). Data were analyzed using General Linear Model ANOVA (Minitab15 Statistical Software) with Tukey's Post-hoc test. Statistical significance was accepted at $P < 0.05$. Correlations were analyzed using the Linear Regression test (Minitab15 Statistical Software).

4.3 RESULTS

4.3.1 Medical Questionnaires

After analyzing the participants' medical questionnaires it was seen that none of the participants presented asthma. Two out of the ten runners presented hay fever as an allergic problem, however as this study was conducted during the autumn and winter period this did not interfere with the participants' health.

4.3.2 Temperature, Humidity and Ozone Levels

The average temperature, humidity and ozone levels for each of the trials were recorded throughout the exposures. They maintained very stable, values are presented in Table 4.2.

Table 4.2. Average values for temperature, humidity and ozone levels in each trial

	Control	Control + O_3	Heat	Heat + O_3
Temperature (°C)	20.0 ± 0.5	20.0 ± 0.5	31.0 ± 0.5	31.0 ± 0.3
Humidity (%)	50.5 ± 1.2	50.9 ± 1.0	69.6 ± 1.0	70.0 ± 0.8
Ozone (ppm)	0.0	0.10 ± 0.02	0.0	0.10 ± 0.02

Values are mean ± SD.

4.3.2 Performance Variables

The results for total time to complete each trial are shown in Figure 4.2. Both the Heat and the Heat + O_3 trials resulted in a significant increase in time required to complete the 8 km trial compared to either the Control or the Control + O_3 trials (P<0.001). Ozone alone did not increase the time required to run the 8 km compared to the Control trial.

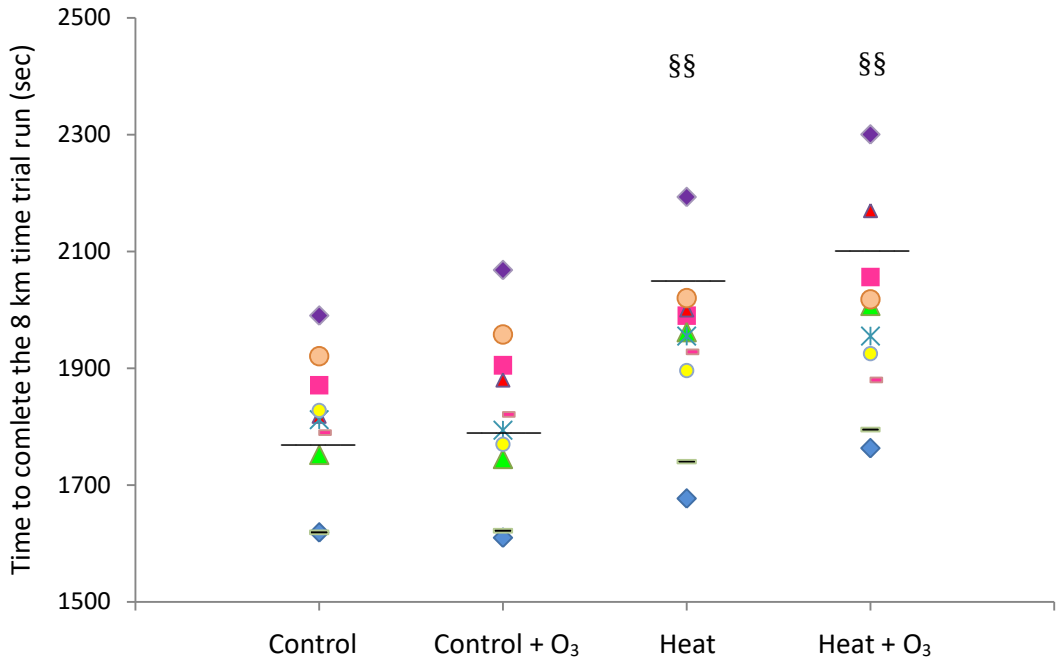


Figure 4.2. Individual time to complete each trial. Mean value for each trial is represented by —. §§ Significantly different from Control and Control + O_3 trial. P < 0.001.

The 95% Confidence Intervals (lower; upper limits) for the time to complete the trials

were:

- Control Trial subtracted from:

Control + O_3 Trial (-45.1; 69.7) Heat Trial (73.9; 188.7) Heat + O_3 Trial (116.5; 231.3)

- Control + O_3 Trial subtracted from:

Heat Trial (61.6; 176.4) Heat + O_3 Trial (104.2; 219.0)

- Heat Trial subtracted from:

Heat + O_3 Trial (-14.8; 100.0)

Results for heart rate, RPE, speed, expired air and $\dot{V}O_2$ for each of the trials are presented in Table 4.3. The Control trial and the Control + O_3 trial presented a significantly higher speed when compared to both the Heat and Heat + O_3 trial (P<0.001). There was no significant difference between the average speed in the Control + O_3 compared with the Control trial. There were no significant differences between the trials for the runners' mean heart rate or the peak heart rate. The Heat and Heat + O_3 trials presented a significant higher peak RPE when compared to the other two trials (P<0.01). At 7.5 kms the average expired air volume for all trials was 120 $l \cdot min^{-1}$. The subjects presented an average decrease of 28 l of expired air at the end of the Heat + O_3 trial when compared to the Control trial, this value was significantly different (P<0.05). The $\dot{V}O_2$ that the subjects were working at during the end of the run (km 7.5) was significantly lower for the Heat and the Heat + O_3 trial when compared to the control trial (P<0.05). There was no significant difference between the Heat and the Heat + O_3 trial for the $\dot{V}O_2$ capacity. Moreover, the difference for this variable between Heat + O_3 and the Control + O_3 trial was borderline significant, with P = 0.056.

Table 4.3. Effect of exercise trial on heart rate, RPE, speed, expired air and oxygen consumption.

	Control	**Control + O_3**	**Heat**	**Heat + O_3**
Average Speed ($km \cdot h^{-1}$)	16.1 ± 0.9	15.9 ± 1.6	14.9 ± 1.2†	14.6 ± 1.2†
Mean Heart Rate ($beats \cdot min^{-1}$)	168 ± 3	170 ± 6	172 ± 3	170 ± 6
Peak Heart Rate ($beats \cdot min^{-1}$)	187 ± 6	190 ± 6	192 ± 3	190 ± 6
Mean RPE	14 ± 0.9	15 ± 0.9	15 ± 0.9	16 ± 0.9*

Peak RPE	17 ± 0.9	17 ± 0.9	18 ± 0.9	19 ± 0.9†
Expired Volume ($l \cdot min^{-1}$)	134 ± 16.7	124 ± 21.1	109 ± 17.4	106 ± 19.2*
$\dot{V}O_2$ consumption ($mlO_2.kg^{-1}.min^{-1}$)	58.1 ± 4.3	56.4 ± 7.4	49.4 ± 7.4*	48.4 ± 5.3*§
O_3 Effective Dose (ED)	0	383.9 ± 86.8	0	366.5 ± 68.5

* Significantly different from Control Trial, $P < 0.05$. † Significantly different from both Control and Control + O_3 trial, $P < 0.01$. § Borderline significance, $P = 0.056$ compared with Control + O_3. Values are mean ± SD.

4.3.3 Respiratory Symptoms Response

An overview of the respiratory symptoms reported by the subjects in response to the trials is given in Figure 4.3. In the Heat trial there is a tendency to increase the severity of respiratory symptoms experienced by the athletes. This increase becomes even more pronounced in the symptoms reported for the Heat + O_3 trial, but it did not reach statistical significance. In response to the question of whether they felt that they would be able to perform maximally in a competition in the environmental conditions tested, all ten runners reported they would not be able to perform maximally in the Heat + O_3 trial.

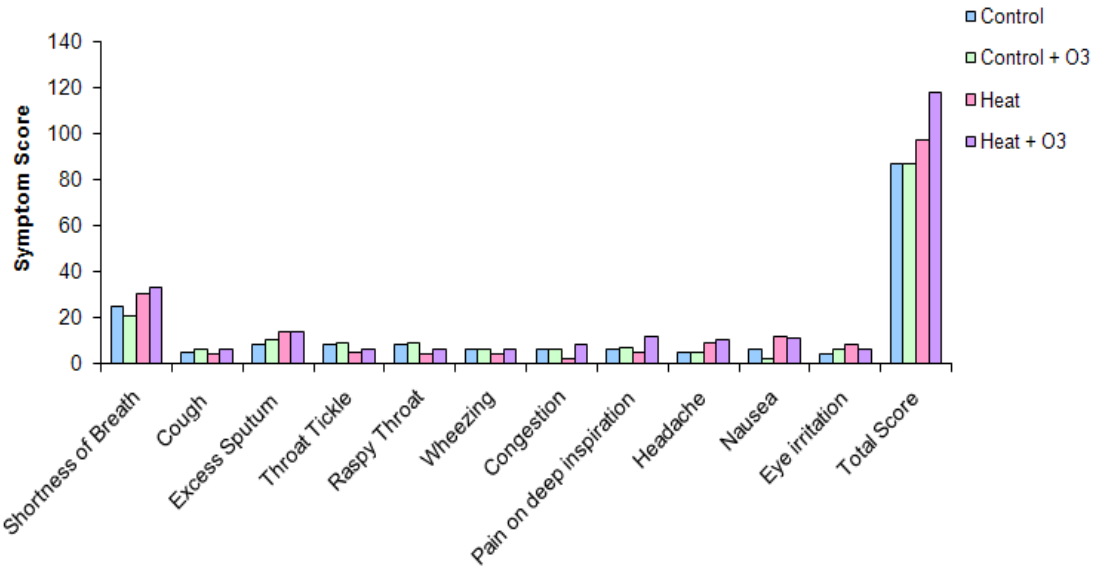


Figure 4.3. Respiratory symptoms presented by the subjects in all 4 trials.

4.3.4 Lung Function

Changes in lung functions pre and post run were compared between trials. No significant differences were found for any of the analyzed variables (FVC, FEV_1, FEF_{25-75} and PEF) within the same trial or between trials (Table 4.4). In addition, when the runners' pre and post FEV_1 was analyzed it was seen that none of them presented exercise induced asthma, which is characterized by 10% or more decrease in this variable (Rundell & Jenkinson, 2002).

Table 4.4. Lung function measures in all four trials.

	Control	**Control + O_3**	**Heat**	**Heat + O_3**
Pre FVC (l)	5.0 ± 0.9	5.2 ± 0.6	5.1 ± 0.9	5.1 ± 0.9
Post FVC (l)	5.2 ± 0.9	5.1 ± 0.9	5.2 ± 0.9	4.9 ± 0.9
Pre FEV_1 (l)	4.4 ± 0.6	4.6 ± 0.9	4.6 ± 0.6	4.5 ± 0.6
Post FEV_1 (l)	4.5 ± 0.6	4.4 ± 0.6	4.7 ± 0.6	4.4 ± 0.9
Pre PEF ($l \cdot min^{-1}$)	611 ± 65	610 ± 66	616 ± 74	607 ± 63
Post PEF ($l \cdot min^{-1}$)	599 ± 66	594 ± 56	621 ± 71	585 ± 53
Pre FEF_{25-75}	4.7 ± 1.2	4.9 ± 0.9	5.1 ± 1.2	4.8 ± 0.6
Post FEF_{25-75}	4.9 ± 0.9	4.7 ± 0.9	5.4 ± 0.9	5.1 ± 0.6

Values are mean ± SD.

4.3.5 Ozone Effective Dose (ED)

The relationship between the ED inhaled by the participants and the average speed developed in both ozone trials is presented in Figure 4.4A. There was a significant negative correlation between these two variable (R=-0.27, P<0.05). When the post-exercise lung function parameters were analyzed a significant positive correlation (R=0.34, P<0.05) was also established for the FVC (Figure 4.4B).

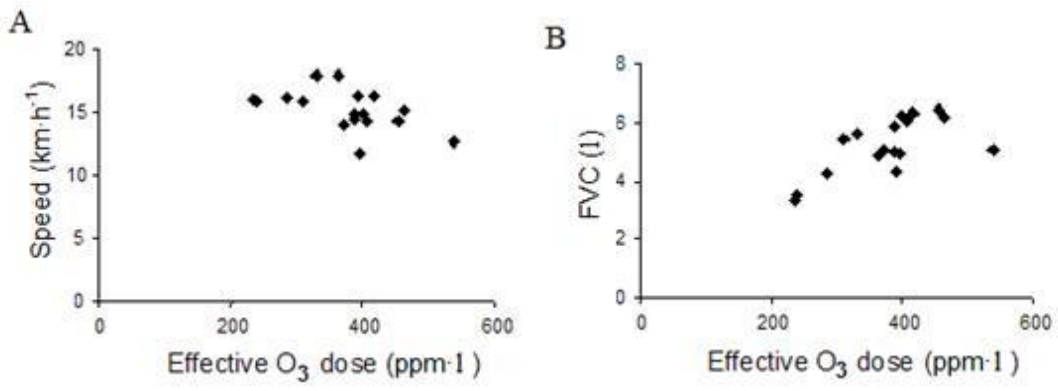


Figure 4.4. Relationship between the ED and the athletes' average speed (A), and FVC (B) during the ozone trials.

4.4 DISCUSSION

This study evaluated the effect of four different environmental conditions in lung function, performance and respiratory symptoms of trained runners performing an 8 km time-trial run. The environmental conditions were: (1) Control: filtered air at 20 ºC + 50% relative humidity (rh); (2) Control + O_3: 0.1 ppm O_3 at 20 ºC and 50% rh; (3) Heat: filtered air at 31 ºC and 70% rh; (4) Heat + O_3: 0.1ppm O_3 at 31 ºC and 70% rh. These conditions were successfully achieved. The main findings described in this chapter were the following. That the environmental conditions did not affect the runners' lung function post-exercise. There was a significant detriment in performance in both Heat and Heat + O_3 trial compared to Control and Control + O_3. Plus, there was a trend towards increased severity in reported respiratory symptoms by the participants after the run in the Heat + O_3 trial.

The first important finding of the present study was that the average speed attained by the runners during the Heat trial (14.9 $km \cdot h^{-1}$) and during the Heat + O_3 trial (14.6 $km \cdot h^{-1}$) was significantly lower when compared to the Control trial (16.1 $km \cdot h^{-1}$). Consequently, this impacted the total time required to complete the 8 km run in both the Heat (32 min 35 sec) and the Heat + O_3 (33 min 09 sec) trials. This drop in performance occurred with all the runners. In relation to the Control trial (30 min 15 sec), the athletes had an average significant increase of 2 min 54 sec in their total time to complete the Heat + O_3 trial. When the performance in the Heat and the Heat + O_3 trials are

compared, the time to complete the Heat + O_3 trial was on average 34 seconds longer than the time to complete the Heat trial. Such difference did not reach statistical significance, but is equivalent to a 1.7% increase in the time to complete the trial with the addition of ozone. When analyzing the individual values, 8 out of 10 runners performed worse in the Heat + O_3 trial when compared to the Heat trial. Contrary to what had been hypothesized ozone alone had no significant impact on either average speed or on total time to complete the trial as a consequence. On average the athletes took an additional 12 seconds, equivalent to a 0.7% increase, to complete the Control + O_3 trial (30 min 27 sec) compared to the Control.

It could be hypothesized that the addition of ozone to the heat and humidity results in a higher strain being placed on the athletes than when the ozone is added in a thermoneutral environment, but this study was not able to fully support this hypothesis. Hopkins and Hewson (2001) demonstrated that, in short duration running events an athlete's performance does not have a large variation between competitions, therefore there is a low coefficient of variation between races. Half marathons and marathons would possibly present a higher variation due to less familiarity of the athletes in competing these distances. Analysing the variation in performance outcome of athletes in endurance running events, the authors suggest that an improvement of 1% for half and full marathons, and 0.5% for shorter endurance events would significantly increase the chances of an athlete obtaining a medal. It is with caution that those results are extrapolated for the present study, but the runners in the present study were all current competitors at a club or national level. In addition, the percentage improvement in the runners' performance time in both environments without ozone is higher than the percentage improvement reported by Hopkins and Hewson (2001) to be medal relevant. Thus, it can be suggested that the ozone had an impact in performance that might be ecologically significant. Moreover, when heat and humidity was added to the ozone, this percentage increased a further 1%. This suggests that heat might exacerbate the toxic effect of ozone in the lungs, resulting in a pronounced increase in the oxidative stress and inflammation process in the lungs. These would in turn increase the runners' perception of discomfort and respiratory symptoms leading to a decrease in the running speed as a protective mechanism. In fact it could be seen that in the Heat + O_3 trial the volume of expired air was significantly reduced compared with the Control. The reduction in ventilatory exchange could be due to respiratory discomfort experienced by

the athletes which might have impaired their maximum effort. The fact that the mean and peak heart rate did not differ between trials suggests that the cardiovascular strain was similar in all four conditions. A total of 10 athletes were used for this study. A power calculation suggests that an n value of 25 would be required to achieve statistical significance.

Other studies have shown a significant impairment of exercise performance when heat is combined with ozone (Gibbons & Adams, 1984; Gong *et al*., 1986). The investigators in the current study used humidity in addition to heat and a lower concentration of ozone, which is more representative of the levels encountered in large urbanized areas. It is very challenging to compare most of the available literature to the results of the present study because of the different exercise protocols, ventilation rates elicited and participants assessed in the studies. In this research the runners were well trained and had a high aerobic capacity (mean value of 64.4 $mlO_2.kg^{-1}.min^{-1}$). As a result, during the exercise trial, they were able to achieve a high minute ventilation and, consequently, inhale more air and more ozone per minute than if non-athletes were exposed for the same exercise duration. In fact, the ventilatory rates (mean value of 120 $l \cdot min^{-1}$) and work intensity elicited by the 8 km time trial run is much higher than that reported in the literature studied to date (Christian *et al*., 1998; Jörres *et al*., 2000; Krishna *et al*., 1998; Ratto *et al*., 2006). Nevertheless, it is important to point out that the motivation and training status of the participants that took part in the present study may be different to those of athletes who are actually in a competitive event. Perhaps most importantly, in big cities, there are a variety of pollutants, such as sulphur dioxide (SO_2), particulate matter (PM_{10}), nitrogen oxides (NO_x) in addition to ozone (Baldasano *et al*., 2003) and it is the combined effect of this cocktail of pollutants that could affect the athlete adversely. It is practically and ethically very difficult to replicate this type of pollution environment and hence this is recognised as one of the limitations of studies conducted in laboratory setting. However, the mechanism of oxidative stress produced by using ozone is similar to that which may occur with a cocktail of pollutants and it is, thus, thought that using ozone alone is an appropriate model to gauge the effect that ambient pollution has on performance.

The decrease in performance, observed in both the Heat and Heat + O_3 trial, can be

explained by the high temperature and humidity of these trials in which we would expect a large stress on the athletes' thermoregulatory system (González-Alonso *et al*., 2008). It has also been shown that a decrease in exercise performance in the heat could be associated with a temperature-induced metabolic perturbation, decrease in neuromuscular activation and stress on the cardiovascular system (González-Alonso *et al*., 2008; Morrison *et al*., 2006; Parkin *et al*., 1999). Physiological variables specific to thermoregulatory mechanisms (i.e. skin temperature, sweat rate) were not measured, as it was not the main aim of this study.

Regarding the mean subjective post-exercise symptom responses, an increase in the overall respiratory symptoms was observed in the Heat trial, with an exacerbation of the symptoms with the further addition of ozone. Some athletes reported feeling additional symptoms such as chest tightness and dizziness after completing the Heat + O_3 trial. Once more, this suggests the differences in individual susceptibility to this pollutant (Passannante *et al*., 1998; Holz *et al*., 1999; Blomberg *et al*., 1999). All runners following the Heat + O_3 trial reported that they would not be able to perform maximally in a competition in such an environment. The increase in subjective discomfort can also be seen when the RPE is analyzed. It has been shown that exercise in the heat can act directly on the central nervous system of the individual reducing motivation and increasing perceived exertion (Nybo & Nielsen, 2001). Nevertheless, the mean RPE was significantly higher only in the trial in which ozone was combined with heat and humidity compared to the Control trial. Even though these values are not very different, the statistical test takes into account the interval of the values in each trial. Thus, the runners were feeling more exhausted, but developed a lower speed, and were working at a significantly lower $\dot{V}O_2$ capacity. The same is true for the peak RPE presented by the runners. Therefore, another explanation for the decrease in performance in the Heat + O_3 trials is related to the athletes' subjective feeling of exhaustion and respiratory symptoms. It could be speculated that the combination of heat and ozone during exercise performance would have an overwhelming effect on the organism - influencing not only performance, but also enhancing subjective limitations.

The lack of changes in the lung function parameters is in accordance with a previous study that used a higher ozone concentration of 0.12 ppm (Holz *et al*., 1999), although they are in contrast with a variety of other studies (Brunekreef *et al*., 1994; Gong *et al*., 1986, Jörres *et al*., 2000; Schelegle & Adams, 1986). It is relevant, however to

highlight that all of those studies used a different exposure protocol to the one utilized in the present study. The O_3 concentration used in this study was environmentally relevant compared to the outdoor concentration (Baldasano *et al.*, 2003). Usually the decrease in lung function is reported after subjects are exposed to higher levels of O_3, such as 0.12 ppm; 0.2 ppm and 0.24 ppm, for a period of time of at least 1 h, performing moderate exercise. In fact, Horstman *et al*. (1990) reported significant decrements in pulmonary responses when subjects were exposed to 0.1 ppm O_3, which is the same concentration as the one in this study, but only after 4.6 h of exposure. This exposure duration is over nine fold higher than the average time of exposure in the current research. Moreover, the findings of Gibbons and Adams (1984) also corroborate the present study in that they reported no significant decrements in pulmonary function when subjects were exercising in an environment where the ozone concentration was 0.15 ppm, performing moderate continuous exercise for 1 h. In the present research, the presence of heat and humidity might have contributed to minimize the transient airway narrowing that can lead to exercise-induced bronchospasm. In fact, it has been shown that athletes' would experience significant airway narrowing mainly when exercising in a dry and cold environment (Rundell & Jenkinson, 2002) and not a hot and humid one. The increase in respiratory symptoms in this study might not have been intense enough to reduce the runners' maximal inspiration volume, or the exercise might not have been long enough to affect airway smooth muscle through hyperventilation. In addition, in this study, we did not measure the respiratory muscle force or mechanic properties of the lung - factors that have been linked to a reduction in lung function post ozone exposure (Passanante *et al*., 1998; Hazucha *et al*., 1989; Scichilone *et al*., 2005). Nevertheless, it is probable that these factors were not affected by the ED of O_3 of this study since no alterations in lung functions were observed.

The magnitude of decrement in lung function has been shown to be related with the ED of ozone, which depends on the ozone concentration, duration of exposure and athletes' ventilatory rate (McDonnell *et al*., 1997). Schelegle and Adams (1986) suggested that lung function impairments would be observed when the ED of ozone is greater than 900 ppm·l. In this present study, the mean ED was 375.2 ppm·l. Some individuals, however, did show a greater decrease in their lung function following the ozone trials. This corroborates with the fact that inter-subject variability in lung function changes is related to differences in individual responsiveness to ozone (Holz *et al*., 1999, Mudway *et al*., 2006). In addition, there was a positive relationship between FVC and ED of

ozone. This relationship, though not very strong (R= 0.34), is statistically significant (P=0.02) and it was an expected result since an athlete that has a larger lung capacity, has higher FVC and can, consequently, inhale more air per minute, receiving a higher ED as a result. Although some studies did not show a strong relationship between ED and lung function parameters, they did show a decrease in lung function post-exercise (Gong *et al*., 1986; Jörres *et al*., 2000; Schelegle & Adams, 1986). The average ED of ozone did not differ between the Control + O_3 (383.9 ppm·l) and the Heat + O_3 trials (366.5 ppm·l). Even though these values are approximately a third of the ED presented in other studies (Gibbons & Adams, 1984; Gong *et al*., 1986), there was a significant correlation with the athletes' performance, measured as the average speed attained by the runners during the trials. While not a strong relationship (R=-0.27), it was statistically significant. This relationship means that the decrease in the athletes' average speed maintained throughout the trial and, hence, a decrease in performance can partially be explained by the amount of ozone, that is the ED, they inhaled during their run. In fact, in both conditions that had ozone, the athletes presented a 1% increase in their performance time irrespective of the temperature. Although this finding is in contrast with the study of Gong *et al.* (1986), who reported a weak and non-significant correlation between performance and ED, it is an important finding because ED had previously only been related to decreases in pulmonary function and increases in airway neutrophilia (Gibbons & Adams, 1984; Gong *et al*., 1986; Mudway and Kelly, 2004). The exercise protocols used in the literature are quite different and this results in different ED exposures between the studies.

This study provides evidence that both heat and heat combined with ozone (0.1 ppm) have a detrimental effect on athletes' performance in an 8 km time trial. Ozone alone had little effect on this variable. Lung function was not impaired after the exercise in any of the environmental conditions tested. The results of this study are important for athletes exercising or competing during summer time in large polluted cities or close to them because they can encounter similar environmental conditions as used in this study. The next chapter will present an analysis of outcome of this study related to the oxidative stress and lung inflammation process.

Chapter 5: Oxidative Stress and CC16 Secretion: Effect of Running in Hot, Humid and Ozone-Polluted Conditions

5.1 INTRODUCTION

The previous chapter showed that while lung function of runners performing an 8 km time-trial run was not affected by environmental conditions – ozone, heat and humidity – their performance was significantly impaired with heat and humidity and with heat, humidity and ozone pollution combined. This chapter presents and discusses the inflammatory responses and oxidative stress of these runners after the exercise trials in these different environmental conditions.

Ozone-Pollution and Airway Inflammation

The literature has shown a lack of consistency in the airway inflammation response of individuals exercising in an ozone-polluted environment (Devlin *et al*., 1996; Krishna *et al*., 1998; Holz *et al*., 1999; Blomberg *et al*., 1999). The conflicting results may be explained by differences in the exercise protocols used, the ED of ozone inhaled by the participants, and the training status of participants in the studies. Moreover, differences in methods and timing of the airway sampling can also influence the results. It seems that when individuals are exposed to a high ED of ozone an increase in airway inflammation markers can be detected as soon as 1 h after exposure, as shown in the study of Devlin *et al*. (1996) in which the participants performed 2 h of high intermittent exercise exposed to a high ozone concentration of 0.4 ppm. Also, 1 h after exposure Holz and colleagues (1999) observed a significant increase in airway neutrophilia and IL-8 when participants completed 3 h of light intermittent exercise 0.25 ppm of O_3. Nevertheless, this response was not seen when the participants performed the same exercise bout exposed to a lower O_3 concentration (0.12 ppm). It has been shown, however, that neutrophil influx reaches its peak in the airways 6 h after exposure (Mudway & Kelly, 2004). Before this time point, some studies using lower ED of ozone have detected increases in inflammatory mediators, such as vascular adhesion molecules, suggesting an initial inflammatory response (Blomberg *et al*., 1999). Table 5.1 gives a general overview of the studies mentioned above.

Table 5.1. Summary of studies evaluating the effect of ozone exposure on lung inflammation.

Study	Subjects	Exercise and Ozone levels	Results
Devlin *et al*., 1996	Healthy male individuals	2 h heavy intermittent cycling 0.4 ppm	1 h post-exposure = ↑ neutrophil, IL-6 and LDH in BAL
Krishna *et al*., 1998	*Healthy individuals	2 h intermittent cycling 0.12 ppm	6 h post-exposure = ↑ neutrophil, IL-8 in BAL
Blomberg *et al.*, 1999	*Healthy individuals	2 h intermittent cycling 0.2 ppm	1.5 h post-exposure: no effect on neutrophil count or percentage in BAL, but ↑ in inflammatory mediators
Holz *et al*., 1999	*Mild asthmatics + nonasthmatics	3 h intermittent cycling 0.12 ppm 0.25 ppm	↑ neutrophil percentage and count in sputum (0.25 ppm O_3) ↑IL-8 in sputum (0.25 ppm O_3)
Broeckaert *et al.*, 2000b	Field study, trained cyclists	Average 35 km cycling Average 0.076 ppm	Post-exercise = ↑ in serum CC16
Blomberg *et al*., 2003	Healthy individuals	2 h intermittent cycling 0.2 ppm	2 h and 4 h post-exposure = ↑ in serum CC16

*Non-smokers, male and female, the study does not report their physical fitness level.

The studies mentioned above used healthy individuals as participants. As mentioned previously the fitness level of the individuals can alter the outcome of the study due to a difference in the airway pool cell composition found in trained individuals. Active individuals that have an endurance training regime, present at baseline a higher neutrophil number in the airways compared with the general population. This has been shown with different sports such as running (Bonsignore *et al*., 2001), swimming (Belda *et al*., 2008) and rowing (Morice *et al*., 2004). This number would also be higher after a competition or after intense training, indicating that lung inflammation can be stimulated by both acute and chronic exercise (Denguezli *et al*., 2008). In sports like swimming, this can be linked to the constant inhalation of chlorine and its derivatives (Belda *et al*., 2008). In non-asthmatic amateur runners, the presence of an increased neutrophil number in the airways, after a marathon, was speculated to be, at least in part, linked to a prolonged period of hyperventilation. The high airflow might cause epithelial damage through shear stress on the epithelial wall which leads to an influx of neutrophils to this region (Bonsignore *et al*., 2001; Morice *et al*., 2004). This may be the

same mechanism that leads to higher neutrophil levels in the airways at rest in individuals that train regularly (Bonsignore *et al*., 2001; Denguezli *et al*., 2008). Nevertheless, this shear stress mechanism is still unclear and requires further investigation.

Lung Inflammation and CC16

Exposure to ozone has also been linked to lung epithelial injury (Christian *et al*., 1998), resulting in an increase in permeability of the lung epithelial barrier. As detailed in *Chapter 2*, for the assessment of lung injury and toxicity, CC16 protein has been shown to be a sensitive and suitable biomarker (Bernard *et al*., 1994; Hermans & Bernard, 1999). This small protein is produced by Clara cells, found exclusively in the pulmonary airways (Hermans & Bernard, 1999), and by similar cells in the nasal mucosal epithelial (Benson *et al*., 2004; Lindahl *et al*., 1999). CC16, measured in the blood, is derived almost exclusively from the airways (Hermans & Bernard, 1999). As a result of pulmonary inflammation and increase in the permeability of the lung epithelial barrier, the concentration of this protein in the blood has been shown to alter (Hermans *et al*., 1999). Few studies have evaluated the effect of exercise alone on this protein. Nevertheless, these studies showed that CC16 levels in the blood are increased after exercise. This occurred in spite of the differences in duration, i.e. 30, 45 or 60 min of exercise; or the type of the exercise, i.e. fire fighting tasks, high intensity exercise and swimming (Nanson *et al*., 2001; Carbonelle *et al*., 2002). More investigation is needed to address specific questions that have resulted from these research studies, such as the mechanism leading to this increase and the influence of exercise intensity and duration.

Because ozone is known to increase lung inflammation and because CC16 is a marker of lung inflammation, most studies investigating the relationship between this protein and exercise have also used an ozone-polluted environment. As described in *Chapter 2*, Broeckaert *et al.* (2000b) and Blomberg *et al.* (2003) found an increase in CC16 levels in the blood following 2 h of exercise in the presence of ozone. One study was a field study and the participants were trained cyclists (Broeckaert *et al*., 2000b), whereas the other study (Blomberg *et al*., 2003) was conducted in the lab with healthy individuals. Even though both studies had the same duration, the intensity varied and so did the ED of ozone - factors which make it difficult to compare them directly. Nevertheless, by

looking at the results, it seems reasonable to conclude that the combination of ozone and an exercise bout is sufficient to affect the lung epithelium integrity. It would have been interesting if these studies had also analyzed if the lung concentration of CC16 had altered. This data would have been useful to strengthen the conclusion of epithelial damage instead of the possibility of an increase in the secretion of this protein into the lungs and its consequential greater diffusion into the bloodstream.

Ozone-Pollution, Heat and Oxidative Stress

As well as stimulating lung inflammation, the inhalation of ozone can increase the reactive oxygen species and free radicals in the lungs. This process occurs mainly by two different mechanisms. The first would be by reacting directly with constituents of the respiratory tract lining fluids (RTLF) and the underlying epithelium (Pryor *et al*., 1995). Antioxidants present in the RTLF, such as glutathione, uric acid and ascorbic acid, have been suggested to have an important role in neutralizing part of the radical generation, hence reducing the ozone induced oxidative stress (Samet *et al*., 2001). The second mechanism by which ozone can increase the oxidation process is indirectly because by exacerbating the inflammatory response in the lungs, there will be an increase in the reactive oxygen species (ROS) production by the immune cells through the respiratory burst process (Corradi *et al*., 2002). These processes combined could overwhelm the local and systemic antioxidant network leading to an increase in the oxidative process. In addition, it is important to remember that an exercise bout can already provide stimulus to the increase in radicals in the body (Fisher-Wellman & Bloomer, 2009). Nevertheless, the literature is still inconclusive in regard to the exacerbation of the lung oxidative stress process following exposure to ozone and physical activities. Therefore, it is difficult to establish the degree of oxidative stress that athletes would have if exercising in such conditions and how it would affect their performance.

The studies mentioned in this section were all conducted in a thermoneutral environment. However, the potential effects of thermally stressful environmental conditions on aspects of lung inflammation, lung injury and oxidative stress also need to be considered. Indeed, hyperthermia has been shown to promote oxidative stress *in vitro* (Mitchell & Russo, 1983; Flanagan *et al*., 1998; Lord-Fontaine & Averill-Bates, 2002)

and *in vivo* (McAnulty *et al*., 2005a). *In vitro* studies, using indirect methods to measure oxidative stress, provided evidence that cells exposed to heat stress can become antioxidant depleted, GSH in particular (Mitchell & Russo, 1983; Lord-Fontaine & Averill-Bates, 2002). The study of Flanagan and colleagues (1998) was the first to directly measure, by the use of electron paramagnetic resonance spin trapping method, the increase in free radical production by cells exposed to hyperthermia. This excess production of free radicals was speculated to be generated at the mitochondrial electron transport chain. Hyperthermia has also been associated with an increase in the conversion of xanthine dehydrogenase into xanthine oxidase which also leads to an increase in free radical production (Mitchell & Russo, 1983). McAnulty *et al.* (2005a) investigated plasma oxidative stress markers of healthy moderately trained males exercising in two different environmental conditions. The control condition was set at 25ºC with 40% humidity, whereas the other environment was hot (35ºC) and humid (70%). The participants ran on a treadmill at 50% of their $VO_{2\ max}$ for the same duration, which was established as the time taken for the participant's core temperature to reach 39.5ºC in the hot and humid environment. Running in the hot and humid environment significantly increased F_2-isoprostane plasma levels, indicating a higher oxidative stress in this condition. Even though the authors did not assess the catecholamine production, these were suggested to have contributed to the increase in oxidative stress since catecholamines are known to contribute to the formation of reactive species and also increase when the person is exercising in the heat. Therefore, also taking into consideration that ozone increases oxidative stress and is a warm season pollutant, it is realistic to analyse these environmental factors together.

This chapter will present the results related to lung inflammation and oxidative stress of runners performing an 8 km time trial run in the four different environmental conditions outlined in *Chapter 4* allowing comparison of these results with the performance and lung function results previously presented. The hypothesis was that ozone alone would increase the analyzed variables of inflammation and oxidative stress and that addition of heat and humidity would increase this impact further. In addition, this study used the nasal lavage procedure in order to assess the upper respiratory airways. Therefore this chapter also presents the methods and results of a second study conducted to evaluate the reliability of the nasal lavage method.

5.2 METHODS

The data for this chapter was collected as part of the same project that originated the data presented in *Chapter 4*. Therefore, most aspects of the methodology, such as the participants' of the study and the exercise protocol are the same. Further tests carried out specifically in this study and that were not described in *Chapter 3* are fully explained in this section.

5.2.1 Experimental Procedures

In summary, the exercise protocol consisted of an 8 km time trial run, where runners controlled their own speed, but they did not have access to the value of the speed. After the exercise trial the participants had blood samples taken and went through the nasal lavage procedure. The environmental conditions were Control: filtered air at 20 ºC + 50% relative humidity (rh); Control + O_3: 0.1 ppm O_3 at 20 ºC and 50% rh; Heat: filtered air at 31 ºC and 70% rh; Heat + O_3: 0.1ppm O_3 at 31 ºC and 70% rh.

To analyze the impact of the different environmental conditions on respiratory inflammation and oxidative stress of the runners, the following variables were assessed:

- Inflammatory markers: nasal lavage neutrophils, IL-8 and IL-6; plasma and nasal lavage CC16 concentrations; plasma fibrinogen; nasal lavage albumin, LDH and C-reactive protein (CRP) concentration;
- Oxidative stress markers: plasma and nasal lavage GSH/protein, nasal lavage uric acid concentration and trolox equivalent antioxidant capacity of plasma and nasal lavage.

Sample Collection: Immediately post-exercise intravenous blood samples were collected in two 6 ml vacuum tubes containing EDTA. The plasma was aliquoted into eppendorfs (500 μl) and immediately stored at -80 °C until further analysis. Nasal lavage was collected around 15 min post-exercise according to methods described in section 3.2.4. The cells were separated, counted and differentiated according to their morphological appearance. The supernatant was aliquoted into eppendorfs (500 μl) and

immediately stored at -80 °C until further analysis

Biochemical Analysis: Full descriptions of the procedures used in this study are given in *Chapter 3*. In brief: Nasal lavage pro-inflammatory cytokines, IL-6 and IL-8, were measured using commercially available enzyme linked immunoabsorbent assay (ELISA) kits (R&D, Abingdon, UK) in accordance with the manufacturer's instructions. Similarly, CC16 was also measured using an ELISA kit (DiaMed EuroGen, Belgium). The total antioxidant capacity of nasal lavage was measured by a colourimetric assay (Cayman Chemical Company, Boldon, UK) that compares the capacity of the antioxidants in the sample to prevent ABTS oxidation with that of a water-soluble tocopherol analogue: Trolox. The result is quantified as milimolar trolox equivalents. The nasal lavage GSH and total protein were both measured. The results are expressed as GSH/protein in order to allow comparisons between samples of different dilutions. In addition, Nasal lavage albumin and uric acid concentrations were measured using quantitative colourimetric assay kits (BioAssay Systems, Cambridge, UK) according to manufacturer's instructions as detailed below. Finally, plasma acute phase proteins were also measured with details of the methods given below.

Albumin: Nasal lavage albumin concentrations were measured using quantitative colourimetric assay kits (BioAssay Systems, Cambridge, UK) according to manufacturer's instructions. After the nasal lavage samples thawed, 5 µl of the samples and albumin standards (0 –5 $g \cdot dl^{-1}$) were added in duplicate into a clear bottom 96 microwell plate. The working reagent was prepared and 200 µl added to each well. After 5 min at room temperature the absorbance was read at 620 nm, the concentrations of each sample were obtained from the standard curve and the average value of the duplicate used.

Uric acid: Uric acid concentrations in nasal lavage were measured using quantitative colourimetric assay kits (BioAssay Systems, Cambridge, UK) according to manufacturer's instructions. The nasal lavage samples were thawed before initiating the experiment. Into a clear bottom 96 microwell plate, 5 µl of the standards (0 – 12 $mg \cdot dl^{-1}$) and samples were added in duplicate. The working reagent was freshly prepared mixing 10 volumes of reagent A, 1 volume of reagent B and 1 volume of reagent C;

200µl of the working reagent was added to each well. The plate was incubated at room temperature for 30 min. Following this period the absorbance was read at 595 nm, the concentrations of each sample were obtained from the standard curve and the average value of the duplicate used.

Acute phase Protein: Plasma content for the acute phase proteins fibrinogen and C-Reactive protein (CRP) were measured by double antibody sandwich ELISA. Samples were thawed and diluted in PBS + 0.1% Tween 20 as follows: 1 in 30,000 for fibrinogen and 1 in 25 for the CRP. For these assays, 96 microwell plate were used. These were coated with the capture antibody for each acute phase protein and left overnight. The following morning the plates were washed (PBS + 0.1% Tween 20) and 100 µl of standard and samples were added to the microwells in duplicate and incubated, at room temperature, for a 2 h period. The plates were then washed and the appropriate detection antibody (100 µl) was added to each microwell. Following a 1 h incubation period, at room temperature and in the dark, the plates were washed again and chromagenic substrate (100 µl) was added. For the colour to develop, the plates were left for 15 min in the dark, after which 0.5 M sulphuric acid (100 µl) was added to stop the colour development. The absorbance was read with a 490 nm wavelength (Dynex MRX II, West Sussex, UK). All rabbit anti-human coating and HRP detection antibodies were purchased from DakoCytomation, Denmark.

Statistical Analysis

All data were checked for normality before statistical analysis. Results are represented as mean values ± standard deviation (SD). Data were analyzed using General Linear Model ANOVA (Minitab15 Statistical Software) with Tukey's *Post-hoc* test. Statistical significance was accepted at $P < 0.05$. In order to verify any association between the variables in the Heat + O_3 trial a correlation matrix was conducted, with a subsequent multiregression analysis (Minitab15 Statistical Software).

5.2.2 Nasal Lavage Reliability Study

5.2.2.1 Participants

Six healthy volunteers took part in this study (mean ± SD, 27 ± 2.5 year; 69.6 ± 10.8 kg; 172 ± 7.5 cm). The participants had to be non-smokers and moderately active. In the same way as in the other studies all participants received explanation about the study's objective, and were aware of possible risks and discomforts they might experience. All participants signed an informed consent form and completed a health history questionnaire before the commencement of data collection.

5.2.2.2 Experimental procedures

The *Nasal Lavage Reliability Study* had a total duration of 4 weeks. First, the participants went through a familiarisation trial were they practiced going through the nasal lavage procedure. After that, once a week, during the first 4 weeks the participants were submitted to the nasal lavage procedure. The nasal lavage samples were collected from the participants at the same time of day.

The nasal lavage procedure was performed as previously described in *Chapter 3*. From the samples, total cell counts, neutrophil counts, albumin, CC16, IL-6 and uric acid were assessed using the methods described previously.

5.3 RESULTS

5.3.1 Neutrophil Counts

In the nasal lavage fluid, the predominant cells were neutrophils and epithelial cells (Figure 5.1). The results for nasal lavage neutrophil counts post-exercise are presented in Table 5.2. There were no significant differences between the trials.

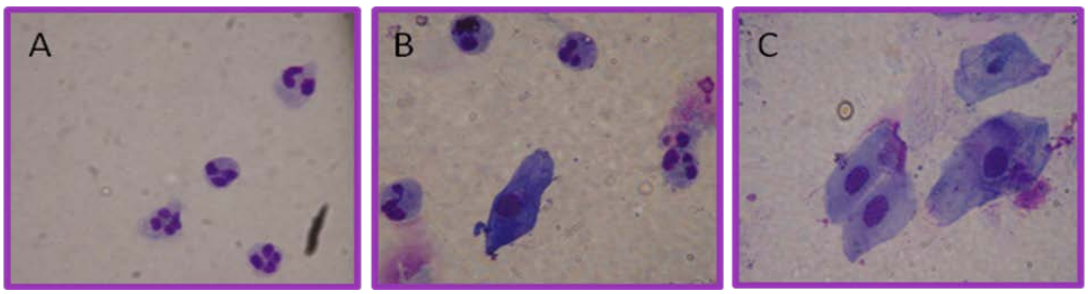


Figure 5.1. Nasal lavage cells. Epithelial cells and neutrophils present in the nasal lavage fluid. A- Neutrophils; B- Neutrophils and an epithelial cell; C- Epithelial cells.

5.3.2 Inflammatory markers

Cytokines and albumin

Nasal lavage IL-6 concentration was not detected with the assay used. The IL-8 concentration, on the other hand, was detected, though it did not vary between trials. Similarly, albumin concentrations did not vary between trials (Table 5.2). The intra-assay coefficient of variation for the duplicate samples was 2.6% for IL-8 and 3.1% for albumin.

Acute Phase Proteins and LDH

. No differences between trials were encountered for plasma CRP, plasma fibrinogen or nasal lavage LDH concentrations (Table 5.2). The intra-assay coefficient of variation for the duplicate samples was 2.9% for CRP, 1.3% for fibrinogen, and 2.5% for LDH.

Clara Cell Protein

CC16 concentration was measured both in the nasal lavage and plasma samples. Figure 5.2 shows the mean values for the nasal lavage CC16 concentration in each trial. One subject exhibited a very high expression of CC16 for all the trials. This participant was considered an outlier and eliminated from the analysis of this protein because he was at least 1.5 times above the interquartile range when the values were placed in a boxplot (Minitab15 Statistical Software). Nasal lavage CC16 concentration in the Heat + O_3 trial was therefore significantly greater compared with the Control trial (P=0.03). The intra-assay coefficient of variation for the duplicate samples was 2.2%.

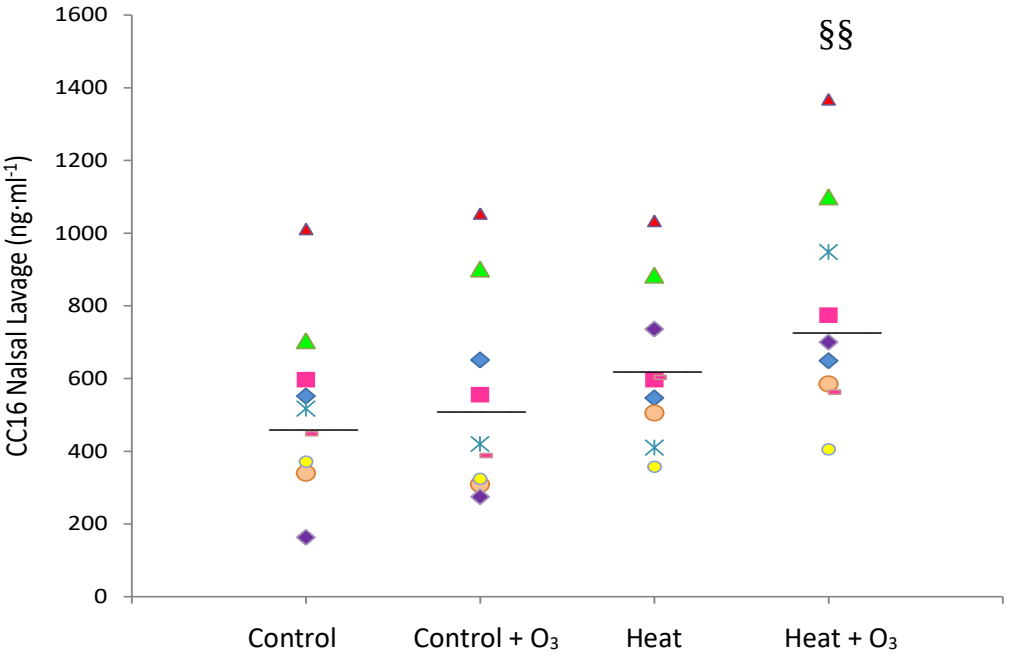


Figure 5.2. Individual nasal lavage CC16 concentration. Mean value for each trial is represented by ——. §§ Significantly different from Control trial ($P < 0.05$).

As for the CC16 concentration in the plasma samples, no differences were found between the trials (Table 5.2). The intra-assay coefficient of variation for the duplicate samples was 2.7%.

Table 5.2. Inflammatory markers from nasal lavage and plasma samples.

	Control	**Control + O_3**	**Heat**	**Heat + O_3**
NL Neutrophil Counts (x10^4)	2.3 ± 3.6	3.4 ± 3.0	3.2 ± 3.6	2.1 ± 2.1
NL IL-8 (pg·ml^{-1})	66.9 ± 56.4	68.2 ± 74.4	53.3 ± 24.0	49.9 ± 35.7
NL Albumin (mg·ml^{-1})	0.48 ± 0.03	0.52 ± 0.06	0.49 ± 0.03	0.49 ± 0.03
Plasma CRP (ng·ml^{-1})	175.8 ± 125.4	199.7 ± 160.9	211.9 ± 112.5	257.3 ± 218.2
Plasma Fibrinogen (mg·ml^{-1})	5.13 ± 0.6	5.45 ± 0.9	5.32 ± 1.5	5.08 ± 0.9
NL LDH (units·ml^{-1})	315.3 ± 218.8	334.6 ± 146.1	261.6 ± 77.1	259.8 ± 122.7

Plasma CC16 (ng·ml^{-1})	46.6 ± 10.5	48.5 ± 12.6	40.7 ± 9.6	44.5 ± 11.8

No differences were observed in any of the variables. Values are mean ± SD.

5.3.3 Oxidative Stress Markers

Nasal lavage and plasma GSH/Protein

There was a significant increase (*P*=0.036) in the nasal lavage GSH/protein concentration when the Heat + O_3 trial was compared with the Control (Figure 5.3). In addition, there was also a significant inter-subject variability (*P*=0.001). The intra-assay coefficient of variation for the duplicate samples was 3.6%. As for the plasma GSH/Protein concentration there were no differences between the trials (Table 5.3). The intra-assay coefficient of variation for the duplicate samples was 3.1%.

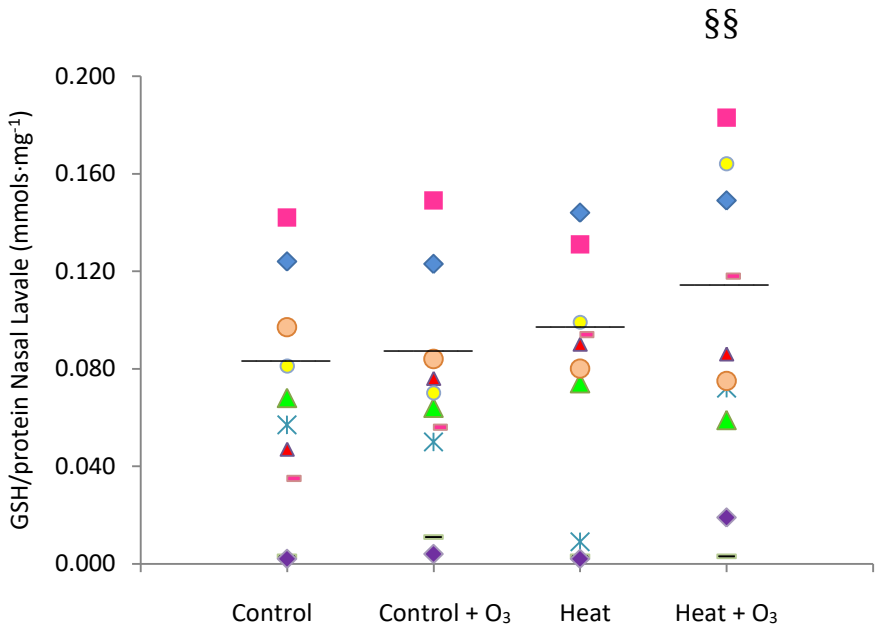


Figure 5.3. Individual GSH/Protein concentration in nasal lavage. Mean value for each trial is represented by ——**.** §§ Significantly different from Control trial, P < 0.05.

Trolox Equivalent Antioxidant Capacity (TEAC)

No significant differences between the trials were observed for either the plasma TEAC or nasal lavage TEAC samples (Table 5.3). In addition, a significant inter-subject variability was found for both the nasal lavage (*P*=0.035) and plasma samples

(*P*=0.001). Intra-assay coefficient of variation was 4.3% for the duplicate samples.

Nasal lavage Uric Acid Concentration

No differences were found between trials for the nasal lavage uric acid concentration (Table 5.3). There was a significant inter-subject variability (P=0.03). The intra-assay coefficient of variation for the duplicate samples in this assay was 1.7%.

Table 5.3. Oxidative stress markers in plasma and nasal lavage.

	Control	**Control + O_3**	**Heat**	**Heat + O_3**
Plasma TEAC (Trolox mM)	1.83 ± 0.78	1.69 ± 0.63	1.74 ± 0.71	2.05 ± 0.24
NL TEAC (Trolox mM)	0.019 ± 0.018	0.014 ± 0.013	0.014 ± 0.012	0.013 ± 0.012
Plasma GSH/Protein (mmoles·mg^{-1})	1.18 ± 0.21	1.09 ± 0.27	1.46 ± 0.21	1.31 ± 0.21
NL Uric Acid (mg·dl^{-1})	0.45 ± 0.06	0.42 ± 0.06	0.45 ± 0.09	0.45 ± 0.09

Values are mean ± SD.

5.3.4 Correlations

A multiregression analysis was conducted to verify if there was any relationship between the percentage change in performance observed in the Heat + O_3 trial compared with the Control trial and the analyzed inflammatory and oxidative stress markers in the Heat + O_3 trial. A significant negative correlation was observed between time to complete the Heat + O_3 trial with the nasal lavage TEAC (*R*=- 0.70, *P*=0.036), and similarly with the nasal lavage GSH/protein concentration (*R*=- 0.69, *P*=0.041) (Figure 5.4).

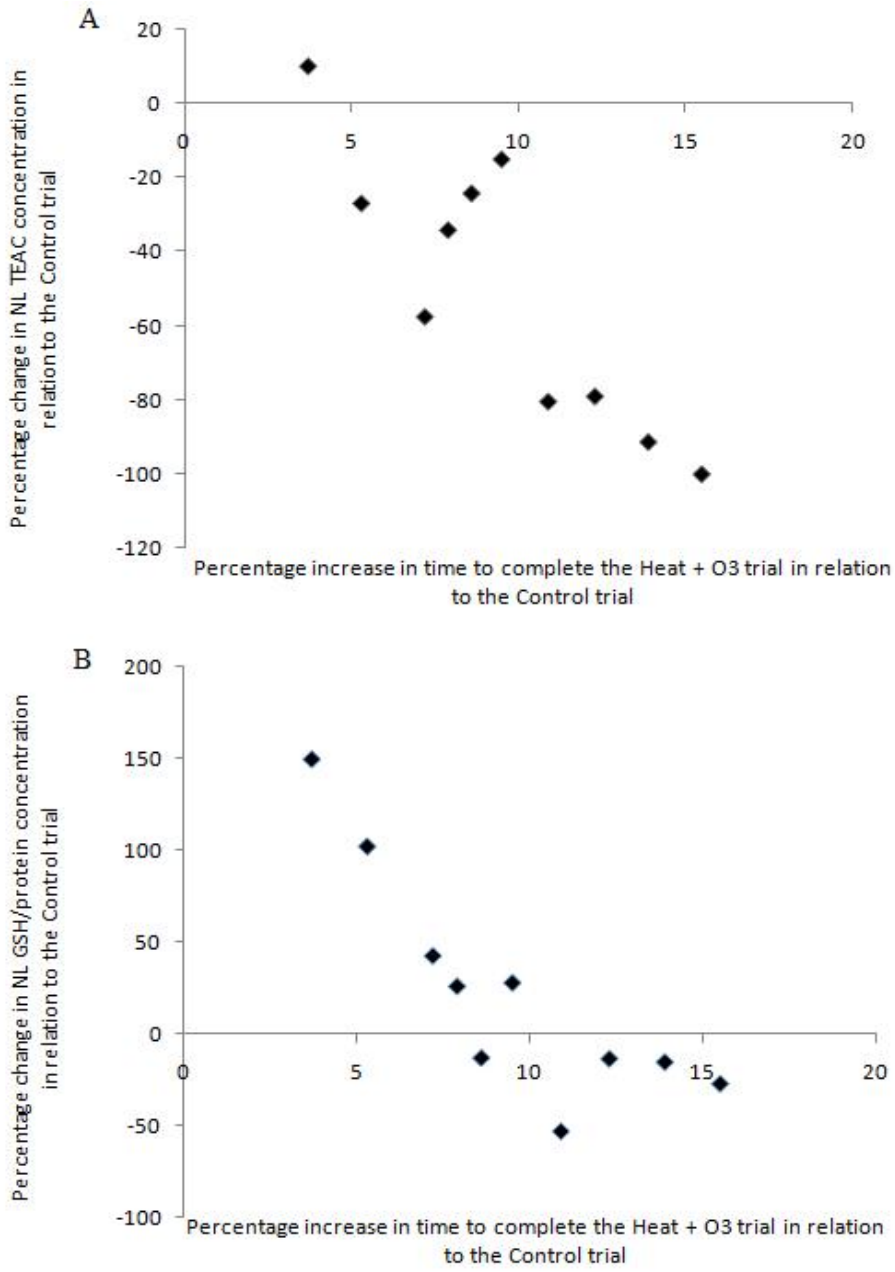


Figure 5.4. Relationship between percentage increase in time to complete the Heat + O_3 trial with the percentage change in nasal lavage TEAC (A) and with the nasal lavage GSH/protein concentration (B).

5.3.5 Nasal Lavage Reliability Study

All participants were highly tolerable to the nasal lavage procedure. The mean recovery of the fluid was 5.2 ± 0.2 ml. This value did not change significantly among the 5 lavages and were highly reproducible in the same individual.

The predominant cells in the nasal lavage were neutrophils and epithelial cells. Occasionally macrophages were also present and in even smaller numbers eosinophils and monocytes. The results for neutrophil counts in the nasal lavage during the 4-week study are presented in Table 5.4. This suggests an initial inflammatory process. Nevertheless, the albumin concentrations did not vary between the 4 weeks of the study nor did the CC16 concentrations (Table 5.4). These were however, reproducible in the same individual. Nasal lavage uric acid and IL-6 could not be detected in the samples. In the case of uric acid this could possibly be due to a low concentration of this antioxidant, whereas the assay for IL-6 might not have been sensitive enough to detect the diluted levels of this cytokine in the lavage.

Table 5.4. Inflammatory markers in nasal lavage (Reliability Study)

	Week 1	Week 2	Week 3	Week 4	CV*
Total cell count ($x10^4$)	1.9 ± 0.9	2.2 ± 0.9	2.2 ± 1.2	1.8 ± 0.6	6.4%
Neutrophil count ($x10^4$)	0.28 ± 0.1	0.22 ± 0.08	0.38 ± 0.15	0.23 ± 0.08	5.8%
Neutrophil percentage	14.7 ± 5.2	10.4 ± 6.3	17.2 ± 8.1	12.7 ± 5.6	6.3%
CC16 ($ng \cdot ml^{-1}$)	381 ± 146	401 ±145	483 ± 78.8	437 ± 189	4.1%
Albumin ($mg \cdot ml^{-1}$)	0.09 ± 0.06	0.10 ± 0.06	0.11 ± 0.09	0.11 ± 0.09	7.2%

Values are mean ± SD.

* Coefficient of variation calculated using the root mean squared error (RMSE) term from the ANOVA (Minitab15 Statistical Software).

5.4 DISCUSSION

The key findings of the main study of this chapter were that there was no significant change in airway inflammation in any condition compared to Control. The nasal lavage CC16 concentration was significantly elevated in the Heat + O_3 trial compared to control, evidence of an early epithelial damage. In addition, in the same trial (Heat + O_3), there was an increase in the nasal lavage GSH/protein concentration suggesting an up-regulation in the antioxidant defences in the upper respiratory airways in response to oxidative stress. Note that this response was greater compared with the Control, but not with heat alone or ozone alone. Neither heat nor ozone alone affected markers of inflammation, epithelial damage or oxidative stress. Furthermore, there was a

significant correlation between percentage decrease in performance, during the Heat + O_3 trial, and decrease in antioxidant status of the athletes' nasal lavage.

There are various different methods that can be used to assess an individual's airways for inflammatory and oxidative stress markers. Some studies in the literature have used bronchoalveolar lavage and bronchial biopsies, procedures that sample the lower respiratory airways. These methods, however, have the disadvantages of being invasive procedures, technically difficult to carryout and they need to be performed in a medical environment where the subject is anaesthetized (Jörres *et al*., 2000; Ratto *et al*., 2006). In addition, bronchoalveolar lavage and bronchial biopsies cannot be repeated at different time points. Nasal lavage and induced sputum have also been used in research studies evaluating the respiratory airways. These two procedures sample the upper respiratory airways and central airways respectively. They have been shown to be easily tolerated by the participants, and the nasal lavage, in particular, can be repeated at multiple time points (Mudway *et al*., 1999; Schock *et al*., 2004). We, thus, decided to use the nasal lavage technique in the present study for those reasons and because the nasal epithelium is the first to come into contact with inspired pollutants (Hernández-Escobar *et al*., 2009) which makes it a relevant area of study.

In the *Nasal Lavage Reliability Study* the main finding was that the nasal lavage sampling method is a reliable technique. Of note is the fact that attention was given to conduct the lavage in a standardized way. Not only was the researcher familiar with all the technical steps, but the same oral instructions were given to all the participants every time they went through this procedure. The reproducibility of the nasal lavage was evaluated in two different ways. First, the reproducibility of the sampling technique was assessed by analysing the total fluid recovered. This result was shown to be highly reproducible in the same individual once they became familiar with the procedure. Second, the reproducibility of the nasal lavage was evaluated by the number of cells and mediators recovered from each lavage. The following parameters were examined: total cell count, neutrophil count and percentage, IL-6, albumin, CC16 and uric acid. The parameters that could be detected were also reproducible. This result is in accordance with the study of Nikasinovic-Fournier and colleagues (2002) that also analysed the reproducibility of the nasal lavage technique for a range of inflammatory markers. This reliability study is of essence to strengthen the outcome of the studies in this thesis,

which used the same protocol for the nasal lavage technique to assess the upper respiratory tract inflammatory and oxidative stress markers.

The present study is, to date, the first to analyse the neutrophil response to ozone and heat challenge combined. There was a large variation between individuals in terms of inflammatory response. For some athletes, an additive effect was found when heat was combined with ozone. For others, however, the mixture of these two variables caused a decrease in the neutrophil count when compared with either O_3 or heat alone. Still others did not show variations between the trials. This reinforces the evidence that people respond very individually to ozone exposure representing their different intrinsic responses to the inflammatory or toxic effects of O_3 (Passanante *et al*., 1998; Holz *et al*., 1999; Blomberg *et al*., 1999; Mudway *et al*., 2006). In addition, this variation in individual susceptibility to ozone may account for the inconsistent neutrophil response observed.

The lack of an increase in neutrophil numbers observed in the current study corroborates other studies (Blomberg *et al*., 1999; Holz *et al*., 1999; Morrison *et al*., 2006; Mudway *et al*., 1999). Morrison *et al.* (2006) did not observe an increase in neutrophil counts post-exposure to 0.1 ppm of O_3 using the bronchoalveolar lavage method. In the same way, lack of early increase in neutrophil counts post-ozone exposure was also observed in other studies using either nasal lavage (Mudway *et al*., 1999) or sputum induced technique (Holz *et al*., 1999) with 0.2 ppm and 0.125 ppm of O_3 respectively. The lack of airway neutrophilia can most likely be explained by the ED of O_3 inhaled by the participants and the airway sampling occurring within the first hour after exercise cessation. Post-exercise changes in blood neutrophil count usually presents a biphasic response, characterized by an initial increase during the exercise, returning back to basal levels 30-60 minutes after, and a greater increase in the neutrophil count peaking around 2-4 h post-exercise (Mackinnon, 1999). Moreover, studies have shown that after exposure to ozone, neutrophil influx into the airways reaches a peak around 6 h post-exposure (Mudway & Kelly, 2004). This occurs due to the necessity of migration, a process that can be summarized by four main events: *1.* rolling; *2.* chemotaxis activated stimulus by chemokines, such as IL-8; *3.* arrest and adhesion; *4.* migration into the tissue (Kuby, 1997) (Figure 2.1). Blomberg *et al*., (1999) observed an increase in the expression of vascular adhesion molecules 1 h post-

exposure to ozone; however, it had not yet resulted in a movement of neutrophils into the airways. Similarly, Morrison *et al.* (2006) reported no changes in neutrophil counts in bronchoalveolar lavage 1 h post-exposure to a high O_3 concentration (0.4 ppm). Nonetheless, 6h after exposure, there was a significant increase in this cell number. On the other hand, Krishna and colleagues (1998) reported that no changes occurred in any kind of inflammatory cells in the bronchial submucosa when measured 6 h following exposure to 0.2 ppm of ozone. Although there is some evidence supporting the lack of airway neutrophilia up to 6 h after exposure to this oxidative gas, there are other studies that did report an early increase in neutrophil numbers in the lungs after exposure to high levels of O_3 such as 0.25 ppm and 0.4 ppm (Devlin *et al*., 1996; Holz *et al*., 1999). These contrasting findings highlight the importance of the airway sampling time and the technique used, as the different techniques sample different airway compartments producing different results. The decision to use nasal lavage as the method to sample the upper respiratory airways in this study, as mentioned previously, was due to the fact that it is a reproducible and well-tolerated procedure as demonstrated in the *Nasal Lavage Reliability Study*, which concurs with other studies (Nikasinovic-Fournier *et al*., 2002; Mudway *et al*., 1999). In addition, the nasal epithelium is the first to come into contact with inspired pollutants and, therefore, protects the lower airways (Hernández-Escobar *et al*., 2009). Even though, during high intensity exercise, such as the one performed in this study, athletes may tend to breathe predominantly through their mouths, the nasal lavage technique is still useful to detect changes in the upper respiratory airways as elucidated further on.

The present study also assessed a variety of inflammatory markers in nasal lavage and plasma samples post-exercise. The IL-6 concentrations were not detected in the nasal lavage samples. Although it has been shown to be detectable in NL (Keman *et al*., 1998), other studies that have analyzed the impact of ozone on this cytokine have used different sampling methods (Krishna *et al*., 1998; Christian *et al*., 1998; Blomberg *et al*., 1999). Moreover, no differences were encountered for the IL-8 concentrations between trials, corroborating the findings of Blomberg *et al.* (1999). This result could be due to the sampling time point. Todokoro *et al.* (2004) conducted an *in vitro* study where they observed that the IL-8 production started to increase after the second hour post ozone exposure and, only after 6 h, did the up-regulation of this cytokine become apparent. Therefore, we might have missed this time point. This could also be true for the other markers measured, such as nasal LDH and albumin concentrations, plasma

levels of fibrinogen and C-reactive protein (CRP). There were no significant changes in these inflammatory markers; however, an increase trend could be observed for the CRP. Similarly, Blomberg *et al*. (1999) did not detect any change post O_3 exposure in either LDH or albumin levels present in the airways. The lack of changes in the nasal lavage concentration of albumin is also in accordance with other studies (Mudway *et al*., 1999; and Morrison *et al*., 2006). It could also be speculated that the O_3 concentration together with exercise were not sufficient to elicit a high level of airway inflammation and lung epithelial damage.

On the other hand, CC16, was the only inflammatory marker that showed a variation due to the environmental condition. This protein has been linked to having an important role in decreasing the inflammation of the respiratory tract and protecting it against the harmful effects of oxidative stress (Broeckaert & Bernard, 2000). Most athletes presented an increase in this protein level after the Heat + O_3 trial when compared with the Control. It is possible that this increase was due to a higher release of this protein by the Clara cells. The majority of studies investigating this protein analyzed mainly its variation in plasma or serum, even though it is easily quantified by the nasal lavage method (Johansson *et al.*, 2005). The assessment of CC16 serum levels can also be used as a specific biomarker of the airway epithelium integrity. If there is an increase in the permeability of the lung epithelial barrier, which can happen following exposure to ozone (Christian *et al*., 1998), there may be an increased diffusion rate of CC16 to the blood (Broeckaert *et al*., 2000b). Nevertheless, in the present study, no alterations in plasma CC16 were observed. It is possible that the ED of ozone was not strong enough to elicit sufficient lung injury to induce CC16 leakage to the blood. Another hypothesis is that there was not sufficient time to detect an increase in this protein due to the blood sampling time point. Blomberg *et al*. (2003) detected an increase in this protein only 2 and 4 h after participants performed 2 h of moderate intermittent exercise exposed to 0.2 ppm of O_3. In the same study, other epithelial permeability markers, such as albumin and total protein concentration, were also assessed; however, they did not show a significant increase. This data supports the idea that serum CC16 is a more sensitive marker of altered lung epithelial permeability than traditional markers. The high inter-subject variability in CC16 concentration, both in plasma and nasal lavage, in the current study can be justified by its diurnal variation (Helleday *et al*., 2006) and possibly by individual sensitivity to ozone.

Once ozone is inhaled, antioxidants present in the RTLF have a significant role in protecting important components of the tract fluids and minimizing the oxidation process of the underlying epithelium (Van der Vliet *et al*., 1995). Studies analyzing the effect of ozone inhalation and airway antioxidant consumption have indeed shown significant alterations in airway antioxidants, such as uric acid, ascorbic acid and GSH (Housley *et al*., 1995; Mudway *et al*., 1999; Blomberg *et al.*, 1999). No changes in nasal lavage uric acid levels were found in the current study. This contrasts to one of the major findings reported by Mudway *et al.* (1999). They observed a significant decrease in the levels of this antioxidant during the exercise period and it remained depressed during the second hour of exercise. The values had, however, been restored to pre-exercise levels 1 h post-exposure. Even though a series of nasal lavages were performed throughout the experimental protocol, the authors reported no washout effect for uric acid. In the Mudway *et al.* (1999) study, there were both female and male participants and they were not trained individuals. They took part in an exercise protocol that consisted of a 2 h intermittent exercise (mean ventilation of 20 $l \cdot min^{-1}$) with 0.2 ppm of ozone exposure. Differences in ED of ozone, exercise protocol, sampling time and subjects taking part in the studies might explain the differences in the results. In the *Nasal Lavage Reliability Study* this antioxidant could not be detected in the nasal lavage samples. This was an unexpected result. However, a possibility would be that, because the participants in the reliability study did not perform an exercise bout this antioxidant might not have been up-regulated in the RTLF. In addition, the runners that took part in the main study might initially have had a high uric acid concentration in the airways in comparison with the non athletic population of the reliability study. This might be possible because athletes are known to have increased antioxidant defence mechanisms (Powers *et al.*, 1999) as detailed in *Chapter 2.*

The trolox equivalent antioxidant capacity was measured both in the plasma and in the nasal lavage fluid of the runners in the current study. Despite a decreasing trend observed for the nasal lavage trolox equivalent antioxidant capacity after the Heat + O_3 trial, the values did not bear significance, a result which corroborates previous evidence (Blomberg *et al.*, 1999). There are different explanations for this outcome. First, measuring antioxidant concentration is an indirect method for analysing oxidative stress and possibly not the most accurate one (Bailey *et al*., 2003). Even though nasal lavage

was collected post-exercise, there was a time period that might have been sufficient for the up-regulation of certain antioxidants. Studies have shown that there are different optimal time points for sampling oxidative stress biomarkers in the plasma (Michilidis *et al.,* 2007), and this is also true for cells *in vitro* (Stone *et al*., 1998) and in the lungs (Rahman & MacNee, 2000). In addition, other researchers have documented how training causes an enhancement in the antioxidant enzyme activity in various tissues and a decrease in free radical generation, which attenuates the degree of exercise-induced oxidative stress (Kanter, 1998; Vollaard *et al*., 2005; Wilson & Johnson, 2000). The type and duration of the training plays an important role in the up-regulation of the endogenous antioxidants, with long-duration high-intensity endurance training having been shown to be most effective (Powers *et al*., 1999). As detailed in the methods, the subjects that took part in the present study were well-trained endurance runners and hence, their antioxidant defence system would have been well-adapted to cope with oxidative stress. The aim of the present study was not to compare the variation of different measures pre and post trial, but to see how a potentially harmful environment combined with an intense exercise affects the athletes. Therefore, it is not possible to establish if there was a change in the oxidative status of the runners' pre and post-exercise - a known limitation of the study.

The lack of changes in the athletes' trolox equivalent antioxidant capacity might also be explained by the increase in the nasal lavage levels of GSH/Protein. These were significantly increased after the Heat + O_3 trial in comparison with the Control trial. Ozone alone or heat alone did not have an effect on this variable, suggesting an additive or potentiative effect of these environmental stressors. It could be speculated that the increase in the GSH/protein concentration in the airways is a reflection of a local up-regulation mechanism or indeed a systemic response to protect the airways against oxidative stress. The large inter-individual variation can be explained by the diurnal variation of GSH (Blanco *et al.,* 2007) and the individual sensitivity to ozone. This finding is in accordance to the study of Blomberg and colleagues (1999) and can be interpreted as a result of increased GSH release from airway epithelial cells (Wu *et al*., 2004) or an unknown storage site. Another possible explanation might be that there was an increase in plasma levels of GSH derived from the liver which, thus, resulted in an augmented diffusion into the lungs. However, a significant increase in the plasma levels of GSH/Protein in the Heat + O_3 trial was not observed, this strengthens the probability

of a local up-regulation mechanism.

A further finding of this study was the relationship between an increase in performance impairment and the decrease in antioxidant status in the airways. Both nasal lavage total antioxidant capacity and nasal lavage GSH/Protein presented this negative correlation with the performance in the Heat + O_3 trial. This is interesting because it suggests that the antioxidants present in the airways have an impact on the performance outcome in a hostile environment such as the one encountered in the Heat + O_3 trial. Although, in this study, we did not assess the athletes' antioxidant status pre-exposure, it could potentially be used as a marker of susceptibility, but for that, further research is required. Moreover, it will be of interest to investigate whether antioxidant supplementation would have any positive effect in a similar exercise and environmental condition.

To conclude, the findings of this research suggest that the nasal lavage procedure used in this study was reliable and reproducible to assess markers of inflammation. In addition, it can be suggested that an 8 km time trial run performed in a hot, humid and ozone-polluted environment (0.1 ppm) elicits an early epithelial damage characterized by increase in CC16 concentration in the airways. Moreover, an increase in the antioxidant concentration in the upper respiratory airways in that same trial, as indicated by the nasal lavage GSH/protein, suggests a protective mechanism against the oxidative stress stimulated by the high intensity exercise in association with ozone, heat and humidity. Ozone or heat and humidity alone did not have an effect on these variables.

Chapter 6: Effect of vitamin supplementation on athletic performance, lung function and antioxidant concentration in an adverse environment

6.1 INTRODUCTION

In *Chapter 5*, two correlations between nasal lavage antioxidant concentration post-exercise and performance were observed. These correlations suggested the possibility that antioxidant availability in the RTLF during an intense exercise bout in an environment that promotes oxidative stress, beyond that stimulated by the exercise alone, might have a beneficial effect on the performance. Hence, based on these correlations and the other results reported in the previous chapters (*Chapters 4* and *5*), the rational for the study presented in this chapter and in *Chapter 7* was elaborated. This chapter will present and discuss the results related with the athletes' performance, lung function and antioxidant status, while the subsequent chapter has as a focus, aspects of the airway and systemic inflammatory responses.

The Indiscriminate Use of Multivitamin Supplementation

Multivitamin supplementation is a practice that is carried out widely and in an indiscriminate manner. It is estimated that around 70% of the USA population, at least occasionally, make use of dietary supplements and 40% intake them on a regular bases. The dietary supplements that are most commonly taken are multivitamins, calcium, vitamin C and vitamin E (Hathcock *et al.,* 2005). In the UK, the consumption of supplements is also a common practice. It was estimated that in the years of 1998 and 1999 an amount of £347 million and £326 million respectively were spent in vitamins and minerals supplements purchase from pharmacies, health stores and grocery stores throughout the UK. This amount does not include products that were sold via the internet. Vitamin E sales alone account for approximately £79 million per year, whereas vitamin C is at the top of the list accounting for an annual sale of around £252 million in the UK (Expert Group on Vitamins and Minerals – EVM – 2003). What is quite intriguing is that these data are from developed countries where the population that has the money to buy vitamin supplementation also has the money to buy good quality food that can provide the recommended daily allowance of vitamin and minerals. A factor that can stimulate the sales of such products is that the benefits of vitamins are quite well established. Vitamin C, a water-soluble antioxidant found in the cytosol of cells and in the extracellular fluid, is most commonly known to help prevent or recover from flu and colds, even though its benefits surpass this. Indeed, the roll of this vitamin in the prevention and treatment of the common cold has been subject to controversy over

the last years. Even today, public interest in the topic is still high and vitamin C continues to be widely sold and used as a preventive and therapeutic agent for this common ailment (Douglas *et al*., 2004). Nonetheless, Hemilä *et al.* (2009) analyzing all previous known publications on the topic, reached a key conclusion: vitamin C supplementation is ineffective in reducing the incidence of colds in the normal population; thus, routine mega-dose prophylaxis is not rationally justified for community use. There were, however, studies that showed consistent and statistically significant minor benefits on duration and severity of the cold for those using regular vitamin C supplementation. This indicates that vitamin C does play some role in respiratory defense mechanisms. Trials which introduced high doses of vitamin C at the onset of cold symptoms, have not been shown to reduce neither the duration nor severity of symptoms. Yet evidence shows that it could be justified in persons exposed to brief periods of severe physical exercise and/or cold environments (Hemilä *et al.*, 2009).

As for vitamin E, it has an important antioxidant property and is considered one of the major inhibitors of lipid peroxidation *in vivo* (Halliwell & Gutteridge, 2007). This is the case because it exerts the important function of maintenance of membrane integrity by protecting virtually all cells of the body against lipid peroxidation (Institute of Medicine, 2000). Besides being a potent antioxidant, this vitamin also has an anti-inflammatory role presenting beneficial effect in cardiovascular diseases, such as attenuating the release of inflammatory mediators related to such diseases (Singh & Jialal, 2004). The necessity of supplementing a well balanced diet with these vitamins is still controversial. In fact, Bruunsgaard *et al.* (2003) conducted a 3-year experiment, with a healthy male cohort population, in which the participants were either taking both vitamin E (136 IU $\cdot day^{-1}$) and C (500 $mg \cdot day^{-1}$) supplementation (n=55) or placebo (n=52). The 36-months of antioxidant supplementation had no detectable effect on systemic inflammation markers. In addition, as shown in *Chapter 2*, a review study, analyzing over 47 antioxidant supplementation research papers on all-cause mortality, showed a negative effect of some supplements, such as vitamin E, which was associated with an increase in mortality. Vitamin C, despite not presenting a negative effect, did not show any evidence that it might increase longevity (Bjelakovic *et al.,* 2007). Therefore, although vitamin C and E have known beneficial effects, healthy individuals should intake supplementation with caution.

Physical Activity and the Use of Vitamins

The matter changes slightly when we add another factor: physical activity. As elucidated previously in *Chapter 2*, it is clear that muscle contraction increases the production of ROS and free radicals, specifically during intense and prolonged physical activity (Powers *et al.,* 1999; Sen & Roy, 2001). Although there is an increase in the radical concentration during exercise, this does not always result in an increase in oxidative stress. This process will only take place, if the oxidant concentration surpasses the antioxidant concentration of the organism (Powers *et al.,* 2004). In addition, the low concentration of radicals in the muscles plays an important signalling role in the muscle contractile function. These radicals are essential for force generation and for exercise adaptation. Nevertheless, as the radicals start accumulating in the contracting muscles the oxidation of proteins and lipids might cause, amongst other things, contractile dysfunction leading to inhibition in force production and contributing to the development of acute fatigue (Reid, 2001). Whether that would justify the supplementation of antioxidant vitamins by exercising individuals or even athletes in order to improve performance and attenuate oxidative stress is a significant matter.

To answer such a question, an aspect that should be taken into consideration is the adaptive antioxidant response to exercise. Studies have shown that the production of reactive species during exercise is important for cellular adaptations, enzyme expression and gene expression all of which lead to antioxidant up-regulation both in the muscle and systemically (Reid, 2001; Gomez-Cabrera *et al.,* 2005; Gomez-Cabrera *et al.,* 2008; Ji, 2008; Radak *et al.,* 2008) independently of the training status of the individuals (Ristow *et al.,* 2009). In an animal study, Gomez-Cabrera *et al.* (2005) reported the importance of reactive species, produced during exercise, to muscle cell adaptations through the regulation of molecular signalling events. They showed that after an exhaustive exercise bout there was a stimulation of mitogen-activated protein kinase (MAP Kinase). Such enzymes and signalling pathways activate transcription factors such as nuclear factor kappaB (NF-kB), responsible for increasing the expression of cellular enzymes involved in antioxidant defence, such as inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and manganese superoxide dismutase (Mn-SOD), but also inflammation (e.g. TNFα). Of practical importance, this

study showed that the mice group that had been administered with allupurinol – an antioxidant inhibitor of xanthine oxidase - did not show such adaptations. It is with caution that we extrapolate results from animal studies to the human population, however, a recent investigation reported similar results with humans. Ristow and colleagues (2009) analyzed the combination of vitamin C (1000 mg·day^{-1}) and E (400 IU·day^{-1}) in muscle enzymes that regulate the antioxidant defence system of the muscles. This study was conducted with two groups: one of previously untrained individuals and another of trained individuals. Both groups underwent a 4-week training period; and, regardless of their previous training status, the study showed that the vitamin supplementation prevented up-regulation of antioxidant expression enzymes (Figure 6.1).

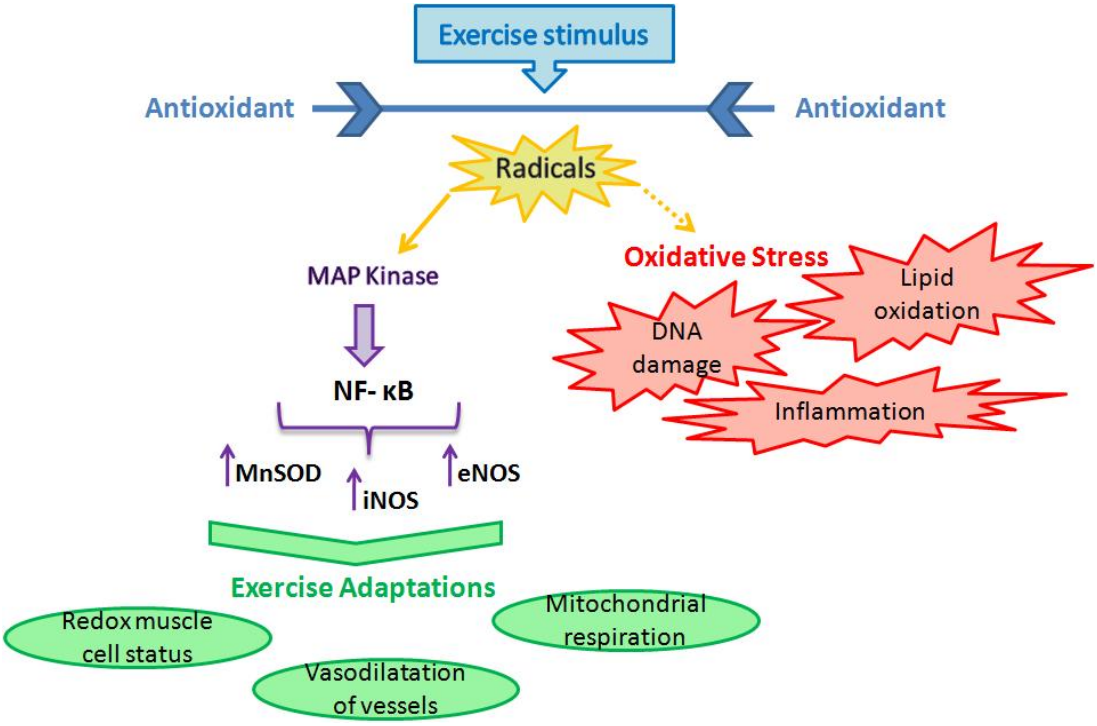


Figure 6.1. Proposed mechanism of the role of exercise in the up-regulation of cellular antioxidant enzymes and the prevention of this process by ingested antioxidants. Adapted from Gomez-Cabrera *et al.* (2005).

On the other hand, antioxidant supplementation might also diminish oxidative stress produced by exercise, but the literature on this topic lacks consistency. Nieman *et al*, (2002) reported no effect on the performance or lipid peroxidation markers of runners that were supplemented with vitamin C (1 week, 1000 mg·day^{-1}) and who completed an ultramarathon race. Traber (2006) reported that a 6-week supplementation of

vitamin C (1000 mg·day^{-1}) and E (400 IU·day^{-1}) had a positive effect in attenuating increases in lipid peroxidation after a long distance running event, but that it did not affect inflammation, muscle or DNA damage. Goldfarb *et al*. (2005) showed that a 2-week supplementation of vitamin E (400 IU·day^{-1}), C (1000 mg·day^{-1}) and selenium (90 μg·day^{-1}) did not attenuate plasma lipid peroxidation (measured as malondialdehyde concentration) or GSSG during the first 24 h after an eccentric bout of exercise, but did attenuate malondialdehyde concentration at the 48 h time point. The participants were non-resistance trained females and the exercise comprised 4 sets of 12 repetitions of elbow flexions. A similar result was found by Davison and Gleeson (2006) who analysed the effect of 2-weeks of vitamin C supplementation (1000 mg·day^{-1}) on plasma malondialdehyde concentration and immunological markers after 2.5 h of cycling at 60% VO_{2max}. The participants of this study were endurance-trained males. In this case, the authors reported that the plasma malondialdehyde concentration was unaffected by the exercise and the temporal response was not influenced by vitamin C supplementation. In a subsequent study, Davison *et al.* (2007), supplemented two groups of recreationally active individuals during a 4-week period with either placebo or a vitamin combination (1000 mg·day^{-1} of vitamin C and 400 IU·day^{-1} of vitamin E). After 2.5 h of cycling (60% VO_{2max}), the plasma F_2-isoprostane concentration was significantly increased post-exercise and the vitamin supplementation had no effect on this marker. However, the lipid peroxidation, measured by plasma TBARS, was lower in the supplemented group compared to the placebo after the exercise bout. The authors explained that because of the lack of specificity of the TBARS assay this result alone should not be considered as evidence of a decrease in oxidative stress in the supplemented group. In addition, the time course of the two oxidative stress markers measured in the study differ. Therefore, perhaps measurements should be made more frequently after exercise and during a longer post-exercise period, and not just immediately and 1 h post-exercise as in that study, although that would obviously be difficult to do practically with human subjects.

Vitamin C and E Supplementation in Performance Outcome

The available evidence does not provide a consensus about the effect of vitamin C and E supplementation and the decrease in oxidative stress following exercise. Most studies have looked mainly into the lipid peroxidation aspect of oxidative stress, however, there are other aspects that could be relevant - such as DNA and RNA damage (Kanter,

1998). Another point of interest is the effect of a decrease in oxidative stress during exercise and its effect on performance. In fact, most studies with a long duration aerobic exercise show no effect on performance. Gomez-Cabrera *et al.* (2006) investigated the effect of acute allupurinol ingestion (300 mg) in runners prior to a marathon competition (23rd Marathon of Valencia). The study showed that the antioxidant abolished the NF-kB activation in peripheral blood lymphocytes of the runners. In addition, malondialdehyde as a marker of lipid peroxidation, was significantly increased after the marathon in the placebo group but not in the allopurinal group. Worthy of note is the fact that, there was no difference in performance between the supplemented and the placebo groups.

The lack of a beneficial effect in performance has also been shown in other studies with vitamin supplementation. Most studies specifically investigating vitamin C supplementation have failed to show its beneficial effects in exercise-induced oxidative stress and in performance. Peters *et al.* (2001), investigating the effect of vitamin C supplementation in athletes performing an ultramarathon race, reported no enhancement in performance compared with a placebo group. The athletes were supplemented for a week (1000 mg·day^{-1}) and presented a significantly higher plasma vitamin C concentration pre-race compared with the placebo group. Nevertheless, after the race the plasma concentration in the placebo group had increased compared to its pre-race values, but in the supplemented group it was similar to the pre-race values. This suggests a possible attenuation of vitamin C mobilization in the supplemented runners. This result diverges partially from the ones presented by Palmer *et al.* (2003) where athletes were supplemented for a 1-week period with vitamin C (1500 mg·day^{-1}) also prior to an ultramarathon race. An increase in the vitamin C concentration post-race in both groups was observed, but the supplemented group maintained a higher level compared with the placebo. In that study, vitamin C supplementation also had no beneficial effect in performance and did not attenuate oxidative stress levels measured by lipid oxidation markers following the race. The authors suggested that the lack of supplementation effect both in oxidative stress and consequently in performance outcome, would be due to the location of this water-soluble vitamin in hydrophilic compartments. Thus, vitamin E intake would be more appropriate to protect against lipid peroxidation.

Nieman *et al.* (2004) tested that theory by supplementing triathletes with 800 IU·day^{-1} of α-tocopherol, during a 2-month period prior to an Ironman competition (Triathlon World Championship in Kona, Hawaii). Plasma α-tocopherol levels were significantly higher before the race, and this difference was maintained post-race. Unexpectedly, however, not only did the vitamin supplementation not attenuate the oxidative stress, measured by the F_2-isoprostane concentration, but after the race it had increased almost two-fold. In addition, the cytokines that were measured - IL-1, IL-6 and IL-8 - were significantly higher post-race when compared to the values of the placebo group. Not surprisingly, there was no significant difference in the performance time between the supplemented and placebo groups. The authors suggested that this unforeseen result may have occurred due to the dualist behavior that vitamin E can have. As explained previously, vitamin E can also act as a pro-oxidant under certain conditions, such as high oxidation and low levels of other antioxidants (Rietjens *et al*., 2002). Moreover, the athletes participating in this study did not refrain from taking other vitamins or nutrients, which could also have influenced the results. The pro-oxidant effect of vitamin E was also shown in another study using the same high-dose supplementation protocol (2 months of 800 IU·day^{-1} of α-tocopherol) and testing highly trained athletes. Although the plasma α-tocopherol levels were elevated in the supplemented group pre and post-race, that did not result in a better performance but increased the lipid peroxidation in the triathletes (McAnulty *et al.,* 2005b). Additionally, a study conducted using an animal model reported that high levels of vitamin E supplementation led to a decrease in muscle force production (Coombes *et al.,* 2001).

In a further attempt to investigate the effect of antioxidant supplementation on an extremely intense and long-duration exercise, Traber (2006) supplemented athletes prior to an ultramarathon trail run. A group of female and male athletes were divided into two groups: supplemented and placebo. Both groups went through a 6-week supplementation period, one receiving both vitamin E (400 IU·day^{-1}) and C (1000 mg·day^{-1}), the other placebo. The supplemented group presented significantly higher levels of plasma antioxidants, but that did not result in an enhanced performance for that group. In addition, there was no difference on exercise-induced DNA damage, muscle damage or recovery. Interestingly, the author did report that the supplemented group had a lower oxidative stress post-run measured by a decreased in lipid peroxidation (F_2-isoprostane), but it had no effect on inflammation. This suggests that the combination of both vitamin C and vitamin E might be beneficial in protecting the runners against lipid

peroxidation when performing such a long and intense exercise. Nevertheless, the authors also reported that the women, in both groups, presented lower F_2-isoprostane concentration when compared with their male counterparts. This is not unexpected since estrogen has shown to have anti-inflammatory and antioxidant properties (Massafra *et al.,* 2000; Bloomer & Fisher-Wellman 2008; Iqbal *et al.,* 2008). This raises the question whether including both men and women in the same study might have influenced the results. It is important to point out that the participants of this study were recreationally trained ($VO_{2\ max}$ 58 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$), making these results relevant to the physically active population but not to elite athletes or sedentary individuals. A summary of the studies mentioned above is provided in Table 6.1.

Table 6.1. Studies of vitamin supplementation on performance and oxidative stress markers

Study	Subjects	Design	Supplement	Exercise	Results
Peters *et al.,* 2001	Trained runners	Placebo (n=6) Supplemented (n=10)	1 week: vit C 1000 ($mg \cdot day^{-1}$)	Ultramarathon (90 km)	No effect on performance
Nieman *et al.,* 2002	Trained runners (male and female)	placebo (n=13) supplemented (n=15)	1 week: vit C 1500 ($mg \cdot day^{-1}$)	Ultramarathon (80 km)	No effect on performance or on lipid peroxidation
Palmer *et al.,* 2003	Trained runners (male and female)	Placebo (n=13) Supplemented (n=15)	1 week: vit C 1500 ($mg \cdot day^{-1}$)	Ultramarathon	No effect on performance or on oxidative stress
Nieman *et al.,* 2004	Triathletes (male and female)	Placebo (n=16) Supplemented (n=16)	8 weeks: vit E (800 $IU \cdot day^{-1}$)	Ironman Triathlon	No effect on performance ↑ oxidative stress in supplemented group
Goldfarb *et al.,* 2005	Non-resistance trained females	Placebo (n =9) Supplemented (n=9)	2 weeks: vit C 1000 mg + vit E 400 IU daily	4 sets of 12 repetitions of elbow flexor	No difference in MDA or GSSG 24 h after exercise ↓ in MDA 48 h after in supplemented group
Davison & Gleeson, 2006	Endurance trained males	Crossover (n=9)	2 weeks: vit C (1000 $mg \cdot day^{-1}$)	2.5 h cycling at 60% VO_{2max}	No difference in MDA post-exercise

Traber, 2006	Recreational runners (male and female)	Placebo (n =11) Supplemented (n=11)	6 weeks: vit C 1000 mg + vit E 400 IU daily	Ultramarathon (50 km)	No effect on performance or DNA damage ↓ lipid peroxidation in supplemented group
Davison *et al.*, 2007	Recreational active males	Placebo (n =10) Supplemented (n=10)	4 weeks: vit C 1000 mg + vit E 400 IU daily	2.5 h cycling at 60% VO_{2max}	No difference in lipid peroxidation post-exercise

While some of the mentioned studies have the advantage of being field studies, they also have the limitation of comparing two different groups of runners. These individuals could present different responses to vitamin supplementation and also to the oxidative stress of the exercise. It would, therefore, be a better study-design to analyse the same runners in both situation: placebo and supplementation. Furthermore, the exercise used in these studies (ultramarathon and ironman) are extremely intense and of long duration. Moreover, some of the studies had elite athletes with a high-training regimen as participants. Hence, their antioxidant defence mechanism was already adapted to protect them against certain levels of oxidative stress (Child *et al.*, 1999; Powers *et al.*, 2004; Bloomer & Fisher-Wellman, 2008), and the natural increase in plasma antioxidants might probably have abolished the effect of additional vitamin supplementation. This, therefore, supports the theory that supplementation of these calibre of athletes is of no benefit.

There is a lack of studies in the literature investigating the effect of these vitamins supplementations on performance outcomes. The majority of studies have focused mainly on the oxidative and inflammatory response after a bout of exercise, though the exercise mode, duration and intensity vary considerably between studies, as well as the antioxidant dosage and duration of supplementation. These facts might account for the inconsistency in the outcomes. The immunological responses will be explored in detail in the next chapter.

Even though more investigation is needed in relation to the benefits, optimal dosage strategy and combination of antioxidant supplementation for active individuals, so far little evidence exists to recommend vitamin intake for the purpose of improving athletic performance. Indeed, from the available literature there has not been a consistent

positive result when it comes to vitamin supplementation improving performance of trained individuals. Nevertheless, it might be more relevant to increase antioxidant intake when, besides the increase in radicals derived from the exercise bout, there are other factors that lead to an increase in the oxidant process which favour oxidative stress. These factors can be a hot environment or a polluted one. In the case of the influence of heat, it has been reported, in studies with animals, *in vitro* studies (Flanagan *et al.,* 1998) and studies with humans (McAnulty *et al.,* 2005a), that both a raise in core temperature and exercising in a hot environment increases the cellular radical production. Such production can contribute to the oxidative process and cellular damage.

Vitamin C and E Supplementation in Individuals Exposed to Ozone-Pollution

As for the oxidant stimulus of air pollution, specifically the potent oxidant gas ozone, a variety of studies have shown that exercising in an ozone-polluted environment can induce oxidative stress, lung inflammation and decrease in lung function as explained in detail in *Chapter 2* (Devlin et al., 1996; Jörres *et al.,* 2000; Ratto *et al.,* 2006). The respiratory tract lining fluid is the first barrier encountered by inspired gases and, therefore, it has a network of antioxidants, such as ascorbic acid, GSH, α-tocopherol and uric acid to provide protection against oxidative stress (Mudway *et al.,* 1999a; van der Vliet *et al.,* 1999; Mudway *et al.,* 2001). For this reason, studies have attempted to investigate the relationship between antioxidant ingestion and increase in antioxidant availability in the respiratory tract lining fluid that would provide additional sacrificial substrates for ozone, which could, consequently, decrease oxidation reactions occurring within this fluid and within the underlining epithelial cells. Unfortunately studies have shown that in practice this relationship is not so simplistic, with factors - such as individual sensitivity to ozone - playing an important role (Mudway *et al.,* 1999b; Mudway *et al.,* 2001). Once inspired, ozone has the potential to cause direct oxidation by reacting with electron donors, which would be the antioxidants present in the RTLF; and by reacting with unsaturated compounds, such as unsaturated lipids (Pryor, 1994). In addition, ozone can also cause indirect oxidation by activating lung inflammation that in turn, would increase the production of ROS which could cause damage beyond the lungs (Pryor *et al*., 1995). Ozone has been shown to deplete the endogenous antioxidant defences, inducing inflammation and oxidative damage in the airways (Mudway *et al.,* 1999a; Mudway *et al.,* 1999b; Mudway *et al.,* 2001).

By increasing the availability of the antioxidants present in the RTLF it can be theorized that there would be an increase in the reaction with ozone, limiting its interaction with the unsaturated molecules as a consequence. In addition, an excess in antioxidants concentration might also confer protection by neutralizing free radical species, derived from these initial reactions or inflammatory cells (Pryor *et al.,* 1995; Mudway *et al.,* 2001). Few studies have investigated this proposed benefit of increasing antioxidant availability in individuals exposed to ozone-pollution by supplementing the participants with vitamins C and E. Indeed, some studies have shown some protective effect when participants are supplemented with a mix of antioxidants. Romieu *et al.* (1998) conducted a field study in which 34 workers (shoe-cleaners) exposed to pollution (Mexico City), participated in a double blind supplementation/placebo crossover design study. The supplementation consisted of a combination of different antioxidants (650 mg vitamin C + 100 IU vitamin E + 15 mg b-carotene) ingested during a 10-week period. The washout period was 2 weeks. The average daily ozone concentration was 0.07 ppm, and on 55% of the days the concentration exceeded the Mexican standard of 0.11 ppm. A protective effect of antioxidant supplementation against ozone exposure on lung function (FEV, FVC, FEF_{25-75}) was reported. The authors, however, did mention that individuals who consumed antioxidants first presented less lung function impairment after consuming placebo than subjects that initially ingested placebo. The authors attributed this result to the short washout period, especially for vitamin E, which accumulates in the tissues, but they did not investigate this issue. Field studies such as this one have some limitation such as the interference of other pollutants and the concentration of the analyzed pollutant throughout the long period in which the study was conducted. In addition, the participants of this study were not exercising while exposed to the pollution.

Two subsequent field studies, conducted with amateur and recreational cyclists (Grievink *et al.,* 1998; Grievink *et al.,* 1999), reached similar results in relation to antioxidant supplementation providing some protection against acute effects of ozone on lung function. Grievink *et al.* (1998) observed two groups of cyclists, during a 3-month period. One group was supplemented with vitamin C (650 $mg \cdot day^{-1}$), vitamin E (100 $IU \cdot day^{-1}$) and β-carotene (15 $mg \cdot day^{-1}$), while the other ingested placebo. This study was conducted during the summer months, and the lung function of the cyclists

was measured before and after training or competition on 4 to 14 occasions. Of note, the supplementation started 1 week before the first measurement and was maintained during the study period, this could have influenced the result as the participants were not all tested on the same occasions. The mean temperature throughout the study period was 23ºC and the ozone concentration averaged 0.05 ppm. In the subsequent study by the same group (Grievink *et al.,* 1999), the same protocol was followed in relation to the exercise and measurements during the summer; however, the supplementation protocol varied slightly (3 months of daily vitamin C 500 mg and vitamin E 150 IU). In this study, even though it was reported that the supplementation was able to partly counteract the decreases of lung function, the authors also mentioned that when participants that had not complied fully with the supplementation were excluded from the analysis the effects of ozone on lung function were no longer seen. The average temperature and ozone concentration in this study were lower than in the previous study: 17ºC and 0.04 ppm respectively. Both studies presented some disadvantages. Firstly, they analysed 2 different groups of individuals, making comparison challenging as the effects of ozone have been shown to vary a lot from one individual to another. Secondly, the placebo group and the vitamin group were not balanced in relation to individuals presenting respiratory allergies or asthma. In addition, in the latter study (Grievink *et al.,* 1998), the placebo group, acting as the control, was not taking any pills, therefore, it was not blinded. Thirdly, some individuals that were in the supplemented group were already taking antioxidants prior to the start of the study. And, finally, it was reported that the adjustment for environmental temperature as a possible confounder was difficult due to the high correlation with ozone. All in all, it is important to view these results with caution because a variety of uncontrolled variables could have influenced the outcome.

In the study of Samet *et al.* (2001), participants first underwent a 1 week period of vitamin C-restricted diet. After this, one group received supplementation (250 mg vitamin C + 50 IU vitamin E + 12 oz of carrot and tomato juice), while the other group received placebo and was still on the restricted diet. The supplementation period consisted of a 2-week period after which the subjects underwent a 2 h low-intensity exercise protocol in a high ozone-polluted chamber (0.4 ppm). After exposure, subjects completed a respiratory symptom questionnaire, performed lung function tests and underwent a bronchoalveolar lavage. There were no differences between the

supplemented group and the placebo group in respect to the respiratory symptom questionnaire. This suggests that dietary antioxidants do not minimize the perceived harmful effects of ozone. In addition, there were no differences in neutrophil counts or other inflammatory markers in the bronchoalveolar lavage fluid. Nevertheless, the authors did report attenuation in lung function impairment in the subjects who ingested the antioxidant mixture. Contrary to this finding, Mudway *et al.* (2006) did not report any changes in lung function when they conducted a double-blind crossover study. The supplementation (500 mg vitamin C + 150 IU vitamin E daily) period used in this study was smaller than most supplementation protocols: just 1 week, with a 2-week washout period. The exposure protocol consisted of 2 h of intermittent cycling in a chamber with 0.2 ppm of ozone. In addition to the lack of changes in lung function, there were no differences in airway inflammation, which was assessed 6 h post-exposure. It is important to point out that, after the supplementation protocol, the subjects did present an increased concentration of plasma ascorbic acid and α-tocopherol. This increased concentration, however, was not observed in the respiratory airways when it was accessed 6 h after the ozone exposure. Nevertheless, the authors did report movement of α-tocopherol from the plasma into the RTLF following ozone the ozone challenge. A summary of the studies presented above is provided in Table 6.2.

Table 6.2. Studies investigating vitamin C and E supplementation and ozone exposure

Study	Subjects	Design	Supplement	Exercise and Ozone levels	Results
Romieu *et al.*, 1998	Shoe-cleaners	Field study Crossover (n=34)	10 wks: vit C 650 mg + vit E 100 IU + b-carotene + 15 mg daily	Daily work Average of 0.07 ppm O_3	Attenuation of lung function decrements with supplementation
Grievink *et al.*, 1998	Amateur and recreational cyclists	Field study Placebo (n =18) Supplemented (n=20)	Started 1 wk before 1st measurement, total of 3 months: vit C 650mg + vit E 100 IU and b-carotene 15mg daily	Training sessions Average of 0.05 ppm O_3	Supplementation provided some protection on lung function

Grievink *et al.*, 1999	Amateur cyclists	Field study Placebo (n =9) Supplemented (n=11)	Started 1 wk before first measurement, total of 3 months: vit C 500 mg + vit E 150 IU daily	Training sessions and competitive races Average of 0.04 ppm O_3	No effect on lung function
Samet *et al.*, 2001	Male and female, physical fitness not specified	Placebo (n =16) Supplemented (n=15)	Placebo: 3 wks vitamin restriction Supplemented: 1 wk vitamin restriction + 2 weeks of 250 mg vitamin C + vitamin E 50 IU + 12 oz of carrot and tomato juice daily	2 h low-intensity intermittent exercise on treadmill or cycling 0.4 ppm O_3	Attenuation of lung function decrements with supplementation No differences in respiratory symptoms or lung inflammation
Mudway *et al.*, 2006	Male and female, physical fitness not specified	Crossover (n=14)	1 week: vit C 500mg + vit E 150 IU daily	2 h intermittent cycling 0.2 ppm O_3	No effect on lung function No effect on lung inflammation

In general, the few studies that have attempted to investigate the effects of vitamin supplementation on exercising individuals exposed to ozone-pollution have not presented a major positive outcome. This could be due to the population investigated, the supplementation protocol, the exercise bout and the study design. In addition, they did not research the influence of these antioxidants on performance in a polluted environment. And, as an increase in ozone-pollution is related to an increase in environmental temperatures, investigating these factors together is needed in order to provide a more relevant environmental condition and applicable results.

The rationale for the study presented in this chapter is based on previous findings related to the relationship between high levels of antioxidant and performance improvement in a hot, humid and ozone-polluted environment (*Chapter 5*). It was hypothesized that supplementation with the antioxidants vitamin C and E would decrease oxidative stress and alleviate respiratory symptoms experienced by trained runners when performing a high intensity exercise in those adverse conditions. This, in turn, would be translated into an improvement in performance when compared with the same athletes ingesting placebo. Results from previous studies investigating the effect of supplementation with these vitamins, alone or combined, have been inconclusive.

Possible reasons for these divergences comprise differences in exercise mode, fitness level of participants and study methodology. Due to the high antioxidant consumption, specifically in the form of vitamins, by the physically active community and by a large portion of the general population, make this an important topic for research, especially when coupled with the fact that large urbanized areas might provide an additional reason, in the form of air pollutants, to increase the antioxidant intake.

6.2 METHODS

Detailed descriptions of certain methods used in this study have been fully elucidated in *Chapter 3*. Any specific methodology used in this study will be fully described here, the others, previously described, will be summarized.

6.2.1. Participants

Ten well trained male runners took part in this study (mean ± SD, age = 29 ± 8.1 years; mass 67.0 ± 4.9 kg; height = 175.5 ± 5.7 cm; $\dot{V}O_{2\,max}$ = 65.8 ± 4.2 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$). The participants were all competitive runners who had to achieve a criterion $\dot{V}O_{2\,max}$ minimum value of 60 $mlO_2 \cdot kg^{-1} \cdot min^{-1}$. The adequacy of each subject's $\dot{V}O_{2\,max}$ was tested during the familiarization trial (see section 3.1.1 for further details). One of the runners dropped out of the study after his first experimental trial due to the severity of respiratory symptoms that he experienced while performing the time-trial run in the ozone polluted environment.

6.2.2 Experimental Procedures

This study took place during the autumn and winter months and had a randomized double-blinded crossover design. After the familiarization trial, the participants were randomly assigned to initiate a 2-week supplementation period of vitamins C (500 $mg \cdot day^{-1}$) and E (100 $IU \cdot day^{-1}$) or placebo (500 $mg \cdot day^{-1}$ of sucrose). They were instructed to take the pills once a day with their breakfast and the last supplementation was taken on the morning of the exercise protocol. After a 1-week washout period the athletes initiated a further 2-week supplementation, with the participants that initially

took the vitamins taking now the placebo and vice-versa (Figure 6.2). The pills for the vitamins and placebo looked exactly the same and when asked if they knew what they were taking all participants said they had no idea. In addition, they were asked to refrain from any physical activity 24 h before the exercise trials.

Figure 6.2. Double-blind randomized crossover study design.

On the exercise trial day the participants arrived at the lab at 8:40am, and remained seated for 5 minutes before their blood pressure was measured to check if it was within the normal range. During this 5-minute period they completed a health questionnaire (Appendix IV) to assess if they were in good health to take part in the exercise trial. If they presented signs of cold or flu they did not take part in the exercise and were asked to return 2 weeks after they recovered. The participants then had blood samples taken, gave a saliva sample, performed the lung function test (FVC, FEV_1, FEF_{25-75} and PEF) and the nasal lavage procedure. The exercise trials took place in the environmental chamber, details are given in section 3.2. The exercise consisted of an 8 km time trial run on a treadmill (Woodway, PPS 55 Med, Germany), which the participants were required to complete as fast as they could. The runners controlled their own speed, but they did not have access to the value of the speed. The environmental condition was 0.1ppm of O_3 at 31 ºC and 70% rh. Immediately post-exercise and 6 h after the exercise the athletes went through the same tests: blood samples were taken, saliva sample, lung function and nasal lavage. The subjects were asked to write down the food they consumed during the first exercise trial day and on the next trial to repeat it. The exercise protocol was the same used previously and has been detailed in section 3.3. The athletes had access to water *ad libitum* during the first trial, however this water intake was measured and the same amount was made available on the second trial.

Assessment of Respiratory Symptoms. The severity of respiratory symptoms was assessed by a written questionnaire after the athletes completed the exercise protocol as described previously in section 3.3.1.

To analyze the impact of the supplementation protocol on the antioxidant status and oxidative stress of the runners, the following variables were assessed:

- In nasal lavage: Vitamin C concentration, vitamin E concentration, trolox equivalent antioxidant capacity, GSH/Protein, and MDA as a marker of lipid peroxidation.
- In plasma: Total antioxidant concentration, vitamin C concentration, vitamin E concentration, GSH/Protein and MDA.

6.2.3.1 Biochemical Analysis

Total antioxidant and Vitamin C

The Vitamin C content in the plasma and nasal lavage samples was measured using a Ferric Reducing Ascorbate assay (FRASC, BioVision). The plasma samples used for this colourimetric assay were collected with a vacutainer containing sodium heparin as an anticoagulant. In this assay Fe^{+3} is reduced to Fe^{+2} by any antioxidant present in the sample, thus allowing the determination of the total antioxidant concentration present in the sample. In addition, the ascorbic acid concentration can also be obtained by the addition of ascorbate oxidase, which is added into parallel samples removing any ascorbic acid present, leaving a background value which is then subtracted from the total (samples without addition of ascorbate oxidase). In brief, 100 µl of samples and standards were added in duplicate to a 96 microwell plate. For the samples, one well of the pair was to determine the total antioxidant present, while the second well was for the ascorbate depletion (background well). The ascorbic acid standards were prepared with distilled water and varied 10 – 2 nmol. Ascorbate oxidase (10µl) was then added to the background well and 10 µl of distilled water to the total antioxidant well. The plates were then incubated at room temperature for 15 min to allow the depletion of the ascorbic acid. Following that, 100µl of the Reaction Mix (80% FRASC Buffer + 10% Ascorbic Acid Probe + 10% $FeCl_3$ solution) was added to each well. The plate was read immediately using a wavelength of 593nm in a 96 well plate reader. The standard curve was obtained on a linear/linear fitted line plot against absorbance and the

concentration of ascorbic acid calculated by the difference between the total antioxidant well and the background well.

Vitamin E

The vitamin E concentration of the plasma and nasal lavage was measured using high performance liquid chromatography (HPLC) with the method described by Siluk et al. (2007). Unfortunately due to problems with the equipment this procedure could not be done accurately. The method used was as follows. Stock solution of the standard was prepared diluting the α-tocopherol (6.0M) standard with the mobile phase (70% acetronitrile + 30% methanol) to a concentration of 1 $mg \cdot ml^{-1}$. For each sample (200µl) were added 20 µl of the standard solution and 200µl of purified water, the resulting solution was vortex for 10 sec. After adding 400µl of ethanol the solution was vortexed for another 10 sec. Hexane (800µl) was added to the solution which was then vortex for 3 min, and then centrifuged at 3500g for 10 min at 10 ºC. After these procedures, 700µl of the hexane layer was transferred to a glass tube and the hexane was evaporated under a stream of nitrogen. The resulting pellet was re-suspended in 200µl of methanol and vortexed for 30 sec. The mixture was transferred to an amber vial and 20µl was injected into the HPLC system for analysis. The chromatography was carried out using a Varian Prostar HPLC System (Varian Prostar AutoSampler, 410, NL) with a Zorbax Eclipse XDB-C18 column (150 mm x 4.6 mm i.d., 5 µm particle size). A Shimadzu UV-160A spectrophotometer was used to determine the absorbance of the solutions. The column was thermostated at 30 ºC, and the flow rate set at 1 $ml \cdot min^{-1}$, the excitation wavelength was set at 295 nm and the emission wavelength set at 330 nm. Unfortunately due to problems with the HPLC equipment this procedure could not be carried out appropriately.

Lipid Peroxidation Marker

In an attempt to measure lipid peroxidation, in plasma and nasal lavage, the assay that quantifies MDA, as a marker of this oxidative stress process, was used. Initially, the samples were thawed and 200µl of each sample was placed into two eppendorfs – one to be the blank sample and the other containing the reagent. MDA (1,1,3,3-tetramethoxypropane, in 20mM Tris-Hcl, pH 7.4) standards were prepared to a final

concentration of 0-20μM. An aliquot of 10μl 0.5M BHT in acetonitrile was added to the samples and standards followed by the addition of 650μl of diluted reagent 1 (three volumes of 10.3mM N-methyl-2-phenylindole in acetonitrile, diluted with one volume of 100% methanol HPLC grade). For the blank samples instead of reagent 1 a solution made up of 75% acetonitrile and 25% methanol was added. Each eppendorf was gently vortexed before adding 150μl of reagent 2 (15.4M methanesulfonic acid). After that, the solution was mixed and incubated at 45ºC for 60min. Samples were then centrifuged at 15,000g for 10 minutes (1K15, Sigma Laboratory centrifuges, Osterode, Germany). The supernatant was then removed and the absorbance was read at 586nm (Dynatech MRX, UK).

Statistical Analysis

All data were checked for normality before statistical analysis. Data were analyzed using General Linear Model ANOVA (Minitab15 Statistical Software) with Tukey's *Post-hoc* test. Statistical significance was accepted at $P < 0.05$. In order to verify any association between the variables a correlation matrix was conducted, with a subsequent multi-regression analysis (Minitab15 Statistical Software). Data are expressed as mean values ± standard deviation (SD).

6.3 RESULTS

6.3.1. Medical questionnaires

One of the participants presented asthma but it was under medical control, and another presented hay fever. This research was conducted during the autumn and winter period therefore, the hay fever problem was not an issue.

6.3.2. Plasma and Nasal Lavage Antioxidant Concentration

The vitamin C and the total antioxidant concentration of the plasma were measured using the FRASC assay. This assay was not sensitive enough to measure the values in nasal lavage. Therefore, the antioxidant concentration of the nasal lavage fluid was measured using the TEAC assay, which was known to work with the nasal lavage

samples as it was successfully used in the previous study.

Vitamin C

The plasma vitamin C concentration was significantly higher when the athletes ingested the vitamin supplementation both pre and post-exercise compared to the placebo values (Figure 6.3). There was a trend for the plasma vitamin C to increase immediately after exercise in both trials; this however was not statistically significant.

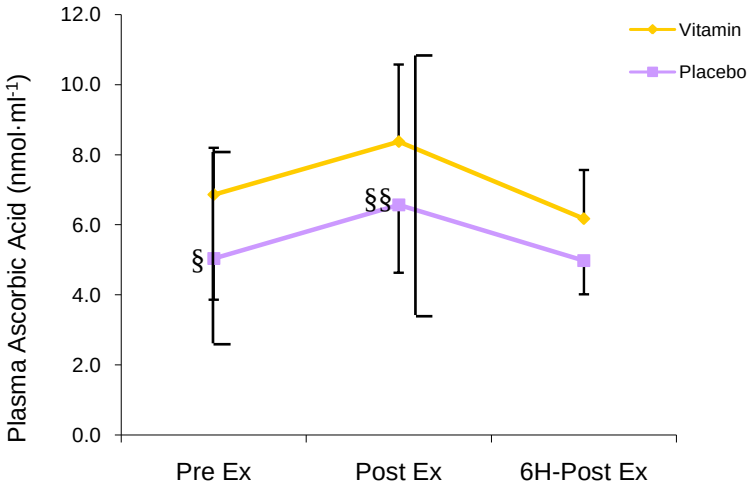


Figure 6.3. Total plasma vitamin C concentration. § Significant differences between trials at the pre-exercise time point ($P < 0.01$); §§ Significant differences between trials at the post-exercise time point ($P < 0.05$). Mean ± SD.

Plasma total antioxidant concentration

Similarly to the previous result, the total antioxidant concentration in the plasma was significantly higher pre and post-exercise in the Vitamin trial compared with the Placebo trial. In addition the post-exercise values for plasma total antioxidant concentrations, in both the Vitamin and Placebo trials were significantly elevated compared with their respective pre and 6 h-post values. This percentage increase did not vary between the trials (Figure 6.4.).

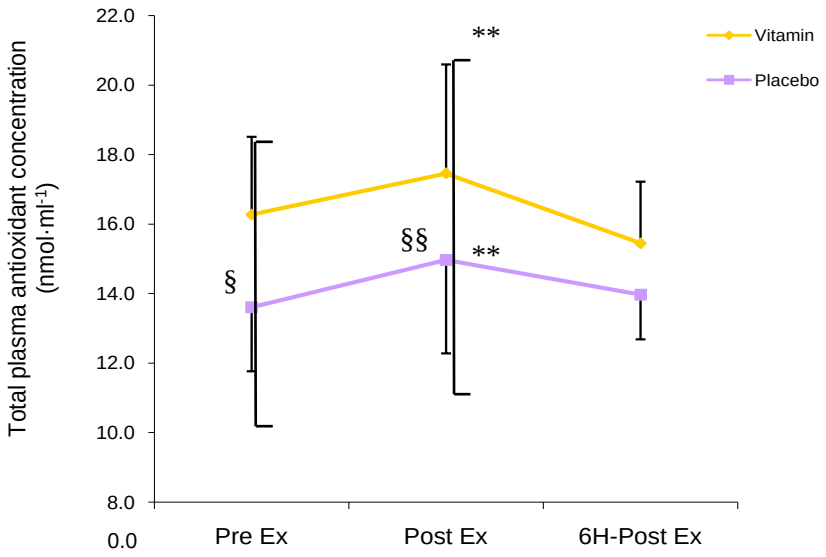


Figure 6.4. Total plasma antioxidant concentration. § Significant differences between trials at the pre-exercise time point (P<0.01); §§ Significant differences between trials at the post-exercise time point (P<0.05); ** Significantly different from pre-exercise and 6 h- Post exercise time points in the same trial (P<0.01). Mean ± SD.

Nasal lavage trolox equivalent antioxidant capacity (TEAC).

The TEAC of the nasal lavage did not vary between the trials (P =0.059) as was seen in the plasma samples. Nevertheless at the post-exercise time point there was a significant increase in the antioxidant capacity of the nasal lavage in the Vitamin trial compared with the pre and 6 h-post values. This pattern was not observed for the Placebo trial. At the 6 h time point in the Vitamin trial the values had returned to baseline (Figure 6.5.).

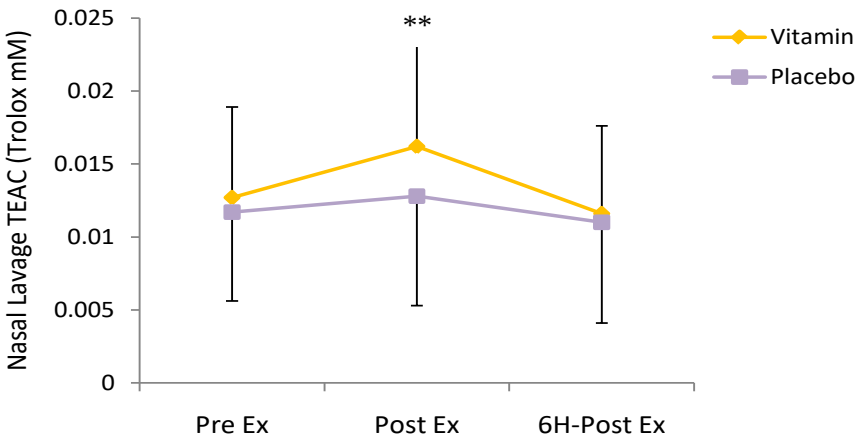


Figure 6.5. Trolox equivalent antioxidant capacity of nasal lavage. ** Significant increase compared to pre-exercise and 6 h-post exercise within the same trial (P<0.05). Mean ± SD.

Plasma and nasal lavage GSH/Protein

No significant differences were observed between trials or between time points for either the plasma or nasal lavage GSH/Protein concentration (Table 6.3.). There was a trend for an increase in this antioxidant concentration post-exercise in the Vitamin trial, but it did not reach statistical significance. The intra-assay coefficients of variation for the duplicate samples were 4.1% and 3.4% for the plasma and nasal lavage samples respectively.

Table 6.3. GSH/protein concentration in plasma and nasal lavage in both trials.

	Vitamin Trial			**Placebo Trial**		
	Pre Ex	Post Ex	6H-Post Ex	Pre Ex	Post Ex	6H-Post Ex
Plasma GSH/protein x10 ^{3} (µmoles·mg^{-1})	1.2 ± 0.7	0.9 ± 0.2	1.1 ± 0.3	1.2 ± 0.6	1.0 ± 0.3	0.8 ± 0.3
NL GSH/protein (µmoles·mg^{-1})	0.08 ± 0.03	0.11 ± 0.03	0.08 ± 0.05	0.09 ± 0.05	0.09 ± 0.04	0.09 ± 0.06

Values are mean ± SD.

Lipid Peroxidation

The MDA assay was carried out in an attempt to measure lipid peroxidation in the plasma and nasal lavage samples. The assay however was not sensitive enough to

quantify the MDA levels of the samples.

6.3.3 Performance variables

The results for total time to complete each trial are presented in Figure 6.6. There was a trend for the participants to complete the Vitamin trial faster (P=0.075).

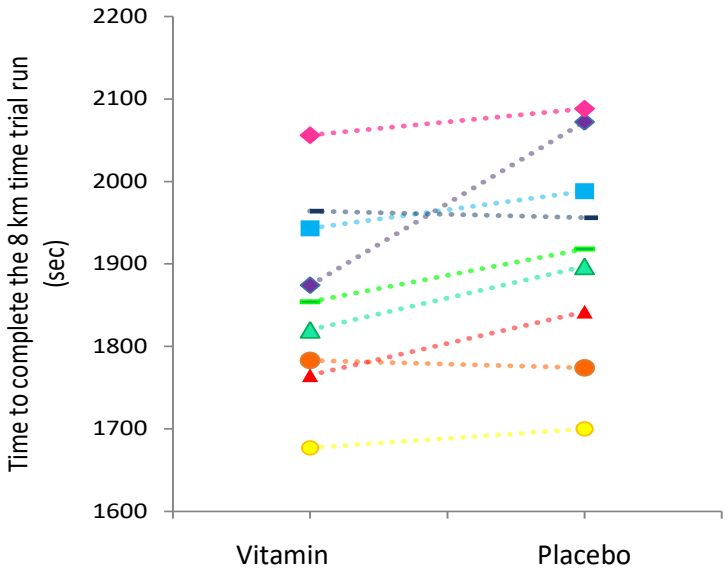


Figure 6.6. Individual time to complete the 8 km run.

There was an average increase of 49 sec in the time to complete the Placebo trial compared with the Vitamin one. This difference is equivalent to a 2.6% improvement in the performance when the runners were taking the vitamin supplementation. The 95% Confidence Interval (lower; upper limits) for the trials for this variable was: (-98.2; 6.0).

Results for heart rate, RPE, speed, expired air and $\dot{V}O_2$ for each of the trials are presented in Table 6.4. There were no differences between the trials for the variables analysed.

Table 6.4. Effect of exercise trial on heart rate, RPE, speed, expired air and oxygen consumption.

	Vitamin Trial	**Placebo Trial**
Average Speed ($km \cdot h^{-1}$)	15.5 ± 1.2	15.1 ± 1.2

Mean Heart Rate (beats·min^{-1})	172 ± 9	167 ± 12
Peak Heart Rate (beats·min^{-1})	191 ± 9	192 ± 12
Mean RPE	15 ± 1.2	15 ± 1.2
Peak RPE	18 ± 1.5	19 ± 1.2
Expired Volume (l·min^{-1})	110.1 ± 13.2	122.6 ± 30.9
$\dot{V}O_2$ (mlO_2·kg^{-1}·min^{-1})	59.0 ± 3.3	61.2 ± 8.1

Values are mean ± SD.

6.3.4 Lung Function

Changes in lung functions pre, post and 6 h-post run were compared within and between trials. No significant differences were found for any of the analyzed variables (FVC, FEV_1, FEF_{25-75} and PEF) within the same trial or between trials (Table 6.5). In addition, when the runners' pre and post FEV_1 was analyzed it was seen that none of them presented exercise induced asthma, which is characterized by a 10% or more decrease in this variable (Rundell & Jenkinson, 2002).

Table 6.5. Lung function measures for both trials.

	Vitamin Trial			**Placebo Trial**		
	Pre Ex	Post Ex	6H-Post Ex	Pre Ex	Post Ex	6H-Post Ex
FVC (l)	5.1 ± 1.2	4.9 ± 1.2	5.0 ± 0.9	5.0 ± 0.9	4.7 ± 1.2	5.0 ± 0.9
FEV_1 (l)	4.1 ± 0.9	4.2 ± 0.9	4.1 ± 0.9	4.2 ± 0.9	4.0 ± 0.9	4.3 ± 0.9
PEF (l·min^{-1})	621 ± 96	616 ± 105	574 ± 108	609 ± 75	576 ± 111	601 ± 108
FEF_{25-75}	4.0 ± 1.2	4.5 ± 1.5	4.2 ± 1.5	4.2 ± 1.2	4.4 ± 1.8	4.9 ± 1.5

Values are mean ± SD.

6.3.5 Respiratory Symptom Questionnaire

An overview of the respiratory symptoms reported by the subjects in response to the trials is given in Figure 6.7. In the Placebo trial there is a trend to increase the severity of respiratory symptoms experienced by the athletes. However, this does not reach significance in any symptom or in the overall score. In response to the question of whether they felt that they would be able to perform maximally in a competition in

the environmental conditions tested, all ten runners reported they would not be able to perform maximally in the Placebo trial and two out of nine subjects reported they would be able to perform maximally in the Vitamin trial. One subjects dropped out of the study after his first trial due to difficulty in breathing. After the completion of the study it was seen that it was his Placebo trial.

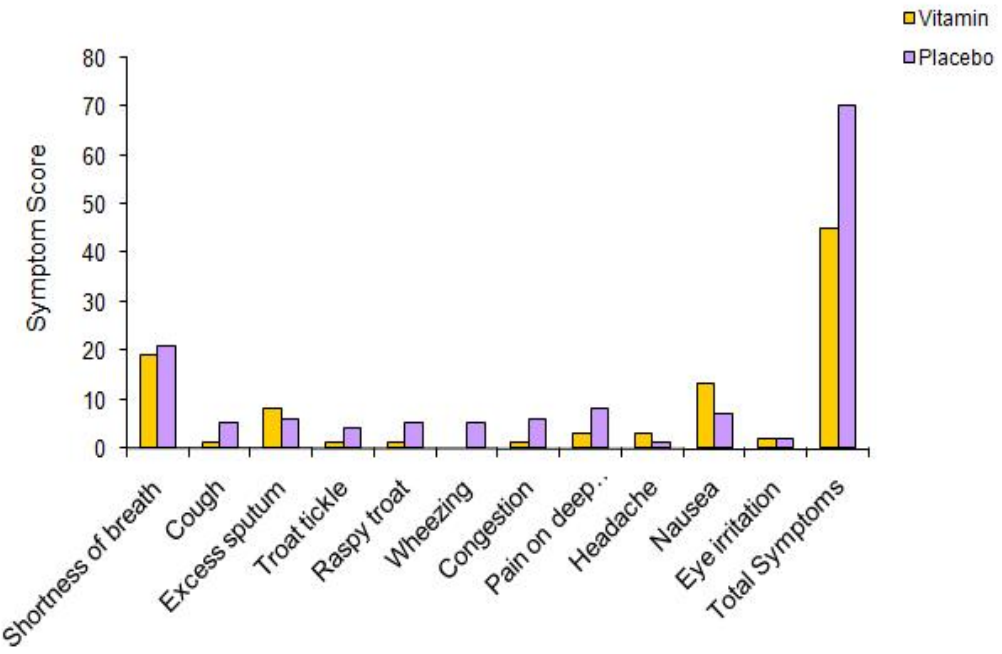


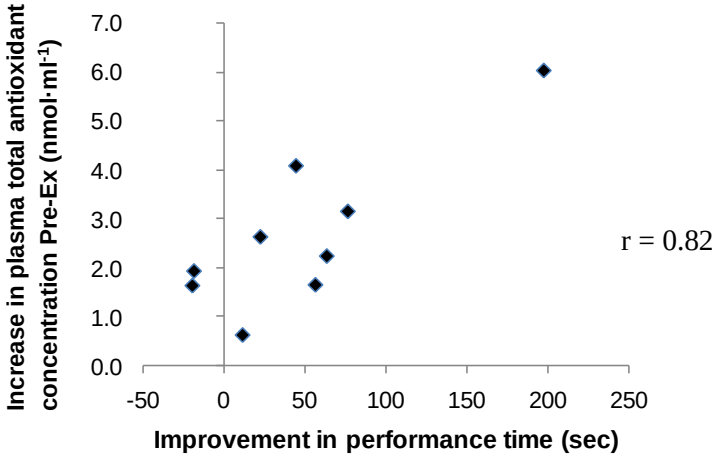
Figure 6.7. Respiratory symptoms presented by the runners after the trials.

6.3.6 Correlations

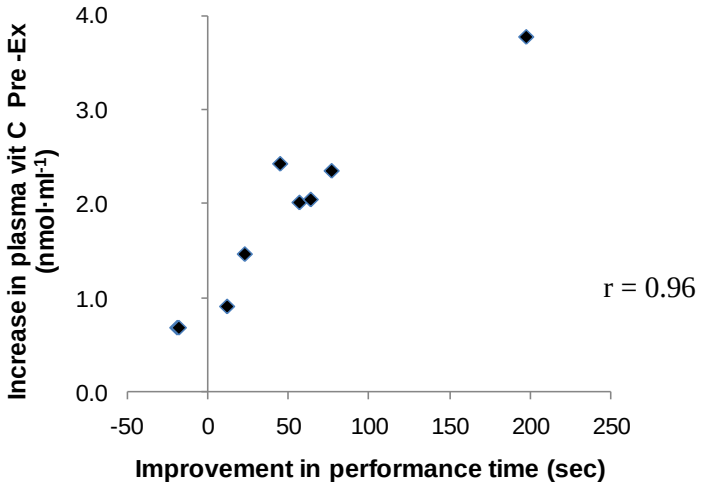
There was a significant positive correlation between the increase in antioxidant concentration after the vitamin supplementation and the difference in performance between the two trials. That is, participants who presented a higher antioxidant concentration had a better improvement in their time to complete the 8 km run. This was true for the plasma vitamin C levels pre exercise, the plasma total antioxidant concentration pre-exercise, and the nasal lavage total antioxidant concentration post-

exercise (Figure 6.8. A, B and C).

A



B



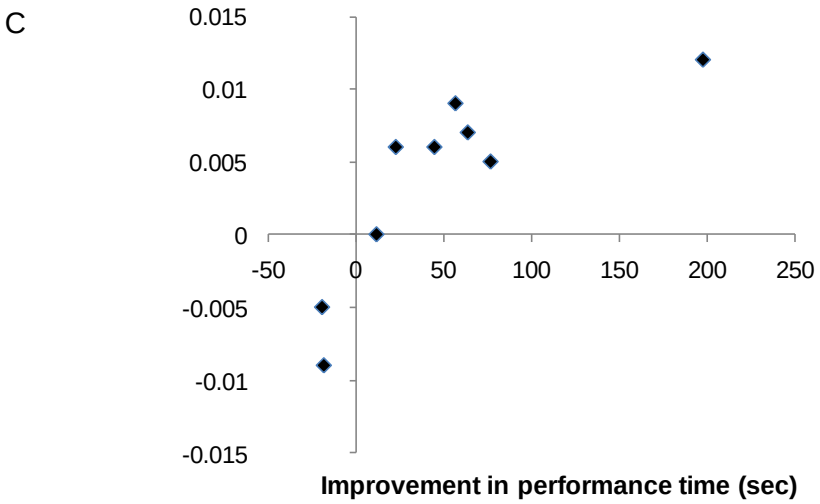


Figure 6.8. Relationship between improvement in performance and increase in the plasma total antioxidant concentration pre-exercise (A), plasma vitamin C concentration pre-exercise (B), and nasal lavage total antioxidant concentration post-exercise (C), $P < 0.01$.

6.4 DISCUSSION

In this chapter the hypothesis tested was that the combination of vitamin C and E supplementation would increase both the systemic and the airway antioxidant concentration. As a consequence, this would decrease the oxidative stress and respiratory symptoms of the participants running in a hot, humid and ozone-polluted environment, thus, resulting in an improvement in their performance when compared with the ingestion of placebo. The main findings of this study were: (1) the supplementation protocol significantly increased the plasma antioxidant concentration pre and post-exercise compared with the placebo ingestion; (2) there was a significant increase in the nasal lavage antioxidant capacity post-exercise in the Vitamin trial; (3) there was a 2.6% improvement in the time to complete the trial when the participants ingested the vitamins; (4) significant positive correlations were found between antioxidant concentration and improvement in time to complete the run in the hot polluted environment.

Vitamins C and E were used as supplements in this study because these two antioxidants are present in the RTLF, they have strong antioxidant properties and together they have been shown to present a synergistic effect in the protection against

oxidative stress (Mudway *et al*., 2001; Rietjens *et al*., 2002). In addition, vitamin E is the major lipophilic antioxidant in human tissues, including in the airways (Cross *et al*., 2006); and vitamin C has been linked to aspects of lung health, such as improvement in lung function (Romieu *et al*., 2006), positive effect on exercise-induced bronchoconstriction in asthmatic individuals and decrease in respiratory symptoms post-exercise (Teckenburg *et al*., 2007). Moreover, the supplementation with these two vitamins has been shown to provide some protection in humans exposed to ozone-pollution (Romieu *et al*., 1998, Grievink *et al*., 1998; Grievink *et al*., 1999; Samet *et al*., 2001; Sienra-Monge *et al*., 2004).

A supplementation protocol of two weeks with 500 $mg \cdot day^{-1}$ of vitamin C was chosen as being able to saturate the plasma and maintain a steady state after 1 week of ingestion (Jacob *et al*., 1987). A dose of 100 $IU \cdot day^{-1}$ of vitamin E was selected, based on the benefits reported by studies which used this concentration (Grievink et al., 1998; Romieu et al., 1998), and the 2-week supplementation period has been shown to be sufficient for plasma concentration of α-tocopherol to reach a steady state (Fisher et al., 2004). These values are higher than the RDA (Institute of Medicine, 2000), but they are also the lowest concentrations that have shown to provide benefits in supplementation studies, as previously described. As the participants of this study maintained their training protocol throughout the duration of the study, a longer supplementation protocol could potentially interfere with their training adaptations (Gomez-Cabrera *et al*., 2008b; Ristow *et al*., 2009). The supplementation protocol used in the present study was shown to be effective in increasing the plasma total antioxidant and vitamin C concentrations at baseline compared with the Placebo trial. This trial difference was maintained for both the total antioxidant and the vitamin C concentrations immediately after exposure, but after 6 h there were no significant differences between the two trials. In addition, post-exercise there was a significant increase in the plasma total antioxidant concentration in both trials compared with their respective pre values. As for vitamin C concentration, there was a similar increasing trend post-exercise in both trials. This pattern of vitamin C change in the plasma is corroborated by other studies (Palmer *et al*., 2003; Traber, 2006), that also show an increase in this vitamin after the exercise in both groups taking vitamin or placebo. A significant increase in plasma total antioxidant concentration after exercise and the lack of a significant increase in the vitamin C levels suggests that the exercise and adverse environmental conditions are stimulating the up-

regulation or mobilisation of a network of antioxidants. Vitamin E could possibly have contributed to that, however we were unable to measure the vitamin E concentration due to technical problems.

In the study of Mudway *et al*. (2006), subjects went through a 1-week supplementation period with 500 mg of vitamin C and 150 IU of vitamin E before being exposed to 0.2 ppm of ozone and performing a 2 h intermittent cycling exercise. Analysis of the plasma showed that vitamin C concentration was significantly elevated before commencing the exposure protocol, and peaked immediately post-exercise, before returning to pre-exercise exposure levels 6 h later. This pattern was not observed for the Placebo trial, which did not show any variation in this antioxidant concentration throughout the trial. As for plasma vitamin E, the 1-week supplementation period was sufficient to significantly increase the basal concentration, and there was a progressive increase over the 8 h experimental period. Bronchial wash and the bronchoalveolar lavage was conducted 6 h after the exposure protocol. When analysing the airways, the authors reported no significant increase in vitamin C in either trial. On the other hand, the vitamin E levels were increased after exposure, and this response appeared more pronounced in the supplementation trial. It is worth noting that, while the 6 h time point is appropriate to measure markers of inflammation, such as neutrophil influx to the airways, it might be too late to analyse the low molecular weight antioxidant concentration in the respiratory tract and evaluate its relationship with the plasma concentration of the same antioxidant.

In the present study the vitamin C and E of the airways were not successfully measured, possibly because of the high dilution of vitamin C in the nasal lavage, and because of technical problems for the vitamin E. It was possible, however, to measure the trolox equivalent antioxidant capacity of the nasal lavage. At baseline there was no difference between the Vitamin and Placebo trial. Immediately after the exercise it was observed that an increase in the nasal lavage trolox equivalent antioxidant capacity occurred only in the Vitamin trial. It can be speculated that the higher antioxidant concentration in the plasma allowed a more efficient up-regulation of the antioxidant defences in the upper respiratory airways. Even though this mobilization from the plasma to the airways might not be so simplistic, it has been suggested to occur in other studies that assessed

different antioxidants, such as a-tocopherol, vitamin C, GSH and uric acid (Mudway *et al*., 1999b; Blomberg *et al*., 1999; Mudway *et al*., 2001; Schock *et al*., 2004; Mudway *et al*., 2006). In fact, an increased vitamin C intake has been shown to boost this antioxidant concentration in the RTLF within 2 h of intake. This fast redistribution of vitamin C between the plasma and the airways, measured by Schock *et al*. (2004) using nasal lavage procedure, most likely occur by passive diffusion, facilitated mainly because of the high vascularisation of the nasal mucosal tissue. This fact might also facilitate the mobilization of other antioxidants from the plasma to the nasal mucosa region. Nevertheless, due to the lack of data on the vitamin C and E status in the nasal lavage it is impossible to speculate how these vitamins actually contributed to the increase in the nasal lavage antioxidant capacity in the present study. The lack of increase of these vitamins in the lower airways reported in studies that have used vitamin supplementation analysing the lower airways could be attributed to a failure of the supplementation to enhance the antioxidant defences in those regions of the airway. In addition, the supplementation protocol and the time point used to assess the airway antioxidant status could also account for those outcomes (Mudway *et al*., 1999b; Mudway *et al.*, 2006).

Most studies that have analysed the performance of subjects after supplementation with vitamin C and E have used as an exercise protocol an actual competition such as an Ironman (Nieman *et al*., 2004; McAnulty *et al*., 2005b), or an ultramarathon (Palmer *et al*., 2003; Mastaloudis *et al.*, 2004; 2006). These studies failed to show a benefit in performance with the use of vitamin supplementation, even though the plasma levels of the supplemented antioxidants were elevated before and during the races. These studies have the limitation of comparing two groups of athletes; in addition, most of them used long supplementation period that varied from 6 to 8 wks and high supplementation dosage, factors which might have interfered with the training adaptations or stimulated the pro-oxidant effect of the vitamins (Gomez-Cabrera *et al*., 2008b; Ristow *et al*., 2009). Moreover, these studies are not directly comparable to the present one because they were not analysing the effect of ozone-pollution and temperature on the outcome of the results, therefore it is with caution that the results are compared. In the present study the time to complete the Vitamin trial (31:05 min) was an average of 49 sec faster compared with the Placebo trial (31:54 min), although this difference did not reach statistical significance (P=0.075). Nevertheless, it is important to point out that while it is not a statistically significant result, the difference in performance observed between

the two trials was of 2.6%. As mentioned previously in *Chapter 4*, according to Hopkins and Hewson (2001) this improvement in performance observed after the supplementation period is over 5 times higher than the necessary improvement (0.5%) suggested to be significant in a short-duration competition environment; or more than twice the necessary improvement (1%) to significantly increase an athlete's chance of winning a medal in a marathon or half-marathon competition. It therefore, seems reasonable to speculate that this improvement in performance might be ecologically relevant. Due to the lack of studies in the literature analysing the performance outcome in a polluted environment after vitamin supplementation these results cannot be compared to others.

Still in regard to the performance outcome, some interesting correlations were observed. First, individuals that presented a more pronounced increase in the baseline plasma total antioxidant concentration with the supplementation protocol also presented a higher improvement in their performance in the Vitamin trial compared to the Placebo ($R=0.82$, $P<0.01$). This correlation was even higher for the increase in baseline plasma vitamin C concentration and performance ($R=0.96$, $P<0.01$). A third important correlation was the positive relationship between the post-exercise increase in nasal lavage trolox equivalent antioxidant capacity in the Vitamin trial and the improvement in performance outcome ($R=0.81$, $P<0.01$). Observing those correlations it can be speculated that the ozone susceptibility of an individual that is exercising could be influenced by the antioxidant availability in their plasma and airways. In addition, the correlation between nasal lavage trolox equivalent antioxidant capacity post-exercise and performance seems to suggest that it is not the initial absolute antioxidant status in the upper respiratory airway which is critical in the performance outcome, but rather the capacity to up regulate a protective response to the oxidative stimulus. In the previous study (*Chapter 5*) this correlation between performance outcome and antioxidant status in the upper respiratory airways was also observed when the participants performed in the Heat + O_3 trial.

It was also observed that even though the runners, when in the Vitamin trial, were all taking the same quantity of daily vitamins, the individual baseline values varied widely from 13.3 $nmol \cdot ml^{-1}$ to 19.2 $nmol \cdot ml^{-1}$ of total antioxidant and from 5 $nmol \cdot ml^{-1}$ to 8 $nmol \cdot ml^{-1}$ for plasma ascorbic acid. This is in agreement with the study of Jacob *et al*. (1987) that showed a difference in vitamin C absorbance in individuals having the same

vitamin intake and in fact the same diet (there values varied from 1 to 1.8 $mg \cdot dl^{-1}$). In the present study we did not control the runners' diet throughout the study, and this might explain the individual differences in the plasma total antioxidant concentration and also in the plasma vitamin C levels. In addition, the individual differences in the plasma ascorbic acid response to the vitamin supplementation could also be attributed to different body pools of this vitamin. That is, the plasma vitamin C levels only represent a portion of the total body ascorbic acid pool, this difference could thus represent a variation of vitamin C binding in different tissues and cells, for instance in red and white blood cells (Jacob *et al*., 1987).

As for differences in plasma vitamin E variation between individuals in response to dietary supplementation, although we did not specifically measure its concentration, this could also contribute to the individual differences in plasma total antioxidant concentration. Studies have shown that the absorption of this vitamin can vary between individuals because of differences in the expression and activity of intestinal vitamin E transporters and the availability of lipids. The absorption efficiency of this vitamin is also influenced by the food with which it is ingested. In addition, its variation in plasma can also be a result of plasma fluctuations in lipoprotein concentration, and selective lipid and vitamin E uptake in different tissues (Traber, 2006; Rigotti, 2007).

A limitation of the present study was the small sample size because of difficulty in recruiting participants. Nevertheless, the crossover design of this study helps to balance this number, as each participant acted as their own control. One of the main reasons for this difficulty was the fitness level criteria established for the runners. On one hand, the high level of the runners presents as an advantage of this research, because most lab studies recruit individuals with low activity levels or moderately active. Therefore, it is possible to extrapolate our results to the athletic community and also to the moderately active individuals. On the other hand, such a lengthy research where the runners have not only to dedicate their time, but also change their habits for the research purposes, does decrease the number of interested individuals. In addition, a couple of runners that were interested in the study and did fit the VO_2 criteria of the study were already taking multivitamin supplementation and did not want to stop taking them. Another individual was training for a marathon and could not fit the trials with his training programme; and one runner dropped out after experiencing severe respiratory distress during his first trial, later it was revealed that he was on his Placebo trial. The individual that dropped

out reinforces that some athletes are more sensitive to the effects of ozone-pollution and heat, and it would be relevant (but difficult ethically) to specifically analyse aspects that might exacerbate this susceptibility, such as the antioxidant concentration in the airways. Analysing his plasma pre antioxidant levels he was within the lower range of the participants values for the Placebo trial: 12.8 $nmol \cdot ml^{-1}$ for total antioxidant concentration and 3.8 $nmol \cdot ml^{-1}$ for ascorbic acid concentration.

To assess the oxidative stress levels in this study GSH concentration was measured in both plasma and nasal lavage, but, we did not manage to measure MDA levels, as a marker of lipid peroxidation, in either the plasma or nasal lavage samples. Palmer *et al*. (2003) reported no differences in markers of lipid peroxidation (F_2-isoprostane and lipid hydroperoxides) in a vitamin C supplemented group compared to a placebo group after an ultramarathon race, despite higher plasma levels of this antioxidant in the supplemented group. Conversely, Traber (2006) reported that individuals supplemented with both vitamin C and E did present a lower F_2-isoprostane level after an ultramarathon compared with individuals that consumed placebo; and the F_2-isoprostane concentrations were inversely correlated with the vitamin C and E levels, providing further evidence that the antioxidant were responsible for preventing the lipid peroxidation. The latter study however, was conducted using two different groups of individuals with both male and female subjects, and the author did report that the female runners presented less oxidative stress after the race. It is difficult to know to which extent this might have influenced the group result. Davison and Gleeson (2006) reported no changes in MDA following a 2-week vitamin C (1000 $mg \cdot day^{-1}$) supplementation protocol and after a 2.5 h of cycling exercise compared with when the participants ingested placebo. Of note, these studies were looking into the effect of vitamin supplementation on oxidative stress after a bout of exercise, but without any additional stressor of ozone or heat and humidity.

As detailed in *Chapter 2*, GSH is a water-soluble low-molecular-weight antioxidant that is synthesized in all types of cells (Anderson, 1998). It is found in high concentration in the airways, and in addition to its important functions in the body, including its antioxidant capacity, GSH also has a role in mediating the immune response (Lu, 1999; Wu *et al.,* 2004). These factors make it relevant to analyse this antioxidant in the context of the present study. There were no differences in the baseline levels of GSH either in the plasma or nasal lavage samples of the runners comparing the Vitamin and

the Placebo trial. Immediately after the exposure protocol there was a non-significant decrease in the plasma GSH/Protein levels, this decrease was accentuated at the 6 h time point in the Placebo trial, but in the Vitamin trial the GSH/Protein levels started to increase. As for the nasal lavage samples, immediately after the exercise there was a non-significant increase in the GSH/Protein levels in the Vitamin trial. After 6 h these values were at the same level as pre-exercise. There was no change in the nasal lavage GSH/protein pattern in the Placebo trial. The increase in the nasal lavage GSH immediately after the exposure might have contributed to the significant increase in the total antioxidant capacity observed in the nasal lavage at this time point, which was statistically significant in the Vitamin trial. It can be speculated that there was a stronger up-regulation of antioxidants in the respiratory tract lining fluid when the runners went through the supplementation period which would thus lead to less oxidative damage. The increase in nasal lavage GSH levels has been reported in other studies where individuals exercised in a polluted environment (Blomberg *et al*., 1999); and was also reported in *Chapter 5* only after the runners exercised in the Heat + O_3 environment.

The present study does not support the protective role of vitamin supplementation against ozone exposure on lung function reported by some studies (Romieu *et al*., 1998; Grievink *et al.,* 1998; Grievink *et al.,* 1999). It was hypothesized however, that the vitamins might improve the participant's lung function especially because of the relationship of vitamin C with lung health and improvement in respiratory symptoms (Romieu *et al*., 2006; Teckenburg *et al*., 2007). Nevertheless, it is not known to what extent the vitamin C concentrations were increased in the RTLF in the present study. In the Vitamin trial the runners had a tendency to experience less shortness of breath, cough, pain on deep inspiration and overall total respiratory symptoms compared to when they performed in the Placebo trial. Although these values did not reach statistical significance it is another result that might help to explain the trend of improvement in the runners' performance. Physiological variables measured throughout the run, such as heart rate and RPE, did not differ between the trials. The participant that had asthma did not differ in any way from the group values. Studies conducted with asthmatic individuals exposed to ozone has shown no evidence of an increased sensitivity in respect to either lung function decrements, magnitude of change in neutrophil numbers in the airways or lipid peroxidation in comparison to non-asthmatics (Mudway *et al*., 2001; Stenfors *et al*., 2002). It was, therefore, based on these facts that we included this runner in our sample.

The novelty of this study lies on the analysis of the effect of vitamin supplementation on the performance, lung function, oxidative stress and inflammation (results will be presented in the next chapter) of well trained runners exercising in an environmentally relevant and challenging condition. Even though there are some studies analysing these factors separately there is a lack of knowledge on how these variables interact together. In addition, methodological differences between studies may account for the inconsistency in the results, making this a challenging area of research. Overall, the analysis of the results in this chapter suggest that 2 weeks of a low dose of vitamin C and E supplementation might present some benefits for the performance outcome of trained individuals when taking part in a race in a ozone-polluted, hot and humid environment. These benefits will however depend on the regulation of the antioxidants uptake and metabolism of each subject. Further mechanisms that might influence this outcome will be discussed in the next chapter.

Chapter 7: Investigating vitamin supplementation on immunoendocrine responses and lung inflammation of runners exercising in a hot, humid and ozone-polluted environment

7.1 INTRODUCTION

The aim of this chapter is to further investigate the effects of vitamin C and E supplementation on well trained individuals performing an 8 km time-trial run in a hot, humid and ozone-polluted environment. In this chapter, the results of lung epithelial injury, airway and systemic inflammation of these runners are presented and analyzed.

Lymphocytes

Lymphocytes constitute around 20-40% of the circulating white blood cells and they are part of the adaptive immunity. When these cells come into contact with a foreign molecule or antigen for the first time they have the ability to recognised and produce a specific response against that pathogen. This response involves the proliferation and differentiation of lymphocytes into their specialized functional roles. Some lymphocytes will turn into effector cells which operate to eliminate the recognized antigen. While others lymphocytes differentiate into memory cells that will be quickly mobilized in the event of a subsequent exposure to that same antigen, allowing for a more rapid immune response. It is the specificity and memory of the adaptive immunity that distinguishes it from the innate immunity. The latter can act against any foreign antigen without prior exposure. In addition, cells from the innate immune system, i.e. neutrophils, contribute to the stimulation of the lymphocytes presenting proteins of the foreign antigen and also secreting a variety of inflammatory mediators, such as cytokines, that contributes to the activation of the adaptive immunity (Makinnon, 1999).

Lymphocytes have a very distinct biphasic response to exercise. During and immediately after exercise these immune cells are mobilized from peripheral lymphoid organs. This results in an increase in the number of lymphocytes in the circulation. The intensity and duration of this lymphocytosis will depend on the type, duration and intensity of the exercise performed (Steensberg *et al.*, 2002.; Mooren *et al.*, 2002; Simpson *et al*., 2006). Nevertheless, around 1 to 3 h after exercise cessation, this lymphocytosis is substituted by lymphopenia, resulting in levels of lymphocyte below the pre-exercise values for several hours (Simpson *et al.*, 2006). The severity and duration of this decrease will again depend on type, duration and intensity of the

exercise performed, as well as the training status of the individual (Steensberg *et al.*, 2002; Mooren *et al.*, 2002; Simpson *et al*., 2006). This decline in lymphocyte count and other alterations in the immune system, observed during early recovery from certain types and high intensity exercise, create an "open window" of decreased host protection. This open window period of weakened immunity, which can last between 3 and 72 h, represents a vulnerable time period for the individual, during which there might be an increased susceptibility to contracting and developing an infection if there is contact with any pathogen (Nieman & Bishop, 2006).

Lymphocytes, neutrophils, and indeed all nucleated cells respond to a stress stimulus by increasing their defence mechanism, such as an up-regulation of protective enzymes and antioxidants. Khassaf *et al*. (2003) reported that an 8 week supplementation period with 500 $mg \cdot day^{-1}$ of vitamin C increased the baseline expression of the lymphocytes' protective enzymes, but blunted the adaptive response of these cells to an exercise bout stimulus. The exercise consisted of 45 min of one-leg cycling at 70% of the subjects' $VO_{2\ peak}$. It is possible that the long supplementation period caused the vitamin C to act as a pro-oxidant, thus, leading to the increased adaptation measured at baseline. Another explanation would be that the vitamin C stimulated modification in the expression of genes which code for specific protective enzymes such as catalase and SOD. After the stress stimulus, the attenuation of the adaptive response of the lymphocytes could be attributed to a reduced local oxidative stress due to the high concentration of vitamin C reducing, hence, the need for further adaptation within these cells. Whether the lack of up-regulation in the protective enzymes after the exercise bout is beneficial or deleterious to the cell is a topic of discussion. It is important to note that Khassaf *et al*. (2003) investigated the response to only one exercise bout after the supplementation period. In addition, the participants of the study were untrained individuals. It would be interesting to see how lymphocytes adapt to the supplementation protocol together with an exercise training programme as training does provide a stimulus to the enhancement of protective enzymes and antioxidant content of tissues (Powers *et al*., 2004; Ji, 2008). A recent study (Gomez-Cabrera *et al*., 2008b) investigated the effect of an 8 week supplementation period with vitamin C ($1000\ mg \cdot day^{-1}$) on muscle cell adaptation and training efficiency in both humans and rats. The results of the study suggested that the supplementation had an adverse effect on the training adaptations affecting the endurance capacity. This was caused mainly by a decrease in mitochondria biogenesis

and an attenuation of the expression of antioxidants enzymes in skeletal muscle that occurs with endurance training.

In addition to vitamin C, lymphocytes and neutrophils also contain vitamin E. These immune cells take up this antioxidant from the plasma in order to increase their antioxidant concentration and, therefore, be able to maintain an optimal functional capacity (Cases *et al*., 2005). A long and intense endurance exercise bout, i.e. half-marathon, has been shown to enhance the vitamin E concentration in lymphocytes and neutrophils of trained individuals. In addition, a month of supplementation with vitamin C (152 $mg \cdot day^{-1}$) and E (75 $IU \cdot day^{-1}$) significantly increased the vitamin E content of neutrophils, after the exercise bout, compared to when the subjects ingested placebo. The increase of this vitamin, observed in the leukocytes, occurred despite any changes in the vitamin E content of the plasma. This suggests that determination of vitamin E in the immune cells can represent a redistribution of plasma vitamin E and might be a useful measure to assess the functional status of this vitamin in the body (Cases *et al*., 2005).

Immunoendocrine Response to Vitamin C and E Supplementation

As mentioned in the beginning of this chapter, usually after a prolonged exercise bout there is a decrease in the number and function of immune cells. Increase in the circulating concentration of the stress hormone cortisol is one of the factors known to contribute to this depression of the immune system observed after exercise (Nieman, 1994; Mackinnon, 1999). Cortisol is a glucocorticoid hormone produced by the hypothalamic-pituitary-adrenal axis, and is considered a stress hormone due to its increased release in response to stressful situations, which include psychological stress, a rise in core temperature and metabolic demands during exercise (Jimenez *et al*., 2007; Peak *et al*., 2007; Webb *et al*., 2008). Moreover, the cortisol release has been well established as a function of the duration and intensity of the exercise, with higher intensity and longer duration exercises, i.e. marathon race, stimulating a greater release of this hormone from the adrenal axis (Tremblay *et al*., 2005, Karkoulias *et al*., 2008).

There have been a number of studies which have investigated the supplementation of vitamin C and E on the inflammatory and cortisol responses to exercise. Most studies

that have used a combination of both vitamins have shown a positive outcome of the supplementation in blunting the cortisol response and decreasing the release of certain inflammatory cytokines such as IL-6 (Fisher *et al*., 2004; Davison *et al*., 2007). Nevertheless, these findings are not unanimous and analysis of the literature has shown that various factors can influence this outcome. These factors include the type of exercise used, which influences the release of reactive species; the supplementation protocol and bioavailability of the vitamins during the exercise trial; and the endogenous antioxidant defences of the participants of the study, which will be augmented if they are trained individuals (Peake *et al*., 2007). In addition, research is still needed to clarify how a decrease in circulating levels of cortisol might be linked with an improved immune function and therefore, a reduction of infection after exercise. It has been speculated that cortisol and some cytokines might contribute to a decreased neutrophil function and also stimulate the release of immature neutrophils with decreased stimulation capacity, from the bone marrow. Circulating cortisol also enhances exercise-induced neutrophilia by inhibiting the ability of the neutrophils to bind to the endothelial and to infiltrate into the tissue (Steensberg *et al*., 2003). Antioxidant supplementation is theorized to be beneficial in decreasing the immune-depression after intense exercise because it would contribute to attenuate cortisol release and neutrophilia; as well as, increasing the antioxidant content of the immune cells which might result in a potentiation of its function together with the protection from oxidative damage.

Peters *et al*. (2001) did indeed show a reduction in post-exercise cortisol levels in athletes who ingested vitamin C (1000 $mg \cdot day^{-1}$) for a 1-week period prior to a 90-km ultramarathon race. Nevertheless, there was no difference in leukocyte counts after the ultramarathon. The effect of vitamin C supplementation on cortisol however, was not observed by Palmer *et al*. (2003) when athletes were supplemented for a 7 day period with vitamin C (1500 $mg \cdot day^{-1}$) also prior to an ultramarathon race. The authors reported that vitamin C had no influence in cortisol increases post-exercise. Also investigating the effect of vitamin C alone, Davison and Gleeson (2006) looked into the immunoendocrine responses to an exercise bout. Contrary to the former study, the supplementation protocol consisted of 2 weeks of vitamin C (1000 $mg \cdot day^{-1}$), and the exercise was shorter and of lower intensity: 2.5 h of cycling at 60% VO_{2max}. The authors reported that even though the supplementation was effective in decreasing the blood

neutrophil count post-exercise, there was only a trend, but not a statistically significant decrease in the cortisol levels (P=0.08) post-exercise compared to when placebo was ingested. In addition, there were no differences between the placebo and the supplemented trials for plasma IL-6. Similarly, Nieman *et al*. (2004) reported the absence of any effect on plasma cortisol levels post-exercise, in this case following an ironman triathlon competition. In this study (Nieman *et al*., 2004), however, the athletes had gone through a 2 month supplementation period with vitamin E (800 $IU \cdot day^{-1}$) alone. In addition to a lack of influence on cortisol release, analysed cytokines, such as plasma IL-6, were elevated in the supplemented group after the race. As explained previously, this could possibly be due to the pro-oxidant effect of vitamin E.

It seems that there is more convincing evidence showing the reduction of plasma cortisol, after an exercise bout, when there is a combined supplementation of vitamin C and E. In the study of Fisher *et al*. (2004), two groups went through a 4 week supplementation period with either a combination of vitamin C (500 $mg \cdot day^{-1}$) and E (400 $IU \cdot day^{-1}$) or placebo. After the supplementation period, the participants performed an exercise protocol consisting of 3 h of dynamic two-legged knee-extensor exercise at 50% of their individual maximal power output. The authors reported an increase in cortisol concentration during recovery in the Placebo group, while this cortisol response was completely blunted in the supplemented group. In addition, other markers of inflammation, including IL-6 and CRP, were reduced post-exercise in the supplemented group. The authors suggested that the decrease in IL-6 might have contributed to the decrease in systemic cortisol concentration as this cytokine is known to stimulate the release of cortisol from the adrenal axis into the circulation (Steensberg *et al*., 2003). Fisher *et al*. (2004), however, did not analyse the neutrophil response to the exercise or supplementation. A similar decrease in cortisol level post-exercise was observed by Davison *et al.* (2007) after a similar supplementation protocol (4 weeks, vitamin C 1000 $mg \cdot day^{-1}$; vitamin E 400 $IU \cdot day^{-1}$). The exercise protocol consisted of a 2.5 h cycle at 60% of the participants' VO_{2max}. Even though there was a decrease in the stress hormone level there were no changes in neutrophil numbers or function compared to the placebo ingestion. The authors explained that, before the exercise, there was a trend for higher cortisol concentration in the supplemented group, and that this could be related to the lack of changes in neutrophil count and function post-exercise in that group. In addition, it is possible that a shorter supplementation period would be more effective in preventing auto-oxidation of the immune cells as shown by Alessio *et al.* (1997). These

suggestions however, are just speculations and more research in this area is necessary. For a summary of the studies mentioned above see table 7.1.

Table 7.1. Summary of studies analysing the effect of vitamin supplementation on cortisol and inflammation markers.

Study	Subjects	Design	Supplement	Exercise	Results
Peters *et al.*, 2001	Trained runners	2 groups: placebo (n=6) supplemented (n=10)	1 week: vit C 1000 ($mg \cdot day^{-1}$)	Ultramarathon (90 km)	Post-exercise: ↓ in cortisol concentration; no difference in leukocyte counts
Palmer *et al.*, 2003	Trained runners (male and female)	2 groups: placebo (n=13) supplemented (n=15)	1 week: vit C 1500 ($mg \cdot day^{-1}$)	Ultramarathon	No difference in cortisol concentration
Fisher *et al*., 2004	Active male non-athletes	2 groups: placebo (n=7) supplemented (n=7)	4 weeks: vit C 500 mg + vit E 400 IU daily	3 h of dynamic 2-legged knee-extensor	Post-exercise: ↓ cortisol, IL-6, CRP concentrations
Nieman *et al.*, 2004	Triathletes (male and female)	2 groups: placebo (n=16) supplemented (n=16)	8 weeks: vit E (800 $IU \cdot day^{-1}$)	Ironman Triathlon	No difference in cortisol concentration; ↑ in IL-6 in the Supplemented group
Davison & Gleeson, 2006	Endurance trained males	Crossover (n=9)	2 weeks: vit C (1000 $mg \cdot day^{-1}$)	2.5 h cycling at 60% VO_{2max}	No difference in cortisol concentration or IL-6
Davison *et al.*, 2007	Recreational active males	2 groups: placebo (n =10) supplemented (n=10)	4 weeks: vit C 1000 mg + vit E 400 IU daily	2.5 h cycling at 60% VO_{2max}	Post-exercise: ↓ cortisol concentration ; no difference in neutrophil count or function

All in all, some of the studies mentioned above support the beneficial role of vitamin E and C supplementation in decreasing cortisol secretion after an exercise bout. As cortisol is released in response to stress it could be speculated that additional stressors such as ozone pollution and heat plus humidity would further stimulate the release of

this hormone. In fact, these multiple stressors might possibly induce immunoendocrine responses that exacerbate the exercise-induced alterations. Therefore, a greater benefit might be seen in the reduction of exercise-induced immune depression if the ingestion of vitamin C and E could blunt the cortisol response to exercise in an adverse environment. There is a lack of available information regarding the effect of vitamin supplementation on inflammation of individuals exercising in an ozone-polluted environment. Most studies have focused mainly on the effect of exercise alone or pollution alone on oxidative stress and/or lung function. In the study of Mudway and colleagues (2006), discussed previously, no benefit of supplementation was observed after exposure to ozone in relation to inflammatory responses. The supplementation consisted of a 1-week period of vitamin C (500 $mg \cdot day^{-1}$) and E (100 $mg \cdot day^{-1}$) ingestion, and the exercise protocol performed after this period was an intermittent cycling exercise for 2 h exposed to 0.2 ppm of ozone. After the exercise, there was no difference between the vitamin group and the placebo group with respect to the increase in neutrophil count in the airways, assessed by BAL. The authors also reported no differences in other inflammation markers in the airways, such as IL-6 and IL-8, and also no differences in indices of altered respiratory epithelial injury measured by total protein and albumin concentration. Of note, the authors did report an increase in the plasma levels of vitamin C and E in the supplementation group, before and after exercise, however, this did not reflect an increase in the vitamin concentration of the airways. It could be speculated that the supplementation did not succeed in increasing the antioxidant defences in the lower airways and, hence, the protection mechanism was not enhanced by the supplementation. The authors did not measure the systemic inflammation of the participants.

This chapter seeks to answer some of the gaps in the literature regarding vitamin supplementation and inflammatory response of trained runners taking part in a time-trial run in a hot, humid and ozone-polluted environment. The hypothesis was that the vitamin C and E would be successful in decreasing the immune depression induced by the exercise bout. In addition, there would be a reduction in the lung epithelial injury, which is usually observed with exposure to ozone. These factors would, hence, contribute to an improvement in the runners' performance, after they went through the supplementation period, compared to when they ingested placebo.

7.2. METHODS

The data for this chapter was collected as part of the same project that originated the data presented in *Chapter 6*. Therefore, most aspects of the methodology, such as the participants' of the study and the study design are the same. Further tests carried out are fully explained in this section.

7.2.1 Experimental Procedures

The experimental procedures have been detailed in section 6.2. In summary, ten well trained male runners took part in this study. After the familiarization trial, the participants were randomly assigned to initiate a 2-week supplementation period of vitamins C (500 $mg \cdot day^{-1}$) and E (100 $IU \cdot day^{-1}$) or placebo. The study's design was double-blinded crossover. On the exercise trial day the participants arrived at the lab at around 8:40am. After having their blood samples drawn, saliva sample collected, lung function tested and nasal lavage procedure conducted, the participants went to the environmental chamber to perform the 8 km time trial run. The environmental condition was set at 31 ºC and 70% rh with 0.1ppm of ozone. Immediately post-exercise and 6 h after the exercise the athletes went through the same tests: blood samples were taken, saliva sample, lung function and nasal lavage procedure.

To analyze the impact of the supplementation protocol on lung epithelial injury and inflammation, and systemic inflammation the following variables were assessed:

- In saliva: cortisol concentration
- In the nasal lavage: neutrophil count and percentage, IL-8, total protein, CC16.
- In plasma: lymphocyte count and percentage, neutrophil count and percentage, CC16.

Variables measured in the blood samples were not adjusted for plasma volume loss, as no significant changes in plasma volume were found between time points. The plasma volume of the blood samples was calculated according to the method described by Dill and Costill (1974).

7.2.2 Saliva collection and cortisol analysis

All participants were seated during the saliva collection. They were instructed to swallow any fluid in their mouth to remove old saliva before providing unstimulated saliva sample. For the collection of the sample the participants leaned forward, with their head tilted down, allowing the saliva to dribble into pre-weighed collecting tubes (Sterilin, UK) with minimal orofacial movements to avoid stimulation of the salivary glands. Whole saliva was collected for a 5 min period. This procedure was performed according to the method previously described by Allgrove *et al*. (2008). Once collected the saliva samples were immediately put on ice and stored in a freezer with the temperature below -80°C until analysis.

Salivary Cortisol Assay

Salivary cortisol concentrations were measured using an RnD Systems kit (Abigdon, UK) in accordance with the manufacturer instructions. This assay is based on the competitive binding technique in which cortisol present in a sample competes for sites on an antibody with a known amount of cortisol (Cortisol Conjugate: horseradish peroxidise labelled cortisol). The following methodology was used: first, the saliva samples were thawed and diluted 1 in 5 with the Calibrator Diluent RD5-43. Cortisol standards were also prepared with the Calibrator Diluent and the concentration varied from 10 – 0.156 $ng{\cdot}ml^{-1}$. Samples and standards were added (100 µl) in duplicate in the 96 microwell plate provided by the manufacturer. The Cortisol Conjugate (50 µl) was then added into each well, followed by 50 µl of the Primary Antibody Solution. The plate was then incubated for 2 h at room temperature on a microplate shaker. After this, the plates were washed 4 times and 200 µl of the Substrate solution added to each well. Again the plate was incubated for 30 min and protected from light. Following this, 50 µl of Stop solution was added to each well and the plate was read with a 450 nm wavelength with correction set to 570 nm. Cortisol concentration from each sample were obtained from the standard curve and the average value of the duplicate used.

Statistical Analysis

All data were checked for normality before statistical analysis and if necessary

transformed. Data were analyzed using General Linear Model ANOVA (Minitab15 Statistical Software) with Tukey's *Post-hoc* test. Statistical significance was accepted at $P < 0.05$. Data are expressed as mean values ± standard error of the mean (SEM).

7.3. RESULTS

7.3.1 Nasal Lavage Cells and IL-8

The nasal lavage neutrophil count and percentage are shown in Table 7.2. There were no trial differences or time differences for the neutrophil count. In the Placebo trial the neutrophil count at the 6 h-post exercise time point seemed to increase but did not reach statistical significance. This increase pattern was not seen in the Vitamin trial. Similarly, there were no group differences in nasal lavage neutrophil percentage, but there was a significant increase at the 6 h time point for the Placebo trial ($P < 0.05$).

For the IL-8 concentration a significant time and trial difference was observed. At the 6 h time point the Placebo trial presented a significantly higher IL-8 concentration compared to the Vitamin trial ($P < 0.01$). In addition at this same time point, in the Placebo trial, the IL-8 concentration was significantly higher compared with the pre and post-exercise time points ($P < 0.001$) within the same trial. In the Vitamin trial this cytokine also presented significantly higher values at the 6 h time point compared with the pre-exercise values ($P < 0.01$) (Table 7.2).

Table 7.2. Neutrophil and IL-8 in the nasal lavage samples of both trials

	Vitamin Trial			**Placebo Trial**		
	Pre Ex	Post Ex	6H-Post Ex	Pre Ex	Post Ex	6H-Post Ex
Neutrophil count (10^4)	0.43 ± 0.46	0.49 ± 0.48	0.55 ± 0.65	0.32 ± 0.41	0.51 ± 0.52	1.04 ± 1.25*
Neutrophil percentage	16.3 ± 20.3	17.5 ± 12.3	22.6 ± 17.2	13.1 ± 13.5	19.9 ± 17.8	25.2 ± 22.6
IL-8 ($pg \cdot ml^{-1}$)	10.5 ± 9.2	50.8 ± 27.4	75.8 ± 43.2*§	9.7 ± 8.8	57.9 ± 31.0	126.6 ± 103.2**

§ Significant difference between trials at that time point ($P < 0.01$). *Significantly higher compared to Pre-exercise values in the same trial ($P < 0.05$). ** Significantly higher compared to Pre and Post-exercise value, Placebo trial ($P < 0.001$). Values are mean ± SD.

7.3.2 Plasma immune cells

Table 7.2 presents the values for white blood cell counts, lymphocyte counts and percentages as well as neutrophil counts and percentages for both trials. There were no differences between trials at any of the time points analyzed. A significant increase, compared to pre and post-exercise values, was observed for the white blood cell count 6 h after the exercise in both trials. The same is true for the neutrophil count and percentage. Lymphocyte count was significantly increased post-exercise compared with pre-exercise values only in the Placebo trial. Nevertheless, in both the Vitamin and Placebo trials, the lymphocyte percentage was significantly decreased at the 6 h time point compared with the post-exercise percentage.

Table 7.3. Immune cells in the plasma samples of both trials

	Vitamin Trial			**Placebo Trial**		
	Pre Ex	Post Ex	6H-Post Ex	Pre Ex	Post Ex	6H-Post Ex
White blood cells ($10^9 \cdot l^{-1}$)	5.04 ± 0.93	5.80 ± 0.84	8.63 ± 2.70**	5.56 ± 0.69	7.01 ± 1.29	8.73 ± 1.77**
Lymphocyte count ($10^9 \cdot l^{-1}$)	1.72 ± 0.36	2.06 ± 0.42	1.72 ± 0.33	1.95 ± 0.45	2.58 ± 0.90*	2.16 ± 0.30
Lymphocyte percentage	32.8 ± 9.27	35.7 ± 6.39	22.3 ± 8.61†	35.3 ± 7.08	36.3 ± 8.25	25.5 ± 5.46†
Neutrophil count ($10^9 \cdot l^{-1}$)	2.59 ± 0.78	3.06 ± 0.75	6.08 ± 2.88**	2.82 ± 0.69	3.68 ± 0.87	5.66 ± 1.62**
Neutrophil percentage	50.8 ± 8.94	52.3 ± 8.61	68.3 ± 11.9**	50.5 ± 8.88	52.7 ± 9.93	64.0 ± 6.99**

No differences between trials. * Significantly higher than pre-exercise in the same trial ($P<0.01$); † significantly lower than post-exercise values within the same trial ($P<0.01$); significantly higher compared to pre and post-exercise values within the same trial ($P<0.001$). Values are mean ± SD.

7.3.3 Salivary Cortisol

One third of the runners were unable to provide an adequate saliva sample, that is an unstimulated saliva sample produced with little orofacial movements. Therefore, the

data regarding cortisol has only been analyzed for the same six runners in both of their trials. The cortisol data is presented in Figure 7.1. There was no difference between trials for cortisol concentration in any of the analyzed time points. Nevertheless, in the Placebo trial there was a significant increase in this hormone immediately after the time-trial run (post-exercise time point), with its values returning back to baseline 6 h after the exercise ($P<0.05$). The intra-assay coefficient of variation for the duplicate samples was 5.6%.

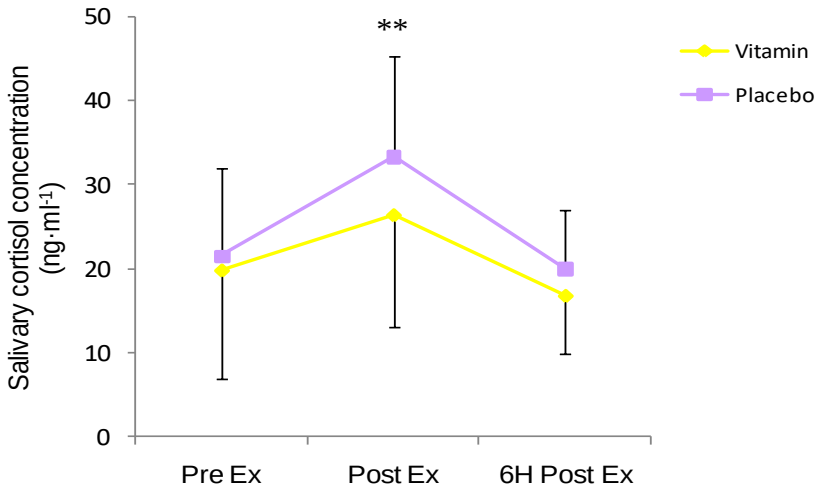


Figure 7.1. Salivary cortisol concentration. ** Significantly different from pre and 6 h-post-exercise values within the Placebo trial ($P<0.05$). Values are mean ± SD.

7.3.4 Clara Cell Protein in Nasal Lavage and Plasma

To analyse lung inflammation and lung epithelium injury CC16 was measured both in the nasal lavage and in the plasma. There were no differences between the Placebo and the Vitamin trial in any of the analyzed time points. Both trials presented the same pattern of CC16 variation across time. However, it was only in the Placebo trial that a significant increase in this protein concentration was observed at the post-exercise time point. This occurred both in the plasma samples and the nasal lavage samples of the Placebo trial (Figure 7.2). The intra-assay coefficient of variation for the duplicate samples was 3.2% and 1.8% for the nasal lavage and plasma samples respectively.

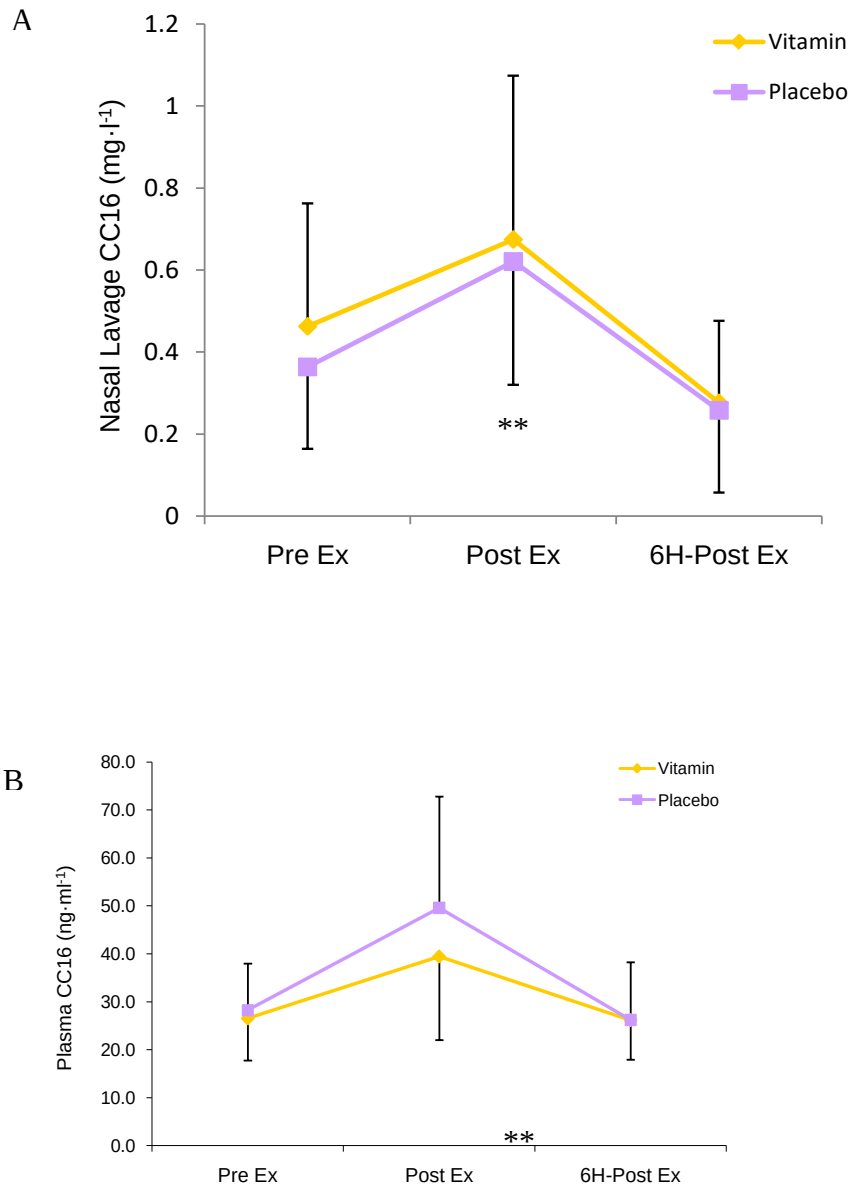


Figure 7.2. CC16 concentration in nasal lavage (A) and plasma (B) samples. ** Significantly higher compared with pre-exercise and 6 h-post exercise time points in the Placebo Trial (P < 0.01). Values are mean ± SD.

7.4 DISCUSSION

In this chapter the effect of vitamin C and E supplementation on the immunoendocrine responses and lung epithelial injury in runners taking part in an 8 km time-trial run in a hot, humid and ozone-polluted environment was investigated. The main findings were

that the vitamin supplementation provided some protection against lung epithelial injury and lung inflammation. In addition, there was an attenuation of the salivary cortisol and plasma lymphocyte increase immediately after the exercise protocol when the runners were taking the vitamins. Nevertheless, there were no other differences in the plasma immune cells.

As reported in Chapter 6, the supplementation protocol that the participants in this study went through was sufficient to increase their plasma baseline levels of vitamin C and total antioxidant concentration. In addition, the supplementation also allowed for an up regulation of the antioxidant capacity in their upper respiratory airways. That was observed immediately after the exercise protocol. When the plasma immune cells were analysed, no difference in the neutrophil counts or percentage was found when the Vitamin trial was compared with the Placebo trial. Nevertheless, this immune cell was increased at the 6 h time point in both trials. There is a lack of studies in the literature analysing the systemic inflammation in response to vitamin supplementation combined with exposure to ozone. Mudway *et al.* (2006) analysed the inflammatory markers in the lower respiratory airways in response to vitamin C and E supplementation and exposure to ozone. Similarly, they reported that there was an increase in the neutrophil count 6 h after the exercise and exposure protocol, but no difference was observed between the Vitamin and the Placebo trial. In the present study, the airway inflammation was analysed using the nasal lavage method. Although we did not encounter differences between the trials for neutrophil count, there was an increase in the neutrophil percentage in the Placebo trial at the 6 h time point compared with the pre-exercise values. It seems possible that the increase in antioxidant capacity, observed in the nasal lavage immediately after the exercise, might have provided some protection against the local oxidative stress, which resulted in a smaller inflammatory response. Another factor that corroborates this result was the IL-8 concentration in the nasal lavage. This cytokine, as described earlier, is an important mediator of the immune function and is chemotactic for neutrophils, thus, an increase in IL-8 is usually accompanied by an increase in this immune cell (Holz *et al.*, 1999). In the present study, an increase at the 6 h time point was observed for nasal lavage IL-8 concentration in both trials. Nevertheless, the values in the Placebo trial were significantly higher when compared with the Vitamin trial; and, as described previously, so was the neutrophil percentage.

It seems, therefore, possible to speculate that the vitamin supplementation did provide some protection against the inflammatory response in the upper respiratory airways of the runners. Another result that reinforces this speculation is related to the CC16 concentration in the nasal lavage and plasma. As mentioned in *Chapter 2* and *5*, this small sized protein secreted by the Clara cells has a role in protecting the respiratory tract against inhaled particles, such as pollutants (Robin *et al.,* 2002). In addition, their quantification is also used as a marker of lung epithelial integrity (Broeckaerte *et al.,* 2000a). An increase in CC16 was observed, both in the plasma and in the nasal lavage fluid, immediately after the exercise protocol, with its values returning back to baseline after 6 h. This increase was only statistically significant in the Placebo group. It is possible that this increase, both in plasma and nasal lavage, was the result of two different but synergistic mechanisms. As previously explained, ozone can cause lung epithelial injury (Christian *et al.,* 1998); therefore, it is possible that the higher antioxidant concentration in the airways of the runners after the supplementation might have reacted with the ozone, partially neutralizing its oxidative effects on epithelial cells. This would not have occurred when the athletes where taking the placebo and, as a result, there would have been damage to the lung epithelial cells, including the tight junctions . This would allow for a greater diffusion of CC16 into the plasma, hence, the higher plasma concentration of this protein immediately after the exercise in the Placebo trial. The mechanism responsible for a significant increase in the nasal lavage CC16 concentration in the Placebo trial would be that the lower antioxidant concentration in the airways in this trial allowed oxidative stress and hence an early inflammatory process to be initiated. This might have stimulated the Clara cells to secrete more CC16 into the respiratory tract lining fluid. To date there is little information on the effect of exercise and pollution on CC16, and this is the first study (to my knowledge) investigating the additional effect of vitamin on this protein. Thus, it is difficult to establish the exact mechanisms by which these vitamins affect the secretion and production of CC16. Mudway and colleagues (2006) also investigated the lung epithelial permeability in their previously-mentioned study. They assessed the concentration of albumin in the bronchoalveolar lavage, but reported no differences between the vitamin and the placebo trial. It has been shown, however, that CC16 would be a better and more sensitive marker to assess the lung epithelial injury (Hermans & Bernard, 1999).

Another immune-modulator effect observed for the vitamin supplementation was that it blunted the cortisol increase post-exercise. This result was observed despite the fact that only a limited number of participants were able to provide an adequate saliva sample for analysis. This effect of vitamin supplementation on cortisol levels is in accordance with some studies (Peters et al., 2001; Fisher et al., 2004; Davison et al., 2007), but not all (Palmer et al., 2003; Nieman et al., 2004; Davison & Gleeson, 2006). Most studies that have not demonstrated an attenuation of the exercise-related increase in circulating cortisol used only vitamin C or only vitamin E as supplements. Taken together, these findings suggest that a combined supplementation is more appropriate in reducing the cortisol responses, and this study provides further evidence for that. Even though it has been suggested that cortisol contributes to an increase in circulating levels of immature neutrophils (Steensberg *et al*., 2003), this was not verified in the present study - as reported at the beginning of this section there was a similar increase in plasma neutrophils in both trials 6 h after the exercise protocol. We did not, however, investigate the neutrophil function, which might have been different between the trials. Davison et al. (2007) also reported a decrease in cortisol levels after an exercise bout, in participants that underwent a supplementation protocol (4 weeks, vitamin C 1000 $mg \cdot day^{-1}$; vitamin E 400 $IU \cdot day^{-1}$). In addition, the authors also reported no change in plasma neutrophil count and no differences in neutrophil function. Even with similar results, it is important to keep in mind the differences between studies in relation to supplementation protocol, exercise protocol, subjects' fitness levels, and most importantly for the present study the additional stress of ozone, heat and humidity.

Cortisol has also been shown to stimulate the lymphopenia response of lymphocytes observed after an intense exercise bout, contributing to the "open window" period (Nieman, 1994). In the present study, at the 6 h time point there was a significant decrease in lymphocyte percentage in both trials, and no difference in the lymphocyte total count. This result is in line with the cortisol response, in that at the same time point, there were no significant differences between trials. In fact, in the Placebo trial, there was a significant increase in the cortisol levels immediately after the exercise bout when compared to the pre-exercise levels, but there were no differences between the trials in any time point. It seems possible that the higher cortisol concentration post-exercise, in the Placebo trial, was not sufficient to cause a decrease of the circulating lymphocytes at the 6 h time point. A significant increase in lymphocyte count immediately after the exercise was found, however, only in the Placebo trial. This

increase would be influenced by another hormone, adrenalin. Strong increases in circulating levels of adrenalin, observed in exercise with intensities above 60% VO_{2max}, (as in the current study) influence the increase in circulating levels of lymphocytes (Nieman, 1994). It is hard to say if this was the case in the present study and if vitamin supplementation has any effect on adrenalin secretion, as we did not investigate this hormone.

The cortisol levels in the present study were measured in the saliva, while in the studies mentioned above it was measured in serum or plasma. Salivary cortisol has been shown to be significantly correlated with cortisol levels in the blood (Gozansky et al., 2005; Cadore et al., 2008). Therefore, this difference in methodology between studies most likely does not affect the results here. The present study suggests that it is possible that vitamin C and vitamin E have an effect on the hypothalamic-pituitary-adrenal axis influencing cortisol secretion as a consequence. This, however, appears to be independent of changes in the immune cell responses. Nevertheless, other immune markers, such as IL-6 and the immune cell functions, were not measured in the present study. Additionally, in spite of the fact that vitamin plasma levels are the most used parameter to determine the vitamin status, these may not reflect the functional status of vitamins. The redistribution of these vitamins from plasma into immune cells, such as lymphocytes and neutrophil, is an important mechanism that can help to enhance the immune response (Cases et al., 2005). The same would apply for the vitamin redistribution in the immune cells in the respiratory airways. As reported, in this chapter, there was a smaller increase in the neutrophil percentage and IL-8 concentration in the upper respiratory airways in the Vitamin trial, suggesting that the increase in local antioxidant defences was indeed protective. However, it would be interesting to understand the redistribution of antioxidants present in the respiratory tract and those present within the airways inflammatory cells and respiratory epithelium cells.

The lack of effect of vitamin supplementation in relation to the plasma markers of inflammation could possibly be because the ED of ozone inhaled by the participants of the present study was not enough to induce a systemic inflammation. This would occur because most of the time ozone is not able to infiltrate from the lung-air interface further than the RTLF and the membranes of cells (Pryor *et al.,* 1995), due to its initial contact and neutralization by antioxidants in the RTLF. It could be speculated that only

when there is a high level of lung oxidative stress and inflammation would ozone result in a systemic response. In addition, if the immune cell variation in the plasma was due to the exercise effect and not the heat and ozone, it is possible that if the exercise duration was longer we would have observed higher levels of inflammation and hence, the higher plasma antioxidant concentration in the Vitamin trial could have helped to counteract the pronounced inflammation. On the other hand, the participants of this study were well trained runners who were used to taking part in competitions. Thus, it is possible that their antioxidant defences were already up-regulated to respond to the oxidative stress and inflammation experienced in the trials. In addition, it is likely that the participants had a balanced daily diet, ingesting the recommended levels of vitamins and minerals. The benefits of ingesting a higher level of antioxidant, through the vitamin supplementation, would mainly be beneficial for the protection of their lungs against inflammation, especially for the individuals capable of a higher up regulation of their antioxidant defences.

To conclude, it has been demonstrated that 2 weeks of vitamin C and E supplementation helped to diminish the early inflammatory response observed in the upper airways of runners taking part in an 8 km time-trial run in a hot, humid and ozone-polluted environment. In addition, the supplementation also had an effect in blunting the cortisol increase and the blood lymphocyte count immediately after the exercise. Nevertheless, no effect of the supplementation was observed for total white blood cell count and neutrophils. Therefore, it seems that the supplementation with the vitamins has only a small beneficial effect on the systemic inflammation of the runners. Based on the results from this chapter and *Chapter 6* it can be speculated that the availability of antioxidants and the up-regulation of these in the upper respiratory airways is linked to a better performance in an environment with various oxidant stimulus.

Chapter 8: General Discussion

This final chapter contains the main results of the studies comprised within this thesis, followed by a general discussion that ties them together and discusses the relevance of these findings and the practical implications within the field of Sports Sciences and Exercise Immunology. Furthermore, the limitations of the studies, within this thesis are outlined. Finally, the importance and direction of future studies is discussed.

The general aim of this study was twofold: to investigate how ozone-pollution, heat and humidity would affect well-trained runners after performing an intense exercise bout and to examine whether antioxidant supplementation could have any positive effect on them. The main findings of the studies comprised within this thesis were:

- The nasal lavage procedure used in this study was reliable and reproducible to assess markers of inflammation and oxidative stress in the upper respiratory airways.
- Ozone-pollution, heat and humidity alone or combined did not affect the lung function of the runners immediately after performing an 8 km time-trial run.
- Both a hot and humid, and a hot, humid and ozone-polluted environment have a detrimental effect on athletes' performance in an 8 km time-trial run. Ozone alone has little effect on this variable.
- A hot, humid and ozone-polluted environment elicits an early epithelial damage characterized by increase in CC16 concentration in the airways after an 8 km time trial run. Ozone or heat and humidity alone did not have an effect on this variable.
- Performing an 8 km time trial run in a hot, humid and ozone-polluted environment resulted in a significant increase in the GSH/protein concentration in the upper respiratory airways, suggesting an up-regulation in the antioxidant defences in response to an increase in oxidative stress stimulated by the high intensity exercise in association with ozone, heat and humidity.
- After the Heat + O_3 trial, a significant negative correlation was observed between performance impairment and antioxidant status in the athletes' nasal lavage.

- A 2-week supplementation protocol with vitamin C and E significantly increased the plasma antioxidant concentration at baseline and also after an 8 km time trial run in a hot, humid and ozone-polluted environment. In addition, there was also a significant increase in the antioxidant capacity in the upper respiratory airways after the run.

- When taking part in the Vitamin trial, the participants' time to complete the run improved 2.6% compared to the Placebo trial. Additionally, significant positive correlations were found between antioxidant concentration and improvement in time to complete the run.

- The supplementation protocol protected the upper respiratory airways of the runners by decreasing lung injury, observed because of the lower CC16 levels both in the plasma and nasal lavage immediately after the run. In addition, 6 h after the run there was a lower IL-8 concentration and neutrophil percentage in the upper respiratory airways of the runners which also suggests that the supplementation was helpful in diminishing lung inflammation.

- In addition, the supplementation also had an effect in blunting the cortisol increase and the blood lymphocyte count immediately after the exercise. No effect of the supplementation was observed for the total white blood cell count and neutrophils count, immediately after the run, or for any plasma immune cell 6 h after the run.

The results of this thesis suggest that individuals taking part in a competition in a hot, humid and polluted environment will possibly experience adverse effects related to their performance and lung toxicity. These can be minimized if the individuals go through a short supplementation period with a low dosage of vitamin C and vitamin E. It is important to highlight that some people can respond differently to the pollutant exposure and also to the vitamin supplementation. Factors that can increase the susceptibility to ozone-pollution include disease status, genetic predisposition and age. In the studies within this thesis, we could control for disease status and age, however, the genetic predisposition might have accounted for the large standard deviation of some results. In fact, it has been reported that polymorphisms in oxidative stress genes and inflammatory genes influence the response to ozone-exposure (Yang *et al.*, 2009). In *Chapter* 4, a significant negative relationship was established for antioxidant

concentration in nasal lavage, after the run and the percentage of performance impairment when the runners exercised in the hot, humid and ozone-polluted environment. This suggests that how an individual's body copes with oxidative stress and up-regulation of antioxidants also affects their susceptibility to ozone-pollution and their subsequent performance.

Individual variation was also present in response to the vitamin supplementation. As elucidated in *Chapter* 6, some individuals presented a higher concentration of antioxidants in the plasma and nasal lavage after the supplementation protocol and also after the exercise bout. This could be explained by differences in vitamin absorption and metabolism, which can be influenced by dietary intake and possibly by genetic factors (Peake *et al*., 2007). In practical terms, athletes should be aware of long-term antioxidant supplementation interventions because it can act as a double-edged sword: they might provide some benefit to the athletes' immune system, but antioxidant supplementation has also been shown to decrease training adaptation. The studies presented in Chapters 6 and 7 used a shorter supplementation period and vitamin dose compared to the majority of research studies that have analysed the effect of vitamin supplementation on exercising individuals. The results demonstrated that this shorter supplementation does provide some protection for individuals who are already well-adapted to the oxidative stress burden of a high intensity exercise bout, but who are not used to having additional stressors such as heat, humidity and ozone-pollution influencing their performance. Therefore, the speculation is that trained individuals might only benefit from antioxidant supplementation when they go through a much higher oxidative stress stimulus than they are used to. In addition, it is possible that individuals who have lower antioxidant defences in their body might benefit more from antioxidant supplementation when they are exposed to a situation which elicits high levels of oxidative stress. These results are of high practical relevance when one considers the high number of individuals who exercise outdoors and/or engage in competitive events and also the millions of pounds spent every year on antioxidant supplements, especially vitamin C and E.

Another relevant finding was that ozone-pollution alone (at this low concentration) does not appear to majorly affect well-trained runners taking part in an intense exercise bout.

Even though some studies have suggested the contrary, there are various factors, which were previously described, that can influence this outcome. This result might not have a large practical implication because usually people are not exposed just to ozone but to a mixture of pollutants. It is ethically difficult to investigate higher levels of ozone and also the cocktail of pollutants that an athlete would normally experience in a polluted environment. Nevertheless, that result suggests that some well-trained individuals are prepared to cope with some degree of exposure to oxidant gases without developing major respiratory or performance distress.

8.1 LIMITATIONS OF THE STUDIES COMPRISED WITHIN THIS THESIS

One of the key limitations of the studies in this thesis was the use of an acute ozone exposure model. This does not reflect what occurs with active individuals who live in a polluted city or athletes who spend a period of days competing in a polluted city. Nevertheless, the aim of the studies was to analyse an acute exposure, this has provided insight to the early events that occur once ozone is inspired by exercising individuals. Another limitation, that has been mentioned previously, is the number of individuals that took part in the study. This, however, is a limitation that occurs with most research in the Sports Science area, and specifically in the field of Exercise Immunology. The use of high level runners besides adding to the novelty of the studies can also been seen as a limitation because it makes it challenging to extend these results to the general population. The use of the nasal lavage technique, in spite of its numerous advantages over the other techniques used to assess the respiratory airways, presents as a limitation the lack of research studies evaluating the similarity and difference in response between the upper and lower respiratory airways. In addition it would be interesting to investigate if the nasal lavage procedure can detect differences when individuals breathe predominantly through their mouth or nose.

For *Chapters* 4 and 5, the study design presented the limitation of only obtaining blood and nasal lavage samples immediately post-exercise. It would have been useful to also get samples at the 6 h time point to be able to have a better understanding of the development of the inflammatory process over time. This limitation was resolved in the

study design for *Chapters* 6 and 7.

Chapters 6 and 7 present as a limitation the lack of knowledge of the vitamin E concentration in the plasma and nasal lavage. This limitation was due to technical issues and unfortunately this procedure could not be conducted with another assay as was done with the vitamin C. In addition, there was an interest to investigate other end points related with oxidative stress and inflammation, however, lack of time and funding restricted this.

8.2 RECOMMENDATIONS FOR FUTURE STUDIES

Most studies reviewed in this thesis and the studies developed within the thesis have investigated the effect of an acute exposure to air pollution on exercising individuals. An important issue that needs to be addressed in further studies is the effect of chronic exposure to pollutants. This would have practical relevance for individuals who live in large industrialized areas and are exposed daily to pollution. In addition, it would provide information about adaptation processes that might occur, in the lungs, due to the repeated exposure to pollutants. Nevertheless, studies involving human exposure to a mixture of pollutants or multiple exposures would have severe ethical implications. A solution, however, would be for these studies to be conducted in cities with high pollution levels. Yet there would then be the limitations associated with field studies as contrasted with chamber exposures. Therefore, it is important to always keep in mind that all studies present limitations and that it is up to the researchers to minimize these limitations.

In vitro studies are also necessary to investigate the specific mechanisms of antioxidant up-regulation and secretion from lung epithelial cells when in contact with pollutants. These studies would help to clarify the defence processes that occur in the lungs, the cell adaptation processes and the biochemical pathways that are activated when an individual is exposed to high level of pollution. A further step would be to analyse if chronic exposure would lead to gene modification as occurs with chronic exercise.

In respect to vitamin intake, it would be interesting to investigate how a lower supplementation period or even a high dose intake, before a competitive event in a polluted environment, would affect athletes' performance, lung inflammation and oxidative stress. The idea behind this acute supplementation protocol is to cancel out the influence of the antioxidant ingestion affecting training adaptations, but to maintain its protective effect. Still in relation to vitamin supplementation, there is a continuous need for more in-depth investigation into the mechanisms involved in the protective role of vitamin E and C in mediating the ozone-induced damage and changes to molecules in the lungs. For this, the measurement of vitamin content in the immune cells, before and after vitamin supplementation together with the investigation to see if these immune cells are activated using flow cytometry method would be of essence to achieve a better understanding of this intervention.

Another interesting point for future studies is the prevention of illnesses, mainly upper respiratory tract infections, by vitamin supplementation of athletes training or participating in events that are located in high pollution areas. This would clarify if the role of vitamin supplementation is just to protect the lung against oxidative stress and inflammation or if its benefits are longer term and clinically significant in providing protection in the respiratory airways. In addition, the investigation of different sports and different combinations of antioxidant supplementation would also provide better practical implications.

Finally, taking into consideration that the ozone-pollution levels together with the temperature might continue to gradually increase throughout the years, it is very important to investigate the impact of ozone exposure in more vulnerable population groups. In these groups, which include the children and the elderly, the inhalation of ozone is of clinical relevance and, thus, the ingestion of vitamin supplementation might have a significant beneficial impact.

A Final Note

Hopefully the results of the studies within this thesis have helped clarify and expand on a topic that still has many aspects to be investigated. The immune system is very complex and has a large network of cells and mediators regulating its response. This makes research in this area very challenging especially when other factors - which include exercise protocol, individual susceptibility and pollutant exposure - that have an array of variability are added to the investigation. Therefore, the outcomes of the studies are usually very specific to the factors analyzed and, in order to be able to generalize more investigation would be necessary.

REFERENCES

Adams, W. C. & Schelegle, S. E. (1983). Ozone and high ventilation effects on pulmonary function and endurance performance. *Journal of Applied Physiology: Respiratory, Environmental and Exercise Physiology, 55,* 805-812.

Alessio, H. M., Goldfarb, A. H. & Cao, G. (1997). Exercise-induced oxidative stress before and after vitamin C supplementation. *International Journal of Sport Nutrition, 7,* 1-9.

Alessio, H. M., Hagerman, A. E., Fulkerson, B. K., Ambrose, J., Rice, R. E. & Wiley, R. L. (2000). Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. *Medicine & Science in Sports & Exercise, 32* (9), 1576-1581

Allgrove, J. E., Gomes, E. C., Hough, J., Gleeson, M. (2008). Effects of exercise on salivary antimicrobial proteins and markers of stress in active men. *Journal of Sports Sciences,* 26(6), 653-661.

An, X., Zhu, T., Wang, Z., Li, C. & Wang, Y. (2006). A modeling analysis of a heavy air pollution episode occurred in Beijing. *Atmosmospheric Chemistry and Physics Discussion, 6,* 8215–8240.

Anderson, M. E. (1998). Glutathione; an overview of biosynthesis and modulation. *Chemico-Biological Interactions, 111,* 1-14.

Ashton, T., Young, I. S., Peters, J. R., Jones, E., Jackson, S. K., Davies, B. & Rowlands, C. C. (1999). Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. *Journal of Applied Physiology, 87*(6), 2032–2036.

Bailey, D. M., Davies, B., Young, I. S., Jackson, M. J., Davison, G. W., Isaacson, R. & Richardson, R. S. (2003). EPR spectroscopic detection of free radical outflow from an isolated muscle bed in exercising humans. *Journal of Applied Physiology, 94,* 1714-1718.

Bailey, D. M., Young, I. S., McEneny, J., lawrenson, L., Kim, J., Barden, J. & Richardson, R. S. (2004). Regulation of free radical outflow from an isolated muscle bed in exercising humans. *American Journal of Physiology - Heart Circulatory Physiology, 287* (4), H1689-99.

Baldasano, J. M., Valera, E. & Jiménez, P. (2003). Air quality data from large cities. *Science of the Total Environment, 307,* 141-165.

Barnes, P J. (1992). Neural mechanisms in asthma. *British Medical Bulletin, 48(1),* 149-168.

Bates, D. V. (2005). Ambient ozone and mortality. *Epidemiology,* 16 (4), 427-429.

Baulig, A., le Garlatti, M., Bonvallot, V., et al. (2003). Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells. *American Journal Physiology Lung Cellular and Molecular Physiology, 285,* L671–L679.

Bell, M. L., Davis, D. L., Gouveia, N., Borja-Aburto, V. H. & Cifuentes, L. A. (2006). The avoidable health effects of air pollution in three Latin American cities: Santiago, Sao Paulo, and Mexico City. *Environmental Research, 100,* 431-440.

Behndig, A. F., Mudwau, I. S., Brown, J. L., stenfors, N., helleday, R., Duggan, s. T., Wilson, S. J., Boman, C., Cassee, R. R., Frew, A. J., Kelly, F. J., Sandrom, T. & Blomberg, A. (2006). Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *European Respiratory Journal, 27,* 359-365.

Belda, J., Ricart, S., Casan, P., Giner, J., Bellido-Casado, J., Torrejon, M., Margarit, G. & Drobnic, F. (2008). Airway inflammation in the elite athlete and type of sport. *British Journal of Sports Medicine, 42,* 244-248.

Benson, M., Carlsson, L., Adner, M., Jerna, M., Rudemo, M., Sjögren, A., Svensson, P. A., Uddman, R. & Cardell, L. O. (2004). Gene profiling reveals increased expression of uteroglobin and other anti-inflammatory genes in glucocorticoid-treated nasal polyps. *Journal of Allergy and Clinical Immunology, 113,* 1137-1143.

Bernard, A., Gonzales-Lorenzo, J. M., Siles, E., Trujillano, G. & Lawerys, R. (1994). Early decrease of serum Clara cell protein in silica-exposed workers. *European Respiratory Journal, 7,* 1932-1937.

Bernard, A., Hermans, C., Van Houte, G. (1997). Transient increase of Clara cell protein (CC16) after exposure to smoke. *Occupational and Environmental Medicine, 54,* 63-65

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G. & Gluud, C. (2007) Mortality in Randomized Trials of Antioxidant Supplements for Primary and Secondary Prevention: Systematic Review and Meta-analysis. *Journal of the American Medical Association,* 287**,** 842-857.

Blanco, R. A., Ziegler, T. R., Carlson, B. A., Cheng, P. Y., Park, Y., Cotsonis, G. A.,

Accardi, C. J. & Jones, D. P. (2007). Diurnal variation in glutathione and cysteine redox states in human plasma. *The American Journal of Clinical Nutrition, 86,* 1016-1023.

Blannin, A. K., Chatwin, L. J., Cave, R. & Gleeson, M. (1996). Effects of submaximal cycling and long-term endurance training on neutrophil phagocytic activity in middle aged men. *British Journal of Sports Medicine, 30,* 125-129.

Blomberg, A., Mudway, I. S., Nordenhäll, C., Hedenström, H., Kelly, F. J., Frew, A. J., Holgate, S. T. & Sandström, T. (1999). Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. *European Respiratory Journal, 13,* 1418-1428.

Blomberg, A. (2000). Airway inflammatory and antioxidant responses to oxidative and particulate air pollutants – experimental exposure studies in humans. *Clinical and Experimental Allergy, 30,* 310-317.

Blomberg, A., Mudway, I., Svensson, M., Hagenbjörk-Gustafssonz, A., Thomassonz, L., R. Helleday, Dumont, X., Forsberg, B., Nordeberg, G. & Bernard, A. (2003). Clara cell protein as a biomarker for ozone-induced lung injury in humans. *European Respiratory Journal, 22,* 883-888.

Bloomer, R. J. & Fisher-Wellman, K. H. (2008). Blood oxidative stress biomarkers: influence of sex, exercise training status, and dietary intake. *Gender Medicine, 5* (3), 218-228.

Bloomer, J. B., Stehr, J. W., Piety, C. A., Salawitch, R. J. & Dickerson, R. R. (2009). Observed relationships of ozone air pollution with temperatures and emissions. *Geophysical Review Letters, 36,* 1-22.

Bonsignore, M. R., Morici, G., Riccobono, L., Insalaco, G., Bonanno, A., Profita, M., Paternò, A., Vassalle, C., Mirabella, A. & Vignola, A. M. (2001). Airway inflammation in nonasthmatic amateur runners. *American Journal Physiology Lung Celular and Molecular Physiology, 281,* 668-676.

Bonsignore, M. R., Morici, G., Vignola, A. M., Riccobono, L., Profita, M., Bonanno, A., Paternò, A., Giorgi, R., Chimenti, L., Abate, P., Mirabella, F. & Bonsignore, G. (2003). Increased airway inflammatory cells in endurance athletes: what do they mean? *Clinical and Experimental Allergy, 33,* 14-21.

Booker, R. (2005). Best practice in the use of spirometry. *Nursing Standard,* 19, 49-54.

Borg, G. (1998). *Borg's Perceived Exertion and Pain Scales. Champain, IL: Human*

Kinetics.

Boyum, A., Rosen, O., Tennfjord, V.A., Tollefsen, S., Haugen, A. H., Opstad, P. K. & Bahr, R. (2002). Chemiluminescence response of granulocyes from elite athletes during recovery from one or two intense bouts of exercise. *European Journal of Applied Physiology, 88,* 20-28.

Broeckaert, F. & Bernard, A. (2000). Clara cell secretory protein (CC16): characteristics and perspectives as lung peripheral biomarker. *Clinical & Experimental Allergy, 30,* 469-475.

Broeckaert, F., Arsalene, K., Hermans, C., Bergamaschi, E., Brustolin, A., Muff, A. & Bernard, A. (2000a). Serum clara cell protein: a sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. *Environmental health Perspectives, 108,* 533-537.

Broeckaert, F., Clippe, A., Knoops, B., Hermans, C. & Bernard, A. (2000b). The impact Clara cell secretory protein (CC16): features as a peripheral lung biomarker. *Annals New York Academy of Science, 923,* 68-77.

Brunekreef, B., Hoek, G., Breugelmans, O. & Leentvaar, M. (1994). Respiratory effects of low-level photochemical air pollution in amateur cyclists. *American Journal of Respiratory and Critical Care Medicine, 150,* 962-966.

Brunekreef, B. & Holgate, S. T. (2002). Air pollution and health. *The Lancet, 360,* 1233-1242.

Bruunsgaard, H., Poulsen, H. E., Pedersen, B. K., Nyyssönen, K., Kaikkonen, J. & salonen, J. T. (2003). Long-term combined supplementations with α-tocopheroland vitamin C have no detectable anti-inflammatory effects in healthy men. *The Journal of Nutrition, 133,* 1170-1173.

Cadore, E., Lhullier, F., Brentano, M., Silva, E., Ambrosini, M., Spinelli, R., Silva, R. & Kruel, L. (2008). Correlations between serum and salivary hormonal concentrations in response to resistance exercise. *Journal of Sports Sciences, 26* (10), 1067-1072.

Cantin, A. M., North, S. L., Hubbard, R. C., Crystal, R. G. (1987). Normal alveolar epithelial lining fluid contains high levels of glutathione. *Journal of Applied Physiology, 63*(1), 152-157.

Carbonelle, S., Francaux, M., Doyle, I., Dumont, X., de Burbure, C., Morel, G., Michel, O. & Bernard, A. (2002). Changes in serum pneumoproteins caused by short-term exposures to nitrogen trichloride in indoor chlorinated swimming pools. *Biomarkers, 7,* 464-478.

Cases, N., Aguiló, A., Tauler, P. Sureda, A., Llompart, I., Pons, A & Tur, J. A. (2005). Differential response of plasma and immune cell's vitamin E levels to physical activity and antioxidant vitamin supplementation. *European Journal of Clinical Nutrition, 59,* 781-788.

Celli, B. R., MacNee, W., and committee members (2004). Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *European Respiratory Journal,* 23, 932–946.

Cerqueira, F. M., Medeiros, M. H. G. & Augusto, O. (2007) Antioxidantes dietéticos: controvérsias e perspectivas. *Química Nova, 30***,** 441-449.

Child, R., Brown, S., Day, S.,et al. (1999). Changes in indices of antioxidant status, lipid peroxidation and inflammation in human skeletal muscle after eccentric muscle actions. *Clinical Science, 96,* 105-115.

Christian, D. L., Chen, L. L., Scannel, C. H., Ferrando, R. E., Welch, B. S. & Balmes, J. R. (1998). Ozone-induced inflammation is attenuated with multiday exposure. *American Journal of Respiratory and Critical Care Medicine, 158,* 532-537.

Coombes, J. S., Powers, S. K., Rowell, B., Hamilton, K. L., dodd, S. L., Shanely, R. A., Sen, C. K. & Packer, L. (2001). Effects of vitamin E and α-lipolic acid on skeletal muscle contractile properties. *Journal of Applied Physiology, 90,* 1424-1430.

Corradi, M., Alinovi, R., Goldoni, M., Vettori, M., Folesani, G., Mozzoni, P., Cavazzini, S., Bergamaschi, E., Rossi, L. & Mutti, A. (2002). Biomarkers of oxidative stress after controlled human exposure to ozone. *Toxicology Letters, 134,* 219-225.

Cross, C. E., Vasu, V. T., Lim, Y. & Gohil, K. (2006). Combating oxidative stress at respiratory biosurfaces: challenges yet to be resolved, a commentary on "Vitamin supplementation does not protect against symptoms in ozone-responsive subjects". *Free Radical Biology and Medicine, 40,* 1693-1697.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarine, D., Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clinical Chemistry, 52* (4), 601-623.

Davison, G. & Gleeson, M. (2005). Influence of acute vitamin C and/or carbohydrate ingestion on hormonal, cytokine, and immune responses to prolonged exercise. *International Journal of Sport Nutrition and Exercise Metabolism, 15,* 465–479.

Davison, G. & Gleeson, M. (2006). The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man. *European Journal of*

Applied Physiology, 97, 454-461.

Davison, G., Gleeson, M. & Phillips, S. (2007). Antioxidant Supplementation and Immunoendocrine Responses to Prolonged Exercise. *Medicine & Science in Sports & Exercise, 39***,** 645-652.

Dekkers, J. C., van Doornem, L. J. & Kemper, H. C. (1996). The role of antioxidant vitamins in the prevention of exercise-induced muscle damage. *Sports Medicine, 21*, 213-238.

Denguezli, M., Chiekh, I. B., Saad, H. B., Zaouali-Ajina, M., Tabka, Z. & Zbidi, A. (2008). One-year endurance training: effects on lung function and airway inflammation. *Journal of Sports Sciences, 26* (12), 1351-1359.

Devlin, R. B., McDonnel, W. F., Becker, S., Madden, M. C., McGee, M. P., Perez, R., Hatch, G., House, D. E. & Koren, H. S. (1996). Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: a comparison of mediators found in the bronchoalveolar lavage fluid 1 and 18 hs after exposure. *Toxicology and Applied Pharmacology, 138,* 176-185.

Dill, D. B. & Costill, D. L. (1974). Calculation of percentage changes in volume of blood, plasma, and red cells in dehydration. *Journal of Apllied Physiology, 37*, 247-248.

Douglas, R. M., Hemilä, H., D'Souza, R., Chalker, E. B., Tracey, B. (2004). Vitamin C for preventing and treating the common cold. *Cochrane Database System Reviews*, 4, 1-67.

Ely, M. R., Chieuvront, S. N., Roberts, W. R. & Montain, S. J. (2007). Impact of weather on marathon-running performance. *Medicine & Science in Sport & Exercise, 39*, 487-493.

Expert Group on Vitamins and Minerals (2003). Safe Upper Levels for Vitamins and Mineral. Available from: http://www.food.gov.uk/multimedia/pdfs/vitmin2003.pdf

Fogarty, A., Lewis, S. A., Scrivener, S. L., Antoniak, M., Pacey, S., Pringlew, M. & Britton, J. (2003). Oral magnesium and vitamin C supplements in asthma: a parallel group randomized placebo-controlled trial. *Clinical & Experimental Allergy,* 33**,** 1355-1359.

Febbraio, M. A. & Pedersen, B. K. (2002). Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *The FASEB Journal*, 16, 1335-1347.

Fiala, J., Cernikovisky, L., Leeuw, F. & Kurfuerst, P. (2003). Air pollution by ozone in

Europe in summer 2003. Overview of exceedances of EC ozone threshold values during summer season April-August 2003 and comparisons with previous years – Topic Report. *European Environmental Agency.*

Fisher, C. P., Hiscock, N. J., penkowa, m., Basu, S., Vessby, B., Kallner, A., Sjöberg, L. & Pedersen, B. K. (2004). Supplementation with vitamins C and E inhibits the release of interleukin-6 from contracting human skeletal muscle. *Journal of Physiology, 558.2,* 633-645.

Fisher-Wellman, K. & Bloomer, R.J. (2009). Acute exercise and oxidative stress: a 30 year history. *Dynamic Medicine, 8* (1), 1-25.

Flanagan, S. W., Moseley, P. L. & Buettner, G. R. (1998). Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonance spin trapping. *FESB Letters, 431,* 285-286.

Folinsbee, J.L., Bedi, J.F., Horvath, S.M. (1984). Pulmonary function changes after 1 h continuous heavy exercise in 0.21 ppm ozone. *Journal of Applied Physiology: Respiratory Environment and Exercise Physiology*, *57*(4), 984-988.

Frank, R., Liu, M. C., Spannhake, E. W., Mlynarek, S., Macri, K. & Weinmann, G. G. (2001). *American Journal of Respiratory and Critical Care Medicine,164,* 1253-1260.

Galloway, S. D. R. & Maughan, R. J. (1997). Effects of ambient temperature on the capacity to perform prolonged cycle exercise in man. *Medicine & science in Sport & Exercise, 29,* 1240-1249.

Gibbons, S. I. & Adams, W. C. (1984). Combined effects of ozone exposure and ambient heat on exercising females. *Journal of Applied Physiology: Respiratory Environment and Exercise Physiology, 57,* 450-456.

Gioldassi, X. M., Papadimitriou, H., Mikraki, V. & Karamanos, N.K. (2003) Clara cell secretory protein: determination of serum levels by na enzyme immunoassay and its importance as an indicator of bronchial asthma in children. *Journal of Pharmaceutical and Biomedical Analysis, 34*(4), 823-826.

Gohil, K., Vigui, C., Stanley, W. C., Brooks, G. A. & Packer, L. (1988). Blood glutathione oxidation during human exercise. *Journal of Applied Physiology, 64*(1), 115-119.

Goldfarb, A. H., Bloomer, R. J. & Mckenzie, M. J. (2005). Combined Antioxidant Treatment Effects on Blood Oxidative Stress after Eccentric Exercise. *Medicine & Science in Sports & Exercise,* 37**,** 234-239.

Gomez-Cabrera, M., Borras, C., Pallardo, F. V., Sastre, J., Ji, L. L. & Vina, J. (2005).

Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. *Journal of Physiology, 567.1,* 113-120.

Gomez-Cabrera, M., Martínez, A., Santangelo, G., Pallardó, F. V., Sastre, J. & Vina, J. (2006). Oxidative stress in maratón runners: interest of antioxidant supplementation. *British Journal of Nutrition, 96* (S1), 31-33.

Gomez-Cabrera, M., Domenech, E. & Vina, J. (2008a). Moderate-exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radical Biology & Medicine, 44,* 126-131.

Gomez-Cabrera, M., Domenech, E., Romagnoli, M., Arduini, A., Borras, C., Pallardo, F. V., Sastre, J. & Vina, J. (2008b). Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *American Journal of Clinical Nutrition, 87,* 142-149.

Gong, H. J., Bradley, P. W., Simmons, M. S. & Tashkin, D. P. (1986). Impaired exercise performance and pulmonary function in elite cyclists during low-level ozone exposure in a hot environment. *American Review of Respiratory Disease, 134,* 726-733.

González-Alonso, J., Teller, C., Andersen, S. L., Jensen, F. B., Hyldig, T. & Nielsen, B (1999). Influence of body temperature on the development of fatigue during prolonged exercise in the heat. *Journal of Applied Physiology, 86* (3), 1032-1039.

González-Alonso, J.,Crandall ,C. C., Johnson, J. M. (2008). The cardiovascular challenge of exercising in the heat. *The Journal of Physiology, 586,* 45-53.

Gozansky, W. S., Lynn, J. S., Laudenslagert, M. L. & Kohrt, W. M. (2005). Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic-pituitary-adrenal axis activity. *Clinical Endocrinology, 63,* 336-341.

Gregg, I., Nunn, A. J. (1973). Peak expiratory flow in normal subjects. *British Medical Journal, 3,* 282-284.

Grievink, L., Jansen, S. M., Veer, P. V. T. & Brunekreef, B. (1998). Acute effects of ozone on pulmonary function of cyclists receiving antioxidant supplements. *Occupational and Environmental Medicine, 55***,** 13-17.

Grievink, L., Zijlstra, A. G., Ke, X. & Brunekreef, B. (1999). Double-Blind Intervention Trial on Modulation of Ozone Effects on Pulmonary Function by Antioxidant Supplements. *American Journal of Epidemiology, 149* (4), 306-314.

Halliwell, B. & Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*, 3rd ed. Oxford University Press, New York.

Hack, V., Strobel, G., Weiss, M. & Weicker, H. (1994). PMN cell counts on phagocitic activity of highly trained athletes depend on training period. *Journal of Applied Physiology, 77* (4), 1731-1735.

Hathcock, J. N., Azzi, A., Blumberg, J., Bray, T., Dickinson, A., Frei, B., Jialal, I., Johnston, C. S., Kelly, F. J., Kraemer, K., Packer, L., Parthasarathy, S., Sies, H. & Traber, M. G. (2005). Vitamins E and C are safe across a broad range of intakes. *American Journal of Clinical Nutrition, 81*, 736-745.

Hazucha, M. J., Bates, D. V. & Bromberg, P. A. (1989). Mechanism of action of ozone on the human lung. *Journal of Applied Physiology, 67* (4), 1535-1541.

Helleday, R., Segerstedt, B., Forsberg, B., Mudway, I., Nordberg, G., Bernard, A. & Blomberg, A. (2006). Exploring the time dependence of serum clara cell protein as a biomarker of pulmonary injury in humans. *Chest, 130*, 672-675.

Hemilä, H., Chalker, E. B., Tracey, B., Douglas, R. M., (2009). Vitamin C for preventing and treating the common cold. *Cochrane Database System Reviews*, 3, 1-67.

Hellsten, Y., Hansson, H. A., Johnson, L., Frandsen, U. & Sjodin, B. (1996). Increased expression of xanthine oxidase and insulin-like growth factor I (IGF-I) immunoreactivity in skeletal muscle after strenuous exercise in humans. *Acta Physiologica Scandinavica, 151*, 191-197

Hermans, C. & Bernard, A. (1999). Lung epithelium-specific proteins. Characteristics and potential applications as markers (State of art). *American Journal of Respiratory and Critical Care Medicine, 159,* 646-678.

Hermans, C., Knoops, B., Wiedig, M., Arsalene, K., Toubeau, G., Falmagne, P. & Bernard, A. (1999). Clara cell protein as a marker of clara cell damage and bronchoalveolar blood barrier permeability. *European Respiratory Journal, 13*, 1014-1021.

Hernández-Escobar, S. A., Avila-Casado, M. C., Soto-Abraham, V., López Escudero, O. L., Soto, M. E., Veja- Bravo, . L., van der Goes, T. F., Reyes-Maldonado, E. (2009). Cytological damage of nasal epithelium associated with decreased glutathione peroxidise in residents from a heavily polluted city. *International Archives of Occupational and Environmental Health,* 82, 603-612.

Hissin, P. J., Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry, 74,* 214-226.

Holz, O., Jörres, R. A., Timm, P., Mücke, M., Richter, K., Koschyk, S. & Magnussen, H. (1999). Ozone-induced inflammatory changes differ between individuals and are reproducible. *American Journal of Respiratory and Critical Care Medicine, 159,* 776-

784.

Hopkins, W. G. & Hewson, D. J. (2001). Variability of competitive performance of distance runners. *Medicine & Science in Sports & Exercise, 33*, 1588–1592.

Horstman, D. H., Folinsbee, L. J., Ives, P. J., Abdul-Salaam, S. & McDonnell, W. F. (1990). Ozone concentrations and pulmonary responses relationships for 6.6-hour exposures with five hours of moderate exercise to 0.08, 0.10 and 0.12 ppm (ozone). *American Review of Respiratory Disease, 142*, 1158-1163.

Housley, D. G., Mudway, I., Kelly, F. J., Eccles, R. & Richards, R. J. (1995). Depletion of urate in human nasal lavage following in vitro ozone exposure. *International Journal of Biochemistry and Cellular Biology, 27*, 1153-1159.

Howals, H. & Segesser, B. (1975). Ascorbic acid and athletic performance. *Annals of the New York Academy of Sciences,* 30, 458-464.

Institute of Medicine (2000). Dietary intakes for vitamin C, vitamin E, selenium, carotenoids. Washington, DC: National Academy Press. Available from: http:www.nap.edu/catalog/9810.htlm.

IPCC (2007). Intergovernmental panel on climate change: fourth assessment report, Geneva, Switzerland.

Iqbal, S., Thomas, A., Bunyan, K. & Tiidus, P. M. (2008) Progesterone and estrogen influence postexercise leukocyte infiltration in overiectomized female rats. *Applied Physiology, Nutrition, and Metabolism, 33* (6), 1207-1212.

Jackson, M. J., Khassaf, M., Vasilaki, A., McArdle, F. & McArdle, A. (2004). Vitamin E and the Oxidative Stress of Exercise. *Annals New York Academy of Science,* 1031**,** 158–168.

Jacob, R. A., Skala, J. H. & Omaye, S. T. (1987). Biochemical indices of human vitamin C stauts. *American Journal of Clinical Nutrition, 46*, 818-826.

Jain, P., Kavuru, M. S., Emerman, C. L. & Ahmad, M. (1998). Utility of peak expiratory flow monitoring. *Chest, 114*, 861-876.

Jeukendrup, A. E. & Gleeson, M. (2004). *Sport Nutrition: An introduction to energy production and performance.* Champaign, IL, Human Kinetics.

Ji, L.L. (1999). Antioxidants and oxidative stress in exercise. *Proceeding of the Society*

for Experimental Biology and Medicine, 222(3), 283-92.

Ji, L.L. (2008). Modulation of skeletal muscle antioxidant defence by exercise: Role of redox signalling. *Free Radical Biology and Medicine, 44,* 142-152.

Jimenez, C., Melin, B., Savourey, G., Launay, J. C., Alonso, A. & Mathieu, J. (2007). Effects of passive hyperthermia versus exercise-induced hyperthermia on immune response : hormonal implications. *European Cytokine Network, 18* (3), 154-161.

Johansson, S., Keen, C., Stahl, A., Wennergren, G. & Benson, M. (2005). Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis. *Allergy, 60,* 638-642.

Jörres, R. A., Holz, O., Zachgo, W., Timm, P., Koscyk, S., Müller, B., Grimminger, F., Seeger, W., Kelly, F. J., Dunster, C., Frischer, T., Lubec, G., Waschewski, M., Niendorf, A. & Magnussen, H. (2000). The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. *American Journal of Respiratory and Critical Care Medicine, 161,* 1855-1861.

Kanter, M. (1998). Free radicals, exercise and antioxidant supplementation. *Proceedings of the Nutrition Society, 57,* 9-13.

Karkoulias, K., Habeos, I., Charokopos, N., Tsiamita, M., Mazarakis, A., Pouli, A. & Spiropoulos, K. (2008). Hormonal responses to marathon running in non-elite athletes. *European Journal of Internal Medicine, 19,* 598-601.

Katsouyanni, K. (2003). Ambient air pollution and health. *British Medical Bulletin, 68,* 143-156.

Kelly, F. J. (2004). Dietary antioxidants and environmental stress. *Proceedings of the Nutrition Society, 63,* 579–585.

Keman, S., Jetten, M., Douwes & Borm, P. J. A. (1998). Longitudinal changes in inflammatory markers on nasal lavage of cotton workers. *International Archives of Occupational and Environmental Health, 71,* 131-137.

Khassaf, M., McArdle, A., Esanu, C., Vasilaki, A., McArdle, F., Griffiths, R. D., Brodie, D. A. & Jackson, J. M. (2003). Effects of vitamin C supplements on antioxidant defense and stress proteins in human lymphocytes and skeletal muscle. *Journal of Physiology, 549.2,* 645-652.

Knowlton, K., Rosenthal, J. E., Hogrefe, C., Lynn, B., Gaffin, S., Goldberg, R., Rosenzweig, C., Civerolo, K., Ku, J. & Kinney, P. L. (2004). Assessing ozone-related health impacts under a changing climate. *Environmental Health Perspectives*, 112 (15),

1557-1563.

Korric, S.A., Neas, L.M., Dockery, D.W., Gold, D. R., Allen, G. A., Hill, L. B., Kimball, K. D., Rosner, B. A. & Speizer, F. E. (1998). Effect of ozone and other pollutants on the pulmonary function of adult hikers. *Environmental Health Perspectives, 106*(2), 93-99.

Krishna, M. T., Springall, D., Meng, Q., Withers, N., Macleod, D., Biscione, G., Frew, A., Polak, J. & Holgate, S. (1997). Effects of ozone on epithelium and sensory nerves in the bronchial mucosa of healthy individuals. *American Journal of Respiratory and Critical Care Medicine, 156,* 943-950.

Krishna, M. T., Madden, J., Teran, L. M., Biscione, G. L., Lau, L. C., Withers, N. J., Sandström, T., Mudway, I., Kelly, F. J., Walls, A., Frew, A. J. & Holgate, S. T. (1998). Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. *European Respiratory Journal, 11,* 1294-1300.

Kuby, J. (1997). *Immunology*. W.H. Freeman and Company, 3rd Ed, New York.

Lagerkvist, B. J., Bernard, A., Blomberg, A., bergstrom, E., Forsberg, B., Holmstrom, K., Lundstrom, N. G., Segerstedt, B., Svensson, M. & Nordberg, G. (2004). Pulmonary epithelial integrity in children: relationship to ambient ozone exposure and swimming pool attendance. *Environmental Health Perspectives, 112,* 1768-1771.

Leaf, D.A., Kleinman, M.T., Hamilton, M., Barstow, T.J. (1997). The effect of exercise intensity on lipid peroxidation. *Medicine and Science in Sports and Exercise, 29* (8), 1036-1039.

Lindahl, M., Svartz, J. & Tagesson, C. (1999). Demonstration of different forms of the anti-inflammatory proteins lipocortin-1 and Clara cell protein-16 in human nasal and bronchoalveolar lavage fluids. *Electrophoresis, 20,* 881-890.

Lodge, J. K., Hall, W. L., Jeanes, Y. M. & Proteggente, A. R. (2004). Physiological Factors Influencing Vitamin E Biokinetics. *Annals New York Academy of Science,* 1031**,** 60-73.

Lord-Fontaine, S. & Averill-Bates, D. A. (2002). Heat shock inactivates cellular antioxidant defenses against hydrogen peroxide: protection by glucose. *Free Radical Biology and Medicine*, 32(8), 752-765.

Lu, S. C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *The FASEB Journal, 13*, 1169-1183.

Mackinnon, L. T. (1999). *Advances in Exercise Immunology.* Champaign, IL, Human Kinetics.

Massafra, C., Gioia, D., Felice, C., Picciolini, E., Leo, V., Bonifazi, M. & Bernabei, A. (2000). Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catase and glutathione peroxidase activities during the menstrual cycle. *Journal of Endocrinology, 167,* 447-452.

Mastaloudis, A., Morrow, J., Hopkins, D., devaraj, S. & Traber, M. (2004) Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. *Free Radical Biology and Medicine, 36,* 1329-1341.

Mastaloudis, A., Traber, M. G., Cartensen, K. & Widrick, J. J. (2006). Antioxidants did not prevent muscle damage in response to an ultramarathon run. *Medicine & Science in Sports & Exercise, 38,* 72-80.

McAnulty, S. R., McAnulty, L. M., Pascoe, D. D., Gropper, S. S., Keith, R. E., Morrow, J. D. & Gladden, L. B. (2005a). Hyperthemia increases exercise-induced oxidative stress. *International Journal of Sports Medicine, 26,* 188-192.

McAnulty S. R., McAnulty, L. S., Nieman, D. C., Morrow, J. D., Shooter, L. A., Holmes, s., Heward, C. & Henson, D. A. (2005b). Effect of alpha-tocopherol supplementation on plasma homocysteine and oxidative stress in highly trained athletes before and after exhaustive exercise. *The Journal of Nutritional Biochemistry, 16* (9), 530-537

McDonnell, W. F., Kehrl, H. R. & Abdul-Salaam, S. (1991). Respiratory response of humans exposed to low levels of ozone for 6.6 hours. *Archives of Environmental Health, 46,* 145-150.

McDonnell, W. F., Stewart, P. W., Andreoni, S., Seal, E. J., Kehrl, H. R., Horstman, D. H., Folinsbee, L. J. & Smith, M. V. (1997). Prediction of ozone-induced FEV1 changes – effects of concentration, duration and ventilation. *American Journal of Respiratory and Critical Care Medicine, 156,* 715-722.

Medved, I., Brown, M. J., Bjorksten, A. R., Leppik, J. A., Sostaric, S. & McKenna, M. J. (2003). *N*-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *Journal of Applied Physiology, 94,* 1572-1582.

Michilidis, Y., Jamurtas, A. Z., Nikolaidis, M. G., Fatouros, I. G., Koutedakis, Y., Papassotiriou, I. & Kouretas, D. (2007). Sampling time is crucial for measurement of exercise-induced oxidative stress. *Medicine & Science in Sports & Exercise, 39,* 1107-1113.

Mitchell, J. B. & Russo, A. (1983). Thiols, thiol depletion, and thermosensitivity. *Radiation Research,* 95, 471-485.

Miyazaki, H., Oh-ishi, S., Ookawara, T., Kizaki, T., Toshinai, K., Ha, S., Haga, S., Ji, L. L. & Ohno, H. (2001). Strenuous endurance training in humans reduces oxidative stress following exhaustive exercise. *European Journal of Applied Physiology, 84,* 1-6

Mooren, F. C., Blöming, D., Lechtermann, A., Lerch, M. M. & Völker, K. (2002). Lymphocyte apoptosis after exhaustive and moderate exercise. Journal of Applied Physiology, 93 (1), 147-153.

Morice, G., Bonsignore, M. R., Zangala, D., Riccobono, L.., Profita, M., Bonanno, A., paterno, A., Giorgi, R., Mirabella, F., Chimenti, L., Benigno, A., Bellia, V., Amato, G. & Bonsignore, G. (2004). Airway cell composition at rest and after na all-out test in competitive runners. *Medicine & science in Sport & Exercise, 36* (10), 1723-1729.

Morrison, D., Rahman, I. & MacNee, W. (2006). Permeability, inflammation and oxidant status in airspace epithelium exposed to ozone. *Respiratory Medicine, 100,* 2227-2234.

Morrison, S. A., Sleivert, G. G. & Cheung, S. S. (2006). Aerobic influence on neuromuscular function and tolerance during passive hyperthermia. *Medicine & science in Sport & Exercise, 38,* 1754-1761.

Mudway, I. S., Blomberg, A., Frew, A. J., Holgate, S. T., Sandström, T. & Kelly, F. J. (1999a). Antioxidant consumption and repletion kinetics in nasal lavage fluid following exposure of healthy human volunteers to ozone. *European Respiratory Journal, 13,* 1429-1438.

Mudway, S. I., Krishna, M. T., Frew, A. J., MacLeod, D., Sandstrom, T., Holgate, S. T., Kelly, F. J. (1999b). Comprised concentrations of ascorbate in fluid lining the respiratory tract in human subjects after exposure to ozone. *Occupational and Environmental Medicine, 56,*473-481.

Mudway, I. S., Stenfors, N., Blomberg, A., Helleday, R., Dunster, C., Marklund, S. L., Frew, A. J., Sandström, T. & Kelly, F. J. (2001). Differences in basal airway antioxidant concentrations are not predictive of individual responsiveness to ozone: a comparison of healthy and mild asthmatic subjects. *Free Radical Biology and Medicine, 31(8),* 962-974.

Mudway, I. S. & Kelly, F. J. (2004). An investigation of inhaled ozone dose and the magnitude of airway inflammation in healthy adults. *American Journal of Respiratory and Critical Care Medicine, 169,* 1089-1095.

Mudway, I. S., Behdig, A. F., Helleday, R., Pourazar, J., Frew, A. J., Kelly, F. J. & Blomberg, A. (2006). Vitamin supplementation does not protect against symptoms in ozone-responsive subjects. *Free Radical Biology and Medicine, 40***,** 1702-1712.

Nanson, C. J., Burgess, J. L., Robin, M. & Bernard, A. M. (2001). Exercise alters serum pneumoprotein concentration. *Respiration Physiology, 127,* 259-265.

Nielsen, B., Strange, S., Christensen, N. J., Warberg, J. & Saltin, B. (1997). Acute and adaptive responses in humans exposed to exercise in a warm, humid environment. *Pflügers Archiv: European Journal of Physiology*, 56, 434-449.

Nieman, D. C. (1994). Exercise, infection, and immunity. *International Journal of Sports Medicine,15*: S131-S141.

Nieman, D. C., Henson, D. A., Butterworth, D. E., Warren, B. J., Davies, J. M., Fagoaga, O. R. & Nehlsen-Cannarella, S. L. (1997). Vitamin C supplementation does not alter the immune response to 2.5 hours of running. *International Journal of Sport Nutrition, 7*, 173-184.

Nieman, D. C. & Bishop, N. C. (2006). Nutritional strategies to counter stress to immune system in athletes, with special reference to football. Journal *of Sports Sciences,* 24 (7), 763-772.

Nieman, D. C., Peters, E. M., Henson, D. A., Nevines, E. I. & Thompson, M. M. (2000). Influence of vitamin C supplementation on cytokine changes following an ultramarathon. *Journal of Interferon & Cytokine Research, 20* (11), 1029-1035.

Nieman, D. C., Henson, D. A., Fagoaga, O. R., Utter, A. C., Vinci, D. M., Davis, J. M. & Nehlsen-Cannarella, S. L. (2002). Change in salivary IgA following a competitive marathon race. *International Journal of Sports Medicine, 23* (1), 69-75.

Nieman, D. C., Henson, D. A., McAnulty, S. R., mcAnulty, L. S., Morrow, J. D., Ahmed, A. & Heward, C. B. (2004). Vitamin E and immunity after the Kona triathlon world championship. *Medicine & Science in Sports & Exercise, 36* (8), 1328-1335.

Niess, A.M., Hartmann, A., Fuchs-Grunert, M., Poch, B. & Speit, G. (1996). DNA damage after exhaustive treadmill running in trained and untrained men. *International Journal of Sports Medicine, 17*, 397-403.

Niess, A.M., Simon, P. (2007). Response and adaptation of skeletal muscle to exercise – the role of reactive oxygen species. *Frontiers in Bioscience, 12,* 4826-4838.

Nikasinivic-Fournier, L., Just, J., Seta, N., Callais, F., Sahraoui, F., Grimfeld, A. & Momas I. (2002). Nasal lavage as a tool for the assessment of upperairway inflammation in adults and children. *Journal of Laboratory and Clinical Medicine, 139,* 173-180.

Nybo, L. & Nielsen, B. (2001). Perceived exertion is associated with an altered brain activity during exercise with progressive hyperthermia. *Journal of Applied Physiology, 91,* 2017-1023.

O'Neil, C. A., Stebbins, C. L., Bonigut, S., Halliwell, B. & Longhurst, J. C. (1996). Production of Hydroxyl radicals in contracting skeletal muscles of cats. *Journal of Applied Physiology, 81*(3), 1197-1206.

Packer, L. (1997). Oxidants, antioxidant nutrients and the athlete. *Journal of Sports Sciences, 15*, 353-363.

Palmer, F. M., Nieman, D.C., Henson, A. D., McAnulty, S. R., McAnulty, L., Swick, N. S., Utter, A. C., Vinci, D. M. & Morrow, J. D. (2003). Influence of vitamin C supplementation on oxidative and salivary IgA changes following an ultramarathon. *European Journal of Applied Physiology,* 89, 100-107.

Passannante, A., Hazucha, M. J., Bromberg, P. A., Seal, E., Folisbee, L. & Koch, G. (1998). Nocioceptive mechanisms modulate ozone-induced human lung function decrements. *Journal of Applied Physiology, 85 (5),* 1863-1870.

Parkin, J. M., Carey, M. F., Zhao, S. & Febbraio, M. A. (1999) Effects of ambient temperature on human skeletal muscle metabolism during fatiguing submaximal exercise. *Journal of Applied Physiology, 86,* 902-908.

Peake, J. M., Katsuhiko, S. & Coombes, J. S. (2007). The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. *Journal of Nutritional Biochemistry, 18,* 357-371.

Pearson, P. J. K., Lewis, S. A., Britton, J. & Fogarty, A. (2004). Vitamin E supplements in asthma: a parallel group randomised placebo controlled trial. *Thorax, 59,* 652-656.

Peden, D.B., Hohman, R., Brown, M. E., Mason, R. T., Berkebile, C., Fales, H. M. & Kaliner, M. A. (1990). Uric acid is a major antioxidant in human nasal airway secretions. *Proceedings of the National Academy of Science USA, 87,* 7638-7642.

Pedersen, B. K., Ullum, H. (1994). NK cell response to physical activity: possible mechanisms of action. *Medicine & Science in Sports & Exercise, 26,* 140-146.

Peters, E. M., Anderson, R. & Theron, A. J. (2001). Attenuation of increase in circulating cortisol and enhancement of the acute phase protein response in vitamin C-supplemented ultramarathoners. *International Journal of Sports Medicine, 22,* 120-126.

Petersen, E. W., Ostrowski, K., Ibfelt, T., Richelle, M., Offord, E., Halkjaer-Kirstensen, J. & Pedersen, B. K. (2001). Effect of Vitamin Supplementation on Cytokin response and on Muscle Damage after strenous exercise. *American Journal of Physiology and Cell Physiology*, *280,* C1570-1575.

Peiser, B. & Reilley, T. (2004). Environmental factors in the summer Olympics in historical perspective. *Journal of Sports Sciences, 22,* 981-1002.

Pellegrino, R., Viegi, G., Brusasco, V., Crapo, R. O., Burgos, F., Casaburi, R., Coates, A., van der Grinten, C. P. M., Gustafsson, P., Hankinson, J., Jensen, R., Johnson, D.C., MacIntyre, N., McKay, R., Miller, M. R., Navajas, D., Pedersen, O. F. & Wanger, J. (2005). ATS/ERS task force: standardization of lung function testing. *European Respiratory Journal,* 26, 948-968.

Peters, E. M. (1997). Exercise, immunology and upper respiratory tract infections. *International Journal of Sports Medicine*, *18,* suppl 1:S69-77.

Powers, S. K., Ji, L. L. & Leewenburgh, C. (1999). Exercise training induced alterations in skeletal muscle antioxidant capacity: a brief review. *Medicine & Science in Sports & Exercise, 31,* 987-997.

Powers, S.K., DeRuisseau, K. C., Quindry, J., Hamilton, K. L. (2004). Dietary antioxidants and exercise. *Journal of Sports Science, 22,* 81-94.

Pryor, W. A. (1994). Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radical Biology & Medicine, 17* (5), 451-465.

Pryor, W. A., Squadrito, G. L. & Friedman, M. (1995). The cascade mechanism to explain ozone toxicity: the role of lipid ozonation products. *Free Radical Biology and Medicine, 19,* 935-941.

Pyne, D. B. (1994). Regulation of neutrophil function during exercise. *Sports Medicine, 17* (4), 245-258.

Rahman, I. & MacNee, W. (2000). Oxidative stress and regulation of glutathione in

lung inflammation. *European Respiratory Journal*, *16,* 534-554.

Radák, Z. (2000). *Free Radicals in Exercise and Aging*. Champaign, IL, Human Kinetics.

Radak, Z., Chung, H. Y. & Goto, S. (2008). Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radical Biology & Medicine, 44,* 153-159.

Ratto, J., Wong, H., Liu, J., Fahy, J., Boushey, H., Solomon, C. & Balmes, J. (2006). Effects of multiday exposure to ozone on airway inflammation as determined using sputum induction. *Environmental Health Perspectives, 114,* 209-212.

Reid, M. B. (2001). Redox regulation of skeletal muscle contraction. *Medicine and Science in Sports and Exercise, 33* (3): 371–376.

Rietjens, I. M. C. M., Boersma, M. G., Haan, L., Spenkelink, B., Awad, H. M., Cnubben, N. H. P., van Zanden, J. J., van de Woude, H., Alink, G. M. & Koeman, J. H. (2002). The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. *Environmental Toxicology and Pharmacology*, *11,* 321-333.

Rigas, M. L., Catlin, S. N., Ben-Jebria, A. & Ultman, J. S. (2000). Ozone uptake in the intact human respiratory tract: relationship between inhaled dose and actual dose. *Journal of Applied Physiology, 88,* 2015-2022.

Rigotti, A. (2007). Review: Absorption, transport, and tissue delivery of vitamin E. *Molecular Aspects of Medicine, 28,* 423-436.

Ristow, M., Zarsea, K., Oberbachc, A., Klötingc, N., Birringera, M., Kiehntopfd, M., Stumvollc, M., Kahne, R. & Blüherc, M. (2009). Antioxidants prevent health-promoting effects of physical exercise in humans. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(21), 8665-8671.

Robin, M., Dong, P., Hermans, C., Bernard, A., Bersten, A. D. & Doyle, I. R. (2002). Serum levels of CC16, SP-A and SP-B reflect tobacco-smoke exposure in asymptomatic subjects. *European Respiratory Journal, 20,* 1152-1161.

Romieu, I., Meneses, F., Ramirez, M., Ruiz, S., Padilla, R. P., Sienra, J. J., Gerber, M., Grievink, L., Dekker, R., Walda, I. & Brunekreef, B. (1998). Antioxidant Supplementation and Respiratory Functions among Workers Exposed to *High Levels of Ozone. American Journal of Respiratory and Critical Care Medicine, 158,* 226-232.

Romieu, I., Varraso, R., Avenel, V., Leynaert, B., Kauffmann, F. & Clavel-Chapelon, F. (2006). Fruit and vegetable intakes and asthma in the E3N study. *Thorax, 61,* 209-215.

Rundell, K. W. & Jenkinson, D. M. (2002). Exercise-induced bronchospasm in the elite athlete. *Sports Medicine, 32* (9), 583-600.

Samet, J. M., Hatch, G. E., Horstman, D., Steck-Scott, S., Arab, L., Bromberg, P. A., Levine, M., McDonnell, W. F. & Devlin, R. B. (2001). Effect of Antioxidant Supplementation on Ozone-Induced Lung Injury in Human Subjects. *American Journal of Respiratory and Critical Care Medicine, 164*, 819-825.

Sastre, J., Asensi, M., Gascó, E, Pallardó, F. V., Ferrero, J. A., Furukawa, T. & Vina, J. (1992). Exhaustive physical exercise causes oxidation of glutathione status in blood: Prevention by antioxidant administration. *American Journal of Physiology, 263*, 992-995.

Schelegle, E. S. & Adams, W. C. (1986). Reduced exercise time in competitive simulations consequent to low level ozone exposure. *Medicine & science in Sport & Exercise, 18*, 408-414.

Scichilone, N., Morice, G., Marchese, R., Bonanno, A., Profita, M., Togias, A. & Bonsignore, M. R. (2005). Reduced airway responsiveness in nonelite runners. *Medicine & science in Sport & Exercise,37* (12), 2019-2025.

Sen, C. K., Rankinen, T., Väisänen, S. & Rauramaa, R. (1994). Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *Journal of Applied Physiology, 76*(6), 2570-2577.

Sen, C.K. (1995). Oxidants and antioxidants in exercise. *Journal of Applied Physiology, 79*, 675-686.

Sen, C. K. (2001). Antioxidant and redox regulation of cellular signaling: introduction. *Medicine & Science in Sports & Exercise, 33*(3), 368–370.

Sen, C. K. & Roy, S. (2001). Antioxidant regulation of cell adhesion. *Medicine & Science in Sports & Exercise, 33* (3), 377-381.

Schock, B. C., Koostra, J., kwack, S., Hackman, R. M., van der Vliet, A. & Cross, C. E. (2004). Ascorbic acid in nasal and tracheobronchial airway lining fluids. *Free Radical in Biology & Medicine*, 37 (9), 1393-1401.

Sienra-Monge, J.J., Ramirez-Aguilar, M., Moreno-Macias, H., Reyez-Ruiz, N. I., Del Rio-Navarro, B. E., Hatch, G., Crissman, K., Slade, R., Devlin, R. B. & Romieu, I. (2004). Antioxidant supplementation and nasal inflammatory responses among young asthmatics exposed to high levels of ozone. *Clinical and Experimental Immunology, 138*, 317-322.

Siluk, D., Oliveira, R. V., Rosas, M. ER, Ling, S., Bos, A., Ferrucci, L. & Wainer, I. W. (2007). A validated liquid chromatography method for the simultaneous determination of vitamins A and E in human plasma. *Journal of Pharmaceutical and Biomedical Analysis, 44*, 1001-1007.

Simpson, R. J., Florida-James, G. D., Whyte, P. G. & Guy K. (2006). The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (B2 integrin) and CD53. *European Journal of Applied Physiology, 97,* 109-121.

Shinkai, S., Watanabe, S., Asai, H. & Shek, P. N. (1996). Cortisol response to exercise and post exercise suppression of blood lymphocyte subset counts. *International Journal of Sports Medicine, 17*(8), 597-603.

Singh, U. & Jialal, I. (2004). Anti-inflammatory effects of α-tocopherol. *Annals New York Academy of Science, 1031,*195-203.

Smit, H. A., Grievink, L. & Tabak, C. (1999). Dietary influences on chronic obstructive lung disease and asthma: a review of the epidemiological evidence. *Proceedings of the Nutrition Society, 58,* 309-319.

Stedman, J. R. (2004). The predicted number of air pollution related deaths in the UK during the August 2003 heatwave. *Atmospheric Environment,* 38, 1087-1090.

Steensberg, A., van Hall, G., Osada, T., sacchetti, M., Saltin, B. & Pedersen, B. K. (2000). Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *Journal of Physiology, 529.1,* 237-242.

Steensberg, A., Morrow, J., Toft, A. D., Bruunsgaard, H. & Pedersen, B. K. (2002). Prolonged exercise, lymphocyte apoptosis and F_2-isoprostanes. *European Journal of Applied Physiology,* 87, 38-42.

Steensberg, A. Fischer, C. P., Keller, C., Moller, K. & Pedersen, K. B. (2003). IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *American Journal of Physiology, Endocrinology and Metabolism, 285,* 433-437.

Stenfors, N., Pourazar, J., Blomberg, A., Krishna, M. T., Mudway, I., Helleday, R., Kelly, F. J., Frew, A. J. & Standröm, T. (2002). Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects. *Respiratory Medicine, 96,* 352-358.

Stone, V., Shaw, J., Brown, D. M., MacNee, W., Faux, S. P. & Donaldson, K. (1998). The Role of Oxidative Stress in the Prolonged Inhibitory Effect of Ultrafine Carbon Black on Epithelial Cell Function. *Toxicology in Vitro, 12,* 649-659.

Streets, D. G., Fu, J. S., Jang, C. J., Hao, ., He, K., Tang, X., Zhang, Y., Wang, Z., Li, Z., Zhang, Q., Wang, L., Wang, B., & Yu, C. (2007). Air quality during the 2008 Beijing Olympic Games. *Atmospheric Environment*, 41, 480-492.

Suzuki, K., Totsuka, M., Nakaji, S., Yamada, M., Kudoh, S., Liu, Q., Sugawara, K., Yamaya, K. & Sato, K. (1999). Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *Journal of Applied Physiology, 87*(4), 1360-1367.

Teckenburg, S. L., Mickleborough, T. D., Fly, A. D., Bai, Y. & Stager, J. M. (2007). Ascorbic acid supplementation attenuates exercise-induced bronchoconstriction in patients with asthma. *Respiratory Medicine, 101***,** 1770-1778.

Thurston, G. D. & Ito, K. (2001). Epidemiological studies of acute ozone exposures and mortality. *Journal of exposure Analysis and Environmental Epidemiology*, 11, 286-294.

Todokoro, M., Mochizuki, H., Tokuyama, K., Utsugi, M., Dobashi, K., Mori, M. & Morikawa, A. (2004). Effect of ozone exposure on intracellular glutathione redox state in cultured human airway epithelial cells. *Inflammation, 28*, 105-114.

Traber, M. G. (2006). Relationship of vitamin E metabolism and oxidation in exercising human subjects. *British Journal of Nutrition, 96* (S1), 34-37.

Tremblay, M. S., Copeland, J. L. & Van Helder, W. (2005). Influence of exercise duration on post-exercise steroid hormone responses in trained males. *European Journal of Applied Physiology, 94,* 505-513.

Trenga, C. A., Koenig, J. Q. & Williams, P. V. (2001). Dietary antioxidants and ozone-induced bronchial hyperresponsivenss in adults with asthma. *Archives of Environmental Health, 56***,** 242-249.

Urso, M. L. & Clarkson, P. M. (2003). Oxidative stress, exercise, and antioxidant supplementation. *Toxicology, 189,* 41-54.

Valencia, E., Marin, A., Hardy, G. (2001). Glutathion – nutritional and Pharmacological viewpoints: Part III. *Nutrition*, 17, 696-697.

Van Der Vliet, A., O'Neil, C. A., Eiserich, J. P. & Cross, C. E. (1995). Oxidative

damage to extracellular fluids by ozone and possible protective effects of thiols. *Archives of Biochemistry and Biophysics, 321,* 43-50.

Van Der Vliet, A., O'Neil, C. A., Cross, C. E., Koostra, J. M., Volz, W. G., Halliwell, B. & Louie, S. (1999). Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *American Journal of Physiology (Lung Cellular and Molecular Physiology), 276,* L289-296.

Vollard, N. B. J., Shearman, J. P. & Cooper, C. E. (2005). Exercise-Induced Oxidative Stress Myths, Realities and Physiological Relevance. *Sports Medicine, 35,* 1045-1062.

Varraso, R., Fung, T. T., Hu, F. B., Willet, W. & Camargo, C. A. (2007a). Prospective study of dietary patterns and chronic obstructive pulmonary disease among US men. *Thorax, 62***,** 786-791.

Varraso, R., Fung, T. T., Hu, F. B., Willet, W. & Camargo, C. A. (2007b) Prospective study of dietary patterns and chronic obstructive pulmonary disease among US women. *The American Journal of Clinical Nutrition, 86***,** 488-495.

Wagner, N. L., Beckett, W. S., Steinberg, R. (2006). Using spirometry results in occupational medicine and research: Common errors and good practice in statistical analysis and reporting. Indian Journal of Occupational and Environmental Medicine [serial online], 10, 5-10. Available from: http://www.Ijoem.com/text.asp?2006/10/1/5/22888.

Watson, T.A., Callister, R.,Taylor, R.D., Sibrbritt, D. W., MacDonald,-Wicks, L. K. & Garg, M. L. (2005). Antioxidant Restriction and Oxidative Stress in Short-Duration Exhaustive Exercise. *Medicine and Science in Sports and Exercise, 37* (1), 63–71.

Webb, H. E., Weldy, M. L., Fabianke-Kadue, E. C., Orndorff, G. R., Kamimori, G. H. & Acevedo, E. O. (2008). Psychological stress during exercise: cardiorespiratory and hormonal responses. *European Journal of Applied Physiology, 104,* 973-981.

West, J. B. (2003). *Pulmonary Pathophysiology – The Essentials.* 6th ed: Lippincott Williams & Wilkins.

Wilson, D. O. & Johnson, P. (2000). Exercise modulates antioxidant enzyme gene expression in rat myocardium and liver. *Journal of Applied Physiology, 88,* 1791-1796.

Withers, R., Gore, C., Gass, G., *et al.* (2000). Chapter 8: Determination of maximum oxygen consumption or maximum aerobic power. In: *Physiological tests for elite athletes – Australian Sports Commission.* IL: Human Kinetics

Wu, G., Fang, Y., Yang, S., Lupton, J. R. & Turner, N. D. (2004). Glutathione metabolism and its implication for health. *The Journal of Nutrition, 134,* 489-492.

World Health Organization - WHO - (2006). *Air Quality Guidelines for Particulate Matter, Ozone, Nitrogen Dioxide and Sulphur Dioxide* – Global update 2005 – Summary of risk assessment. 1-33.

Xu, X., Wang, L. & Niu, T. (1998). Air pollution and its health effects in Beijing. *Ecosystem Health*, 4(4), 199-209.

Yang, I. A., Fong, K. M., Zimmerman, P. V., Holgate, S. T., Holloway, J. W. (2009) Genetic susceptibility to the respiratory effects of air pollution. *Postgraduate Medical Journal*, 85 (1006), 428-436.

APPENDICES

APPENDIX I - GENERAL MEDICAL HISTORY QUESTIONNAIRE

HEALTH SCREEN FOR STUDY PARTICIPANTS

Name .....

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

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

   (a) on medication, prescribed or otherwise..... Yes ☐ No ☐

   (b) attending your general practitioner..... Yes ☐ No ☐

   (c) on a hospital waiting list..... Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

   (a) consult your GP ..... Yes ☐ No ☐

   (b) attend a hospital outpatient department..... Yes ☐ No ☐

   (c) be admitted to hospital ..... Yes ☐ No ☐

3. **Have you ever** had any of the following:

   (a) Convulsions/epilepsy..... Yes ☐ No ☐

   (b) Asthma..... Yes ☐ No ☐

   (c) Eczema ..... Yes ☐ No ☐

   (d) Diabetes..... Yes ☐ No ☐

   (e) A blood disorder..... Yes ☐ No ☐

   (f) Head injury..... Yes ☐ No ☐

   (g) Digestive problems..... Yes ☐ No ☐

(h) Heart problems ..... Yes ☐ No ☐

(i) Problems with bones or joints ..... Yes ☐ No ☐

(j) Disturbance of balance/coordination ..... Yes ☐ No ☐

(k) Numbness in hands or feet ..... Yes ☐ No ☐

(l) Disturbance of vision..... Yes ☐ No ☐

(m) Ear / hearing problems ..... Yes ☐ No ☐

(n) Thyroid problems ..... Yes ☐ No ☐

(o) Kidney or liver problems..... Yes ☐ No ☐

(p) Any kind of allergy (i.e food) ..... Yes ☐ No ☐

(q) High blood pressure…….. Yes ☐ No ☐

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

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

.....

.....

5. **Have you** had a cold or feverish illness in the past month? Yes ☐ No ☐

6. **Are you** accustomed to vigorous exercise (1-3 hours per week)? Yes ☐ No ☐

Thank you for your cooperation

APPENDIX II – SPECIFIC MEDICAL QUESTIONNAIRE

Specific medical history related to this study

YES NO

____ ____ 1. Have you ever been diagnosed with asthma? If NO go to question 5.

____ ____ 2. Do you currently use any asthma medication?

Yes, _____

____ ____ 3. Do you have any night-time, early morning or activity related asthma symptoms during the day?

Yes, _____

4. When was your last asthma attack?

_____

____ ____ 5. Do you have any allergies?

Yes, _____

____ ____ 6. Have you ever suffered from any heart and lung problems?

Yes, _____

____ ____ 7. Have you had any respiratory infections in the last 6 weeks?

Yes, _____

____ ____ 8. Have you ever suffered from any other diseases not described above?

Yes, _____

____ ____ 9. Do you smoke? If YES, please specify.

Yes, _____

10. What is your occupation?

_____

____ ____ 11. Are you exposed to any sources of fumes or dust at work or elsewhere?

Yes, _____

____ ____ 12. Do you have any pets (including birds)?

Yes, _____

_____

Print Name

_____

Signature

Date:

APPENDIX III – PHYSICAL ACTIVITY QUESTIONNAIRE

Name:_____

Date of birth: _____

Weight: _____ Height: _____

The following questions are designed to give us an indication of your current level of physical activity.

Are you currently ENDURANCE TRAINING? YES ☐ NO ☐

If yes, how many days each week do you usually train? _____

How many minutes does each session last? _____

Which type of exercise do you perform (ex: running, cycling)?

_____

Are you currently doing WEIGHT TRAINING? YES ☐ NO ☐

If yes, how many days each week do you usually train? _____

How many minutes does each session last? _____

Sign:_____

APPENDIX IV – DAILY HEALTH QUESTIONNAIRE

HEALTH QUESTIONNAIRE

Name: Date:

Trial:

Please complete the following brief questions to confirm your fitness to participate in today's session:

At present do you have any problems for which you are:

1) On medication, prescribed or otherwise? ☐ YES ☐ NO

2) Seeing your general practitioner? ☐ YES ☐ NO

Do you have any symptoms of ill health, such as those associated with a cold or other common infection?

☐ YES ☐ NO

If you have answered yes to any of the above questions, please give further details below:

……

.....

……

.....

Would you like to take part in today's experiment? ☐ YES ☐ NO

Signature…….

APPENDIX V– BLOOD DONATION FORM

EDINBURGH NAPIER UNIVERSITY School of Life Sciences

Subject Declaration for Vene Puncture Blood Donation

You have consented to donate blood in the School of Life Sciences. The School phlebotomists have all undergone an approved training course and have Hepatitis B immunity. The blood you are donating will be used for the project entitled: ***Effect of antioxidant supplementation on athletes' performance in an 8 Km time trial run in a hot, humid and ozone-polluted environment***; but will not be screened for pathogenic organisms that could adversely affect the health of any exposed person. It is therefore important that you do not donate blood if any of the risk factors listed below apply to you. At the end of the experiment the cells will be disposed of and not stored for future experiments.

Please read the list below and think very carefully if any apply to you.

If any factors do apply please do not sign the declaration and do not offer your services as a donor. You do not have to say which risk factors apply.

Risk Factors

Recent –

Ill-Health
Contact with infectious diseases
Vaccinations or immunisations

In the last year-

Tattoo or body piercing
Childbirth
Blood transfusion
Tissue or skin graft
Hormone treatment
Major surgery
Travel to a malarial area or in sub Saharan Africa, Asia or South America

At any time –

If you have lifestyle factors which would pose a risk please do not donate blood.

Declaration

I have read the risk factors and have considered my lifestyle factors and to the best of my knowledge none of them apply to me and I am in good health. I understand that my blood will be used for research purposes.

Name of Donor: ..... Name of Phlebotomist: .....

Signature of Donor:..... Signature of Phlebotomist:.....

Date:..... Date:.....

Post-Trial Questionnaire

Name: Date:

Trial:

Did you experience any of the following during the trial? Please choose one of the following options for each symptom.

0 = Not present 1 = Minimal 2 = Mild 3 = Moderate

4 = Severe 5 = Incapacitating

Shortness of breath	0	1	2	3	4	5
Cough	0	1	2	3	4	5
Excess sputum	0	1	2	3	4	5
Throat tickle	0	1	2	3	4	5
Raspy throat	0	1	2	3	4	5
Wheezing	0	1	2	3	4	5
Congestion	0	1	2	3	4	5
Pain on deep inspiration	0	1	2	3	4	5
Headache	0	1	2	3	4	5
Nausea	0	1	2	3	4	5
Eye irritation	0	1	2	3	4	5

Please detail any other symptoms:

_____

_____

_____

During the test did you feel you would be able to perform maximally in competition?

Please circle your answer

Yes No