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Lung Inflammation, Oxidative Stress and Air Pollution

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1. Introduction

This is a very relevant chapter in the context of “Lung Inflammation” because it details and discusses an important theme in this area: air pollution. The analyses of 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. The whole population is affected, but the active and athletic population is of special concern because of the amount of time they spend training and/or competing outdoors, eliciting high ventilation rates that result in higher pollutants delivery to the lungs.

This chapter addresses the systemic effects that inhaled pollutants have as well as the local pulmonary inflammatory processes and oxidative stress. The consequences that these processes may impose to the health and performance of the active population will also be described. The use of antioxidants to counteract the deleterious oxidative stress of exercise when performed in a polluted environment is discussed here too.

2. Air pollution

Air pollution can be composed by a cocktail of different substances that in large amounts can be harmful to the ecosystem. The industrial revolution marks the beginning of an accelerated global urbanization process. As a result, large urbanized areas suffer from, amongst other problems, a high concentration of air pollutants. The most evident form of air pollution is a dark layer of gas – also known as smog – present above big cities. Nevertheless, there are different kinds of air pollution that are not as visible. Sources include natural processes, such as volcano emissions, and also industrialized sources found in urban centres linked to human activities. The latter includes power plants, factories, burning of fossil fuel and transportation.

2.1. Classification of air pollutants

Air pollutants can be found in different forms and sizes. They can be solid particles, liquid molecules or gases. According to the way they are generated these pollutants can be considered as primary (directly produced) or secondary (formed by the interaction of primary pollutants). Some pollutants can be both produced directly as well as formed by the reaction of other pollutants. In addition, there are indoor and outdoor pollutants, all of which can be detrimental to human health. Although indoor pollution is generally low, its levels can be augmented by the use of several chemical products (e.g. cleaning products, paints), heaters, stoves and indoor smoking. Fortunately, various countries have already adopted policies that ban smoking in closed public spaces. Description and classification of the main pollutants resulting from human activity are listed in table 1 below.

Air Pollutant	Characteristics	Source
Ammonia (NH ₃)	<ul style="list-style-type: none"> • Gas • No colour • Strong pungent smell 	Produced industrially. Mostly used as fertiliser to agricultural crops (over 80%). Also used, for example, in the fermentation and textile industry, as cleaning product, and as antimicrobial agent for food products.
Carbon monoxide (CO)	<ul style="list-style-type: none"> • Gas • No colour • No smell 	A major source of this poisonous gas is vehicular exhaust. It is also a product of incomplete combustion of carbon-containing fuels.
Nitrogen dioxides (NO ₂)	<ul style="list-style-type: none"> • Gas • Brownish-red colour • Strong smell 	Released by internal combustion engines and power-plants. Indoor sources of this pollutant include gas and kerosene heaters. It is also released by electric discharge during storms. NO ₂ is also a precursor for the formation of O ₃ pollution.
Ozone (O ₃) or Tropospheric O ₃ or ground level O ₃	<ul style="list-style-type: none"> • Gas • No colour • Strong smell 	This highly oxidant pollutant is formed by the reaction of nitrogen oxides and volatile organic compounds in the presence of sunlight. As it is easily transported by the wind it can be found in high concentrations not only in large urban areas but also in rural areas.
Particulate Matter (PM)	<ul style="list-style-type: none"> • Solid or liquid particles 	This pollutant can vary in size, form and composition being made up by a mixture of extremely small particles (metals, soil, dust) and liquid droplets (acids, organic compounds). The smaller the PM size the deeper it can penetrate into the lungs and might even be able to pass to the systemic circulation and affect other organs.
Sulfur oxides (SOx)	<ul style="list-style-type: none"> • Gas • No colour • Strong smell 	Amongst the highly reactive gases of this group is the sulphur dioxide (SO ₂) that is produced by the burning of fuels such as petrol, diesel, and coal. It is naturally released into the atmosphere by activated volcanoes.
Volatile Organic Compounds (VOCs)	<ul style="list-style-type: none"> • Gases emitted from solids and liquids • Strong smell 	VOCs are mainly indoor pollutants because they can be released by a variety of materials and products which are used in the households and offices such as paints, disinfectant, air-fresheners and photocopy machines. VOCs are also released by fuels. As mentioned previously they are precursors of O ₃ .

Table 1. Main primary pollutants resulting from human activity

3. General health consequences of exposure to air pollution

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 diseases, chronic obstructive lung diseases (COPD), cancers, cerebrovascular-strokes, cardiovascular diseases and mortality. These occur due to the nature of the pollutants and via their impacts on the respiratory and cardiovascular systems [1-7]. Air pollution does not only affect individuals that are directly exposed to it, but it also has a deleterious effect on foetal development and preterm birth. Various studies have shown that decreases in neonate birth weight are associated with exposure to air pollution [8-10]. Children are also very susceptible to the harmful effects of pollution exposure. This is reflected by an increase in the number of young children admitted to hospitals for acute lower respiratory infection: pneumonia and bronchiolitis. These respiratory infections are the largest causes of mortality among young children worldwide, especially in developing countries. Within these countries a low socioeconomic status potentiates the consequences of air pollution exposure [5, 11, 12].

These adverse effects on health, caused by air pollution, have been shown to occur in both developed and developing countries. However, its health-related burden falls most heavily on developing countries [3, 6, 13-15]. According to the World Health Organisation [16], air pollution is responsible for over 800, 000 premature deaths each year, with more than 6 million years of life lost annually. Asia alone would account for approximately two thirds of these numbers [2]. It goes without saying that this air pollution disease-burden is a great economic issue to countries affected [14].

The World Health Organisation, together with governments of various countries, have proposed guidelines for maximum concentrations of the different pollutants (see table 2). Nevertheless, this does not always translate into national policies and various cities are unable to maintain pollution levels within the suggested guidelines. The implementation of anti-pollution measures is not always straight forward. At times, for example, it means there is a need to change energy sources and invest in green technology. As a consequence, some countries are more reluctant than other to execute such measures [3, 17].

In relation to indoor air pollutants, on March 2004, the Republic of Ireland became the first country to completely ban smoking from workplaces. More and more countries gradually adopted this public-space smoking ban. Nowadays, most countries in the world have some kind of law pertaining to the issue. There is a growing body of evidence on the positive health outcomes and the economic benefits related to the adoption of smoke-free laws. Improvements in lung function, airway and systemic inflammation, and adverse respiratory symptoms were observed in individuals working in bars [18, 19]. Hospital admissions of children and adults presenting asthma and other pulmonary illnesses also decreased as a result of reduced exposure to environmental tobacco smoke [20-22]. Smoking bans also positively impacted individuals with heart diseases which include myocardial infarction, unstable angina and stroke [20, 22-24].

Pollutant	Averaging time	Air quality guideline values ($\mu\text{g}/\text{m}^3$)
PM _{2.5}	1 year	10
	24 h (99 th percentile)	25
PM ₁₀	1 year	20
	24 h (99 th percentile)	50
O ₃	8 h, daily maximum	100
NO ₂	1 year	40
	1 h	200
SO ₂	24 h	20
	10 min	500

Source: (WHO 2012)

Table 2. Updated WHO air guideline values

4. Oxidative stress and air pollution

The mechanism responsible for the adverse effect of oxidant pollutants – ozone, PM – in the lungs, which might possibly also lead to a systemic outcome, would be its direct and indirect oxidative reaction with molecules and cells present in the airways [25, 26]. This might result in oxidative stress in the airway tissues [27]. But what is oxidative stress?

4.1. Oxidative stress

Oxidative stress can be defined as damage that might occur in cell components, such as membranes, RNAs and DNA, as a result from an imbalance in the body's ability to neutralize certain molecules or to repair the resulting damage. Free radicals and reactive species (reactive oxygen species and reactive nitrogen species) are molecules that easily react with other molecules to become more stable. This is an oxidation reaction, because there is transfer of electrons from a substance to the free radicals/reactive species (oxidizing agents). When in our organism these reactions occur with cell components, which could have detrimental effects such as cell function impairment and cell death. Nevertheless, the body has an elaborate antioxidant defence system that neutralizes the reactive species and free radicals so as to maintain a redox homeostasis. This balance, however, can be disturbed if the concentration of pro-oxidants (reactive species and free radicals) overwhelms the available antioxidants or if the antioxidants are depleted due to disease or poor diet. This associated with reduced repairing process, can result in oxidative stress, and impaired cellular function may occur. In humans, oxidative stress has been shown to be the cause and consequence of various diseases (Fig 1). These include, but are not limited to, cancers, atherosclerosis, Alzheimer's disease, Parkinson's disease, dementia, heart diseases and pulmonary disorders [28-30].

On the other hand, free radicals and reactive species are essential to our well-being. This happens because these molecules are necessary for a variety of reactions to occur. For example, they are used by the immune system to attack and kill pathogens in a process called respiratory burst. They are also used for gene stimulation, cell signalling, vasoregulation and inflammatory processes [31, 32].

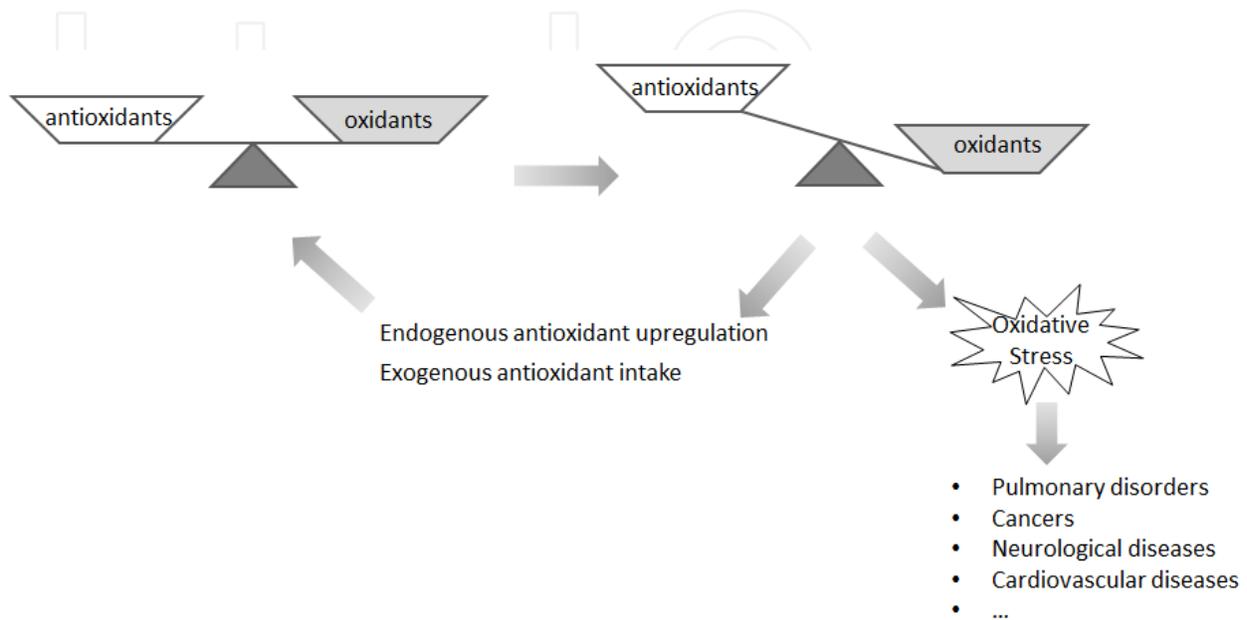


Figure 1. Redox balance

4.2. Antioxidants

Antioxidants are often reducing agents capable of being promptly oxidized by free radicals and reactive species, eliminating their pro-oxidant nature before they react with cell components. There is a wide range of antioxidants in body fluids, tissues and organs. They are present both intracellular and extracellular, working synergistically in a network to balance the free radicals and reactive species. Therefore, the action of one antioxidant may depend on the correct function of other antioxidants in the system.

Antioxidants are both synthesized *in vivo* and absorbed through the diet and they 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 different pro-oxidants and they are located in different cellular compartments. Glutathione, vitamin C, vitamin E, carotenoids, and uric acid are some examples of non-enzymatic antioxidants. Similarly to the enzymatic antioxidant, these are present in different cellular compartments and elicit distinct antioxidant properties which maximize their effectiveness [29]. Antioxidants can also be classified according to their soluble nature: hydrophilic (water soluble) or lipophilic (lipid soluble). It is this characteristic that allows them to be present in different parts of the cells.

Antioxidant	Solubility	Concentration in human serum (μM)
Glutathione	Hydrophilic	4
Lipoic acid	Hydrophilic	0.1-0.7
Melatonin	Hydrophilic and lipophilic	Varies throughout the day
Ubiquinol (coenzyme Q)	Lipophilic	5
Uric acid	Hydrophilic	200-400
Vitamin A (retinol)	Lipophilic	1-3
Vitamin C (ascorbic acid)	Hydrophilic	50-60
Vitamin E (α -tocopherol)	Lipophilic	10-40
β -carotene	Lipophilic	0.5-1

Table 3. Important antioxidants

Most antioxidants undergo redox cycling. This means that once oxidized they can be reduced to their former state and act again as an antioxidant. Nevertheless, this redox cycling allows the antioxidant to act as pro-oxidant promoting free radical formation. This would happen if there is an unbalance in the antioxidant network system. Vitamin C and vitamin E are examples of antioxidants with this characteristic, while melatonin is considered a terminal antioxidant because it cannot be recycled.

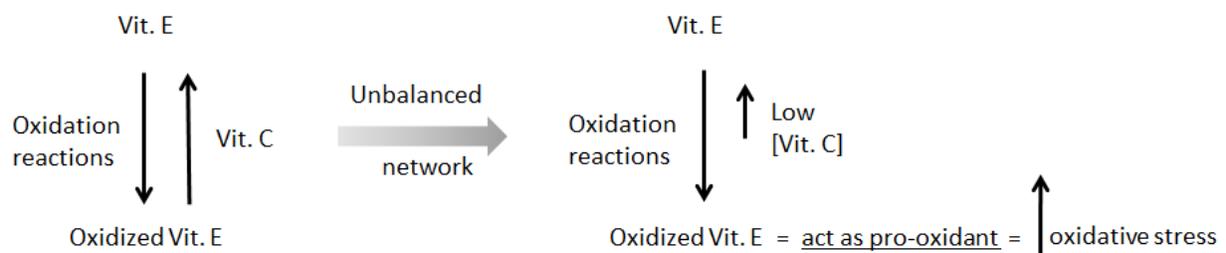


Figure 2. Redox cycling of vitamin E (vit E) by vitamin C (Vit. C)

4.3. Lung oxidative stress

Oxidant air pollutants such as ozone, particulate matter and nitrogen dioxide have been shown to induce lung inflammation through stimulation of the oxidative stress process. Little is known, however, about their effects as oxidant compounds in the lungs or about the role and the effectiveness of the antioxidants present in the respiratory tract lining fluid (RTLFL) in scavenging and protecting against their harmful effects. A great variety of antioxidants can be found in human RTLFL. Their concentration and distribution throughout the airways, however, is not homogeneous, with high levels of GSH in the alveolar epithelial regions and uric acid predominating in the upper airways [33, 34].

Numerous studies have investigated the effects by which O₃ 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 O₃, or indeed any gas, is inhaled, it first comes into contact with the RTLF. Indeed, 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. Once O₃ comes into contact with the RTLF, it induces oxidative stress by two different mechanisms. The first would be by reacting directly with the constituents of the RTLF and the underlying epithelium [26]. It has been suggested that antioxidants present in the RTLF, such as glutathione, uric acid and ascorbic acid have an important role in neutralizing part of the radical generation, hence reducing the ozone induced oxidative stress [35]. Studies analysing 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 [36-38].

The second mechanism by which ozone can increase the oxidation process is indirectly because, even though O₃ 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 reactive species [25, 26]. These processes combined could overwhelm the local and systemic antioxidant network leading to an increase in the oxidative process, the intensity of which varies depending on the O₃ inhaled dose and also on the antioxidants present in the lining fluid. If the oxidative stress is sufficient, the activation of an inflammatory response occurs and is characterized by the 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. The hypothesis is that the antioxidants present in the epithelial lining fluid of the lungs would neutralize the excess production of free radicals and ROS, consequently reducing lung injury induced by air pollution [27, 35]. This will be discussed in the following sections.

5. Lung inflammation and air pollution

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: interleukin-6 (IL-6), interleukin-8 (IL-8), and prostaglandins. 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 in the circulation and are the first cell type to migrate to sites of injury and inflammation. When a tissue is inflamed, an increase in the expression of adhesion molecules of the selectin family (E-and P-selectin molecules) occurs in the local endothelium. This process is mediated by cytokines and other inflammatory mediators. Neutrophils present in the blood recognise the site of inflammation because of these adhesion molecules which bind to the other molecules (mucin-like cell-adhesion molecules, CAM) on the neutrophil surface. This step is referred to as rolling: the first step to the attachment of neutrophils onto 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. Upon arrival at the inflamed tissue, neutrophils release a number of chemoattractants to amplify the inflammatory response by recruiting other cells.

The cytokine IL-8 is a mediator of the immune function and helps 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 [39]. 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 of an inflammatory response. This cytokine – IL-6 – has been thoroughly studied in immunological responses to exercise [40-41].

Inhalation of air pollution has been shown to stimulate airway inflammation due to its oxidative nature. Airway inflammation can be detected by both a local increase in inflammatory cells as well as inflammatory mediators. The depletion of antioxidants found in the airways, characterizes the oxidative process that triggers an inflammatory response due to epithelial damage [42, 43]. Both the inflammatory mediators and the antioxidants can be measured using different techniques, each with its own advantages and disadvantages. Bronchial biopsies and bronchoalveolar lavage (BAL) are quite invasive procedures that require local anesthesia and need to be performed in a medical environment [44]. These two techniques have the advantages of sampling the more distal regions of the airway and the biopsies retrieves tissue samples which can give further information about the local inflammatory process. The sputum induction procedure and the nasal lavage (NL) procedure, on the other hand, are less invasive and less technically difficult procedures than the previously-mentioned bronchial techniques and can be repeated at multiple time points [37, 45]. Sputum induction and NL are techniques that sample the upper respiratory airways.

There has been a variety of studies analysing airway inflammation of individuals exposed to O₃ pollution. Due to the similar oxidative nature of O₃ in relation to other pollutants, the results of these studies can be, in a certain way, generalized to include them too. Nevertheless, when analysing the literature, it is always essential to take into account the total volume of pollutants inspired by the participants, as well as the techniques used to sample the airway compartments. Ideally it would also be relevant to take into account the antioxidant concentrations in the RTLF, which has been shown to vary between individuals. In order to increase the amount of air – and consequently air pollution – inspired in a shorter amount of time, most studies use exercise protocols in association with the exposure.

Airway inflammation can lead to the destruction of the cilia of the epithelial cells that line the respiratory tract. The cilia have an important immune function because they constantly move the mucus up from the lungs to the back of the throat where it is eliminated or swallowed and digested. The mucus serves as a “trap” to infectious agents and small particles, such as pollutants and allergens, preventing them to enter deep into the airways. Gas pollutants are

not trapped in the mucus, though they can exacerbate its production and destroy the cilia making the airways more susceptible to the invasion of other foreign agents. Another consequence of airway inflammation is lung epithelial injury which leads to an open interface between the lung and the blood. This facilitates the dispersion of microbes to the rest of the body, initiating a systemic inflammatory response. If the lung epithelial injury is chronic and the tissue is recurrently going through a repairing process, this can lead to fibrosis with consequential decrease in lung function, chest discomfort, fatigue and weakness.

Injury and toxicity involving the respiratory epithelium can be assessed by a simple and noninvasive way by measuring the concentration of Clara cell protein, also known as CC16, CC10 or Uteroglobin. This protein is secreted, as the name indicates, by Clara cells. The function of these cells is mainly the protection of the respiratory tract. They present a high content of xenobiotic metabolizing enzymes which protect our system against inhaled particles including pollutants [46, 47]. CC16 is a small protein with an important role in decreasing the inflammation of the respiratory tract and protecting it against the harmful effects of oxidative stress [48]. This protein can be measured by the methods used to assess the respiratory airways, including the NL procedure. In addition, CC16 can also be found in the blood, where it is derived almost exclusively from the airways [46]. In normal healthy individuals, the serum level of CC16 ranges, on average, from 10 to 15 $\mu\text{g}\cdot\text{l}^{-1}$ [49]. Yet, the concentration of this protein in the blood has been shown to rise as a result of pulmonary inflammation and increases in the permeability of the lung epithelial barrier. 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 permit its diffusion into the blood [50, 51] where it can easily be detected by conventional enzyme immunoassays [52].

The bi-directional exchange of proteins between lung and blood is regulated by several factors, such as the size of the proteins, the epithelium permeability and the driving force of the transepithelial concentration gradient. The concentration gradient allows the movement of proteins from an area of high concentration to an area of low concentration. In the case of CC16, this is 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 [51].

The changes that occur in serum concentrations of CC16 may result from three different mechanisms. The first mechanism would result from the increase in the permeability of the lung epithelial barrier, and this has as a consequence a higher diffusion of CC16 into the blood. This can happen following exposure to ozone, which causes epithelial lung injury, or more specifically, damage to the tight junctions of the cells (fig. 4) [51, 53]. 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 [54]. The third mechanism that would lead to an enhancement in the levels of serum CC16 would result from a reduction in the clearance of this protein by the kidney. Serum CC16 has a half-life of approximately 2-3

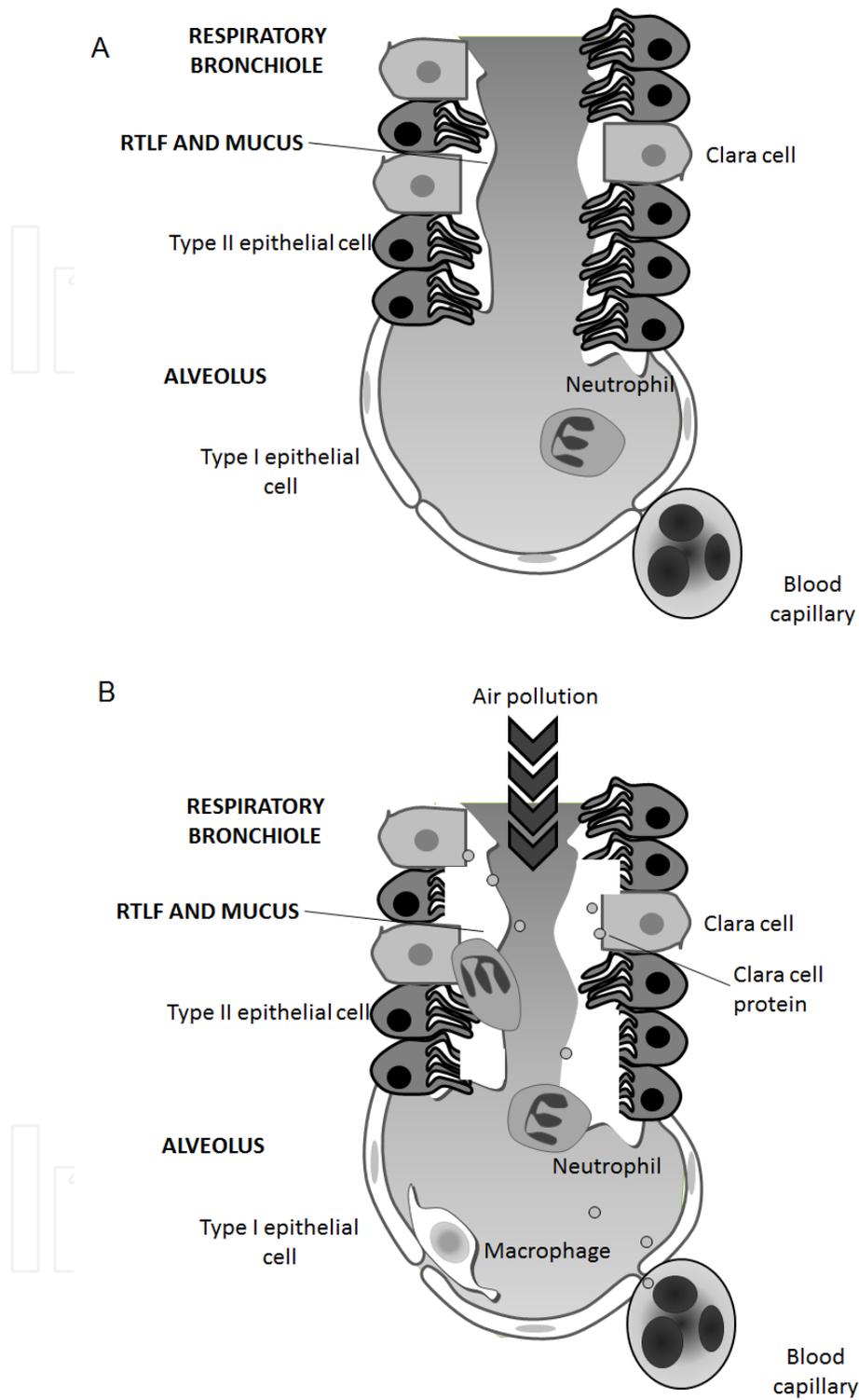


Figure 3. Respiratory bronchiole before (A) and after (B) air pollution exposure.

h due to its rapid clearance through the kidney [46]. Hence, the variation in CC16 serum levels can only be used as a specific biomarker of the airway epithelium integrity if the individual does not present renal dysfunction.

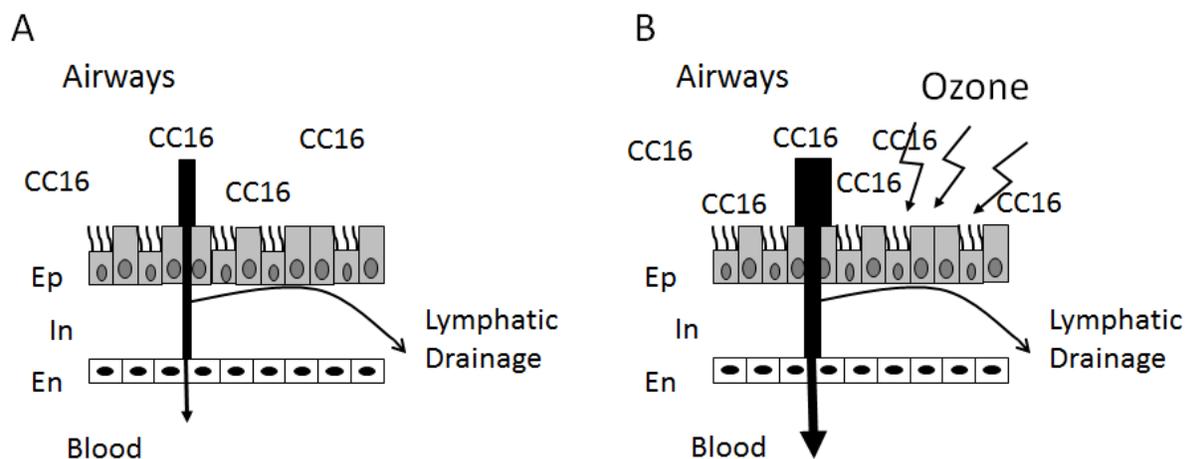


Figure 4. Movement of CC16 from the airways to the blood. (A) Under normal conditions, (B) after exposure to ozone. The thickness of the arrows is used to illustrate the relative permeability of the different barriers and the increase in the CC16 flux after ozone exposure. Abbreviations: Ep – epithelium; In–interstitium; En – endothelium (adapted from Broeckeaert *et al.* 2000b).

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 injury [46, 54]. A study conducted with firefighters [55] showed that acute smoke inhalation significantly increased serum CC16 levels. In addition to smoke inhalation, the firefighters also had to perform physically demanding tasks. Serum CC16 concentration was measured immediately after exposure and was three times higher than that of control participants. The change in serum CC16 concentration was attributed to a transient increase in the lung epithelial permeability, but with no sign of lung function impairment. Ten days after exposure, the CC16 concentration had returned to baseline levels.

When two groups of individuals were compared, a chronic toxic effect on Clara cells was observed in workers inhaling silica-rich dust for an average of 15 months [54]. One group was composed of workers exposed to silica and the other was a control group, matched for age, body mass index and proportion of smokers. After 15 months, the mineworkers showed a significant reduction in 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, but an additional and significant effect of tobacco smoking was 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. Pertaining literature suggests that the toxic metabolites of tobacco smoke not only increase the permeability of the lung epithelium, but also cause a progressive destruction of Clara cells [47].

5.1. Studies investigating the effect of air pollution on performance, lung inflammation and injury

Exercise leads to various physiological changes that can aggravate the effects of air pollutants. At resting conditions our breathing is predominantly nasal. This has various advantages which

includes not only humidifying and heating the inspired air but also filtering it. Once exercise starts becoming more intense, individuals automatically switch the nasal breathing to oral breathing in an attempt to increase the amount inhaled. Nevertheless avoiding the nasal filtration system potentially enhances the pollutant concentration that reaches the lungs.

With the beginning of exercise, the ventilatory exchange rate (VE) starts to increase and, depending on the exercise intensity and the size of the individual, the VE can be higher than 160 l/min which also leads to an increase in air pollution inhaled. For example, it has been shown that, with the start of an exercise bout, there is an increase in the proportion of ultrafine particles (nanosized particulate matter) inhaled and deposited in the airways but not eliminated [56, 57]. This could be due to impaired nasal mucociliary clearance and reduced cilia beat frequency which can occur with strenuous exercise [58, 59]. This impairment of respiratory defences together with a higher VE and deeper breathing makes the active and athletic population of large cities more vulnerable to the harmful effect of air pollution on health and on performance, especially with long-duration high-intensity exercise. Most of the major sports events (e.g. Summer Olympic Games, Football World Cup) take place within or near large cities, e.g. polluted Olympic Games venues Barcelona 1992; Atlanta 1996; Athens 2004; Beijing 2008; London 2012 [60, 61]. Rio de Janeiro, Brazil's second biggest city (population density of 5346 hab/km²) and host of the 2016 Olympic Games, also presents high levels of air pollution [5].

Studies that have investigated the deleterious effects of air pollution on performance do indeed report that athletes have an impaired performance [62-64]. This can be further exacerbated depending on other environmental conditions, such as elevated temperature and humidity [65]. This impairment can be attributed not only to an increase in lung inflammation, which can decrease its function, but also to the increase in respiratory symptoms that the athletes experience, including cough, nausea, pain on deep inspiration and wheezing, amongst others. This would lead to a decrease in maximal inspiration volume via neural stimulation of sensory fibers present in the lungs, affecting. In more reactive individuals, the ozone could activate "irritant" receptors leading to contraction of alveolar smooth muscles and as a result changes in respiratory muscle force and mechanic properties of the lungs would occur [66, 67]. Endurance exercise alone has also been shown to decrease lung function because hyperventilation affects the airway smooth muscles [68].

Table 4 presents a summary of studies that have investigated the effect of O₃ air pollution on lung inflammation, injury and function. It is interesting to see the different markers that were analysed, as well as the protocols used. More details of some studies are described below.

Devlin *et al.* [72] 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 min⁻¹) for 2 h in a chamber where the ozone concentration was 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 [39] 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 min⁻¹ m⁻² of body surface

Study	Subjects	Exercise and Ozone levels	Results
Adams & Schelegle, 1983 [69]	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 [70]	Cyclists	1 h 1 h competitive cycling simulation protocol 0; 0.12; 0.18; and 0.24 ppm	↓ lung function at 0.18 and 0.24 ppm ↑ Respiratory symptoms with higher O ₃ concentration, impairment in performance
Brunekreef <i>et al.</i> , 1994 [71]	Field study with cyclists	75 min cycling 0.04 – 0.1 ppm + 17,9 °C	Small ↓ in lung function Correlation between the O ₃ exposure and the impairment in lung function
Devlin <i>et al.</i> , 1996 [72]	Healthy male individuals	2 h heavy intermittent cycling 0.4 ppm	1 h post-exposure = ↑ neutrophil, IL-6 and LDH in BAL
Krishna <i>et al.</i> , 1998 [73]	*Healthy individuals	2 h intermittent cycling 0.12 ppm	6 h post-exposure = ↑ neutrophil, IL-8 in BAL No effect on lung function
Blomberg <i>et al.</i> , 1999 [38]	*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 ↓ Lung function
Holz <i>et al.</i> , 1999 [39]	*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) No effect on lung function
Broeckert <i>et al.</i> , 2000 [51]	Field study, trained cyclists	Average 35 km cycling Average 0.076 ppm	Post-exercise = ↑ in serum CC16
Blomberg <i>et al.</i> , 2003 [76]	Healthy individuals	2 h intermittent cycling 0.2 ppm	2 h and 4 h post-exposure = ↑ in serum CC16
Lagerkvist <i>et al.</i> , 2004 [77]	Children (10-11 years of age)	2 h of outdoor exercise 0.059 ppm	No effect on serum CC16 or lung function
Gomes <i>et al.</i> , 2010 [64] and 2011 [65]	Elite runners	8 km time trial 0.1 ppm 31°C + 70% rh	Immediately post-exercise: ↑ CC16 in NL and no difference on neutrophil counts No effect on lung function Decrease in performance

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

Table 4. Studies investigating the effect of O₃ after exercise

area-exposed to 0.25 ppm of O₃. Nevertheless, when the participants performed the same exercise bout exposed to a lower O₃ 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.* [38] 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 O₃

in subjects performing intermittent moderate cycling exercise producing an average minute ventilation of $20 \text{ l min}^{-1} \text{ m}^{-2}$ of body surface area. This difference might be explained by the lower exercise and O_3 levels in the latter study which would have as consequence the lowering of the inhaled O_3 dose. Nevertheless, in tissue obtained from bronchial mucosal biopsies, Blomberg *et al.* [38] were able to detect 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.* [74] using the same study design as the previously-mentioned researchers, 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 importance of these adhesion molecules in the inflammatory response since they recruit inflammatory cells into the airways of healthy individuals.

Gomes and colleagues [64, 65] investigated well trained runners performing an intense exercise bout (8 km time trial) in an environment that, in addition to ozone pollution, was warm and humid. This kind of environment is relevant because the formation of ozone is intensified during the summer when there is a high incidence of sun light. Even though they did not report any changes in lung function, there were signs of lung inflammation and lung injury, the latter observed by an increase in NL CC16 concentration. In addition, there was a positive correlation between lung antioxidant concentrations and performance, that is, the athletes who presented lower concentrations of lung antioxidants were the ones who had a higher impairment in their performance in that extreme environment.

Other studies have also looked into changes in CC16 with exercise associated with ozone pollution. Broeckert *et al.* [75] investigated 24 non-smoking cyclists, performing a 2 h of moderate intensity cycling 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 the O_3 concentration and the cyclists' serum levels of CC16 both pre and post-exercise. By contrast, when comparing pre and post rides, no decrease on lung function parameters were found-these are usually impaired by O_3 exposure. Thus, this study showed that short-term exposures to ambient-levels of O_3 induced an early increase in serum CC16 which took 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 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 lung epithelium permeability.

Blomberg *et al.* [76] conducted a lab study where 22 subjects performed 2 h of moderate intermittent exercise. They were exposed to two different environment conditions: 0.2 ppm of O₃ 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, FEV₁ and FVC, were observed immediately post O₃ exposure. However, at 2 and 4 h post-exercise this decrease was no longer observed. Moreover, a significant increase in CC16 serum levels was seen around 2 and 4 h post O₃ exposure. No relationship was noted between CC16 and lung function at any of the analyzed points. Serum CC16 concentrations were shown to have returned to baseline 18 h post-exposure. Other epithelial permeability markers, albumin and total protein concentration, which 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 when compared to traditional markers.

Contrary to the above-mentioned studies, Lagerkvist *et al.* [77] 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 O₃ value reached 0.059 ppm. Blood samples and lung function performance were analyzed 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 O₃ 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. More investigation is needed to establish the effect of ozone and exercise on airway permeability of different populations, as there are still contradictions in the literature using CC16 as a marker of lung injury.

Acute exposure to ozone, as investigated in the studies above, is very relevant and can also be related to some real-life situations when cities experience intense pollution episodes or when individuals travel to more polluted areas. Nevertheless, chronic exposure to air pollution also needs further investigation. Unfortunately, several challenges are present for the development of research on chronic exposure to air pollution on a human exercising population. Christian *et al.* [53] showed an attenuation of the inflammatory response in BAL after four consecutive days of 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.* [44] support the previous finding, with them additionally reporting 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 the attenuation of some inflammatory markers in BAL. It is important to point out that such persistent injury could lead to airway remodeling, which has been observed in several animal studies, but needs further investigation in humans [53].

Using a different technique, sputum induction, to assess airway inflammation, Ratto *et al.* [45] found an increase in the percentage of neutrophils after 4 h of 0.2 ppm O₃ exposure during 4 consecutive days. This finding is in contrast to the afore-mentioned studies using BAL where it was observed an attenuation of the inflammatory response. Nevertheless, the increased recruitment of neutrophils to proximal airway tissue, showed by the analysis of induced sputum, was consistent with the examination of endobronchial biopsies samples taken by Jörres *et al.* [44]. 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 [39, 78].

6. Air pollution, exercise and antioxidant supplementation

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 [42, 78, 79]. For this reason, antioxidant supplementation has been suggested as a benefit for people exercising in an air-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 the oxidant-be it PM, O₃ or any other oxidant gas. These additional sacrificial substrates will, in turn, decrease oxidation reactions occurring within this fluid and within the underlying epithelial cells. In addition, an excess in antioxidants concentration might also confer protection by neutralizing free radical species, derived from these initial reactions or inflammatory cells [26, 78]. As a result, the toxicity of the pollutant would be decreased limiting the inflammation response of the cells from the epithelium tract [80], consequently, lung injury would be minimized and lung function would be maintained.

Some studies have investigated this proposed benefit of increasing antioxidant availability in individuals exposed to ozone-pollution by supplementing the participants with a mix of antioxidants, mostly vitamins C and E. These two antioxidants are present in the RTLFL, they both have strong antioxidant properties and together have been shown to present a synergistic effect in the protection against oxidative stress [78, 81]. Vitamin E is the major lipophilic antioxidant in human tissues, whether in the airways or otherwise; and vitamin C has been linked to maintenance of lung health, as for example, by improving lung function, having a positive effect on exercise-induced bronchoconstriction in asthmatic individuals and decreasing the adverse respiratory symptoms experienced during exercise [82-84]. As will be further elucidated, supplementation with these two vitamins has been shown to provide some protection in humans exposed to ozone-pollution [35, 85-88]. Little is known in relation to the benefits of antioxidants when it comes to exposure to other kinds of air pollutants other than O₃. Nevertheless, the benefits would be expected to be similar for all the oxidant air pollutants.

Romieu *et al.* [85] have shown the existence of some protective effect when participants are supplemented with a mix of antioxidants. These researchers conducted a field study in which

34 workers (shoe-cleaners), who were constantly exposed to pollution (Mexico City), participated in a double blind supplementation/placebo crossover design study. The supplementation consisted of a mix 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. As reported, antioxidant supplementation resulted in a protective effect on the lung function against the ozone exposure. The authors, however, do 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 in relation to vitamin E, which accumulates in the tissues; but they did not investigate this issue. Field studies, such as this one, have some limitations, as for instance, the interference of other pollutants and the change in 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 being exposed to the pollution.

Two subsequent field studies, conducted with amateur and recreational cyclists [86, 87], reached similar results in relation to antioxidant supplementation providing some protection against the acute effects of ozone on lung function. Grievink *et al.* [86] observed two groups of cyclists, during a 3-month period. One group was supplemented with vitamin C (650 mg day⁻¹), vitamin E (100 IU day⁻¹) and β -carotene (15 mg day⁻¹); while the other group 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, and 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 23°C and the ozone concentration averaged 0.05 ppm. In the subsequent study by these researchers [87], 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 later 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 who had not complied fully with the supplementation were excluded from the analysis, the effects of ozone on lung function were no longer observed. The average temperature and ozone concentration in this study were lower than in the previous study: 17°C and 0.04 ppm respectively. Both studies presented some disadvantages. Firstly, they analysed two 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 [87], the placebo group, acting as the control, was not taking any pills, therefore, it was not blinded. Thirdly, some individuals in the supplemented group were already taking antioxidants prior to the start of the study. Lastly, 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 this array of uncontrolled variables could have influenced the outcome.

In the study of Samet *et al.* [35], participants were divided into two groups and all underwent a 1-week period of vitamin C-restricted diet. After this, one of the groups received supplementation (250 mg vitamin C+50 IU vitamin E+12 oz of carrot and tomato juice), while the other group received placebo and continued 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, the 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 answers given in 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 relation to the subjects who ingested the antioxidant mixture.

Contrary to this finding, Mudway *et al.* (89) 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 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. Besides 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 the ozone challenge.

In a 2011 study, Gomes *et al* [90] reported that there was a positive effect of a 2-week supplementation period of vitamin E and vitamin C on the pre-exercise levels of the total antioxidant concentration in the RTLF. In addition, after the 8 km time trial run the participants, when on the vitamins, presented decreased lung injury (higher CC16 levels in both the plasma and NL) compared to when they took the placebos. Participants also ran on average 49sec faster when taking the vitamins. The environment where the exercise took place had, besides the ozone pollution (0.1ppm), also heat and humidity. This study was also conducted in a double-blinded randomized and crossover way, which minimizes biases. A summary of the studies presented above is provided in Table 5.

The inconclusiveness of the literature can possibly be attributed to divergences in the methodologies used in the research, such as different supplementation protocols, exercise modes and participants' fitness levels. Additionally, only one study looked at the effect of antioxidant supplementation on performance in a polluted environment, with more information being necessary in relation to the antioxidant and inflammatory response. Due to the high antioxidant consumption by the physically active community and by a large portion of the general

Study	Subjects	Design	Supplement	Exercise and ozone levels	Results
Romieu <i>et al.</i> , 1998 [85]	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 impairment with supplementation
Grievink <i>et al.</i> , 1998 [86]	Amateur and recreational cyclists	Field study Placebo (n=18) Supplemented (n=20)	Started 1 wk before 1 st 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 <i>et al.</i> , 1999 [87]	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 <i>et al.</i> , 2001 [35]	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 <i>et al.</i> , 2006 [89]	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
Gomes <i>et al.</i> 2011 [90]	Male elite runners	Crossover (n=10)	2 weeks: vit C 500mg + vit E 100 IU daily	8km time trial run	Attenuation of lung injury with supplementation 49 sec improvement in performance

Table 5. Studies investigating vitamin C and E supplementation in ozone exposure

population, this is an important topic for research, particularly 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.

7. Chapter summary

The main points of this chapter are:

- Air pollution can be found both indoor and outdoor. Sources of air pollution are mainly found in urban areas linked to human activities, such as power plants, and locations where there are high concentration of vehicles and burning of fossil fuel. Some pollutants are also generated by natural processes.
- Air pollutants can be found in different forms and sizes and they can exist as solid particles, liquid molecules or gases. Some examples include: Ozone, particulate matter, carbon monoxide, and nitrogen dioxide.
- High levels of air pollutants have been shown to cause and exacerbate various pulmonary and cardiovascular diseases, as well as increase mortality. Low birth weight and baby development have also been shown to occur in areas with high levels of air pollution.
- The active population that exercise and compete outdoors are a susceptible group. This is the case because exercise leads to an increase in the amount and depth of air that is inhaled, which results in higher doses of air pollutants reaching deeper places in the lungs. This also facilitates the passage of the smaller sized PM into the systemic circulation.
- Some air pollutants can trigger an oxidative stress process in the lungs which can lead to cell death, inflammation, injury and loss of function.
- Antioxidants are sacrificial molecules that promptly react with free radicals and reactive species neutralizing them. There is a wide range of antioxidants in body fluids, tissues and organs, working synergistically in a network
- CC16 is a small-sized protein which changes in concentrations can indicate lung injury and toxicity. It can be measured in the upper and lower respiratory tract, as well as in the blood.
- Performance could be affected when individuals exercise in an ozone polluted environment. This, however, depends on the exercise type, intensity, duration, individual susceptibility and other environmental factors, such as heat and humidity.
- Although more studies are necessary, antioxidant supplementation might help to mitigate the adverse effect of ozone pollution on health and performance.

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