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AVALIAÇÃO DE DANO PULMONAR EM RATOS CRONICAMENTE EXPOSTOS A POLUIÇÃO DE ORIGEM VULCÂNICA E ANTROPOGÉNICA

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Dissertação apresentada ao Departamento de Biologia, no âmbito do Mestrado em Ciências Biomédicas para obtenção do título de Mestre em Ciências Biomédicas.

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"Nenhum de nós pode presumir que já aprendeu o suficiente. O fechar da porta de uma etapa da vida é o abrir de outra etapa em que devemos continuar a busca do conhecimento"

Gordon B. Hinckley

Indice:

Chapter 1

General Introduction

1.1 –Overview

The adverse health effects of air pollution have been noted since the middle ages, particularly in the relation to the smoke produced from burning coal. The contribution of vehicular exhausts to atmosphere pollution increased greatly during the twentieth century, although their importance was not recognized for several decades (Gregory & Burr 2011). In fact, the last 50 years have been characterized by a major industrial growth that lead to big developments in processes of agricultural and industrial production. Such phenomena guided to the emerging of large quantities of chemicals in the environment with the ability to accumulate and persist to our days (Duarte, 2008).

Although some authors claim that since the beginning of times, man has become more aware of the harm that represents a polluted atmosphere (Braun *et al*., 2003), it was only after the severe air pollution episodes in northern Europe and North America between 1900 and 1965, on which resulted in thousands of deaths, that it became evident that air pollution had adverse, short- and long-term health effects, including an increase in mortality (Katsouyanni *et al*., 2011).

For the time being, volcanogenic pollution has been widely ignored as a source of pollutants with impacts on health and environment, despite the fact that in nature, levels of certain compounds are much greater than expect and, thus, with elevated toxicity to humans and other living organisms.

1.2 – Objectives

The main goal of this work is to evaluate lung injury associated with exposure to either natural-volcanogenic (Furnas village) or anthropogenic (Ponta Delgada) air pollutants, by using the house mouse – *Mus musculus*, as a bioindicator species.

This work also aims to: (i) to develop histological and cellular biomarkers of effect to air pollution exposure (ii) test the association between the different analyzed biomarkers.

Chapter 2

Main Concepts

2.1 – Anthropogenic air pollution and health effects

Air pollution has been associated with the increasing respiratory health problems in human populations. World Health Organization (WHO) members point out 800 000 cases of cancer caused by direct exposure to pollutants and also a great increase of asthma, bronchitis, coronary diseases, low weight birth and congenital defects (Cohen *et al*., 2003). According to Hildebrandt *et al*. (2009) several studies have established an association between the increase of the atmospheric pollution and the rate of pulmonary diseases.

The main primary pollutants known to cause harm when in high concentrations are carbon compounds, such as carbon monoxide (CO), carbon dioxide (CO_2), methane (CH_4) and Volatile Organic Compounds (VOCs), nitrogen compounds, such as nitrogen dioxide (NO_2), nitrous oxide (N_2O), and ammonia (NH_3) and, sulfur compounds, such as hydrogen sufide (H_2S) and sulphur dioxide (SO_2). The main secondary pollutants are NO_2 and nitric acid (HNO_3) (formed from NO), ozone (O_3) (formed from photochemical reactions of nitrogen oxides and VOCs) and sulfuric acid droplets (formed from SO_2) (Daly & Zennetti, 2007). These pollutants may interfere with health, safety and well-being of living organisms (Cançado *et al*., 2006).

The Portuguese environmental state report for 2009, produced by the Portuguese environmental agency (Vilão *et al*., 2010), mentions that Portugal is already aware of the climate change problem, and that it demands a strong political intervention in the creation of countermeasures to the presented values of environmental pollution. Also announced, are the most important compounds for the evaluation and management of air quality: SO_2, NO_2, lead (Pb), CO_2, CO, Benzene, O_3 (consistent with statements of Daly *et al*.,2007), heavy metals like arsenic (Ar),

cadmium (Cd), nickel (Ni), mercury (Hg) and particulates PM_{10} and $PM_{2.5}$. Particulate matter is recognized as one of the most noxious air pollutants to human health. Among the effects due to inhalation of particulate matter, the following are highlighted: asthma, lung cancer, several cardiovascular diseases and premature death (WHO, 1993).

Also accordingly to Portuguese environmental state report (Vilão *et al*., 2010), in 2008 the energy industries (23%), other industries (23%) and road traffic (22%) were the sectors that gave the major contribution for the emission of air pollutants.

2.2 – Volcanogenic air pollution and health effects

Even though the vast majority of studies focused on health effects related to anthropogenic pollution, since it has been considered the main responsible and with major impact in the environmental quality, the impacts of pollutants of volcanic origin must not be disregarded, particularly in what concerns the health of human populations (e.g. yearly, volcanoes are responsible for the release of almost 150 million tons of CO_2 into the atmosphere (Amaral & Rodrigues, 2011)).

Also, volcanic eruptions may produce a lethal amount of toxic gases. Once again a minor importance has been given to the effects of these gases and fine particulates which affect the biological systems and that are released by volcanoes, not only trough the main crater, but also through soil degassing. The most dangerous gases and aerosols are: CO_2, SO_2, sulphuric acid (H_2SO_4), H_2S) chlroridric acid (HCl), hydrogen fluoride (HF) and Radon gases (^{222}Rd), as well as, As, Hg, aluminum (Al), rubidium (Rb), Pb, magnesium (Mg), copper (Cu), zinc (Zn) (Durand et al. 2004).

2.2.1 – Radon gas

Radon-222 is a chemically inert radioactive gas with a half-life of 3.8 days, giving rise to a series of short-lived progeny. It arises from the decay chain of uranium-238, which is present throughout the earth's crust, and seeps out of rocks and soil. If radon itself is inhaled, some will be absorbed through the lung, but the majority will be exhaled. However, the progeny are solid and form into small molecular clusters or attach to aerosols in the air and these may be deposited on the bronchial epithelium. Two of the short-lived progeny in the commonest decay chain, polonium-218 and polonium-214, decay by emitting alpha particles. These have a limited range of penetration into tissues but are highly effective at causing genetic damage in the cells they reach (Darby et al. 2001). Radon becomes especially dangerous when the emitter substances enter the organism by an open wound, ingestion or inhalation (Villalobos-Petrini et al., 1998; Brooks et al., 1997). In fact, radon inhalation is the main source of exposure to radioactivity for most people throughout the world (Catelenois *et al.,* 2006), and several studies have proven a significant lung cancer risk after domestic radon exposure (Darby *et al.,* 2005; Krewsky *et al.,* 2005; Lubin 2003).

2.2.2 – Sulphur dioxide

Sulphur compounds like SO_2 are also of volcanogenic origin, and can promote oxidative stress and some alterations in the tissues of respiratory organs, like metaplasia. When SO_2 contacts the atmosphere it is oxidized to sulphur trioxide (SO_3), which then reacts with water to produce H_2SO_4. According to the International Agency of Research in Cancer (IARC), SO_2 isn't considered a carcinogenic substance to humans, unlike H_2SO_4. However, Meng *et al*. (2005) referenced several epidemiologic

occurrences on which there is a relation between SO_2 exposure and pulmonary diseases and lung cancer. Findings from other authors also refer SO_2 concentrations can cause acute and chronic effects in the respiratory system (Amaral *et al.*, 2006; Iwasawa *et al*., 2009).

2.2.3 – Heavy metals

Some heavy metals, from volcanic origin, are known to be carcinogenic, such as Cd, chromium (Cr), Pb and As (Smith *et al*., 1992; Shukla *et al*., 1998; Waalkes, 2003). A recent study for the development of cancer risk, by Duntas *et al*. (2009) revealed that lithium (I), As, Zn and sodium (Na), are positively related to an increase in cancer risk, whereas selenium (Se) and vanadium (V) are inversely related.

2.3 – Air pollution and CO_2

When discussing either natural or anthropogenic pollution, the CO_2 concentrations are the main concerning values, since CO_2 has gained the most attention of all the greenhouse gases because it has the highest radiative force. Radiative force can be described as the change in the balance between radiation coming into the atmosphere and radiation going out. On average, a positive radiative force warms the surface of the earth and a negative radiative force cools the surface (Mandlebaum & Nriagu, 2011). CO_2 is considered an inert asphyxiant gas, which means it replaces oxygen, but it does not cause direct toxic effects over biological tissues. However, some recent studies have suggested that CO_2 may be a molecular species with significant bioregulatory properties, by altering the neutrophils activity and behavior (Coakley *et al.,* 2002; Norozian *et al.,* 2011).

2.4 - Biomonitorization

2.4.1 - Biomonitoring

Biomonitoring is a scientific technique used for assessing human exposure to natural and synthetic chemicals, based on sampling and analysis of an individual's tissues and fluids (Bocca *et al*., 2010).

By using living organisms it is possible to study the effect of all air contaminants acting jointly, because biomarkers are integrators opposed to the instrumental automatic stations that only sample for quality of air.

According to Salegui (2002), the ecological variations, due to atmospheric contamination that are produced in an organism, can be disclosed at 3 levels:

- Accumulation of contaminants in the organisms;
- Morphologic or structural modifications in the organisms;
- Alterations in the composition of the animal and/or vegetal community.

Biomonitoring allows evaluating the effects of the pollutants throughout time, for the effect of the pollutants go on being restrained in the organism. In this way, when the organism is analyzed, the result obtained is of an exposure throughout weeks, months, etc., and not only of the exposure at the moment preceding the monitorization; thus, the main difference between biomonitorization and monitorization arrests with the development in the space and/or time.

2.4.2 - Bioindicators

A bioindicator is defined as a measurement, indices of measurements or model of biological answers which characterize an ecosystem or one of its critical components, and that may reflect biological, chemical or physical attributes, of certain

ecological condition. The main purpose of a bioindicator is to characterize the state of a certain population, community or ecosystem, and to predict any significant changes in that state (Walker *et al*., 2000). The use of these indicators allows increasing the sensitivity of analytical procedures and summarizes a complete sign of pollution (Beeby, 2001).

According to Landis & Yu (1995) the chosen bioindicator to be used in monitoring programs should have, if possible, the following criteria:

-Be sedentary and abundant in the field of the work;

-Be easy to breed in laboratory, as to obtain in the environment;

-Its genetic parameters should be known;

-Its response to different class of xenobiotics should be known, regarding the measurements objective;

-Should be fairly tolerant to the xenobiotic analyzed;

-Its sensitivity should be class representative.

As it is difficult to match all the mentioned criteria, frequently the chosen bioindicator species is the one that matches some of the above requirements, but usually has great abundance and can be somehow related to humans, by either food chain or genetic resemblances. Some algae, mollusks, fish and small rodents, and sometime humans, are some examples of species used in environmental biomonitorization (Solbé, 1993; O'Connor, 1998; Minier *et al*., 2000; Debenay *et al*., 2003).

For this present study the bioindicator species *Mus musculus* was used. According to Timm (1994) *Mus musculus* is considered a good bioindicator species, since:

-it is ubiquitous and can be found in both active and non-active volcanic areas

-it shares the same houses as humans and eats their food

-it occupies a middle position in many food chains

-it contacts with soil, not only by living in soil made holes, but also ingesting soil

-it has small ranges of action, usually an area of 64 m2

2.4.3 - Biomarkers

Through the interaction between chemical agents and the organism, many are the biological parameters that can be modified; however, the quantitative determination of these parameters used as Biological markers or Biomarkers it is only possible if exists correlation with the intensity of the exposition and/or the biological effect of the substance. In this way, the biomarker contains the substance or a product from its biotransformation, as well as any precocious biochemical alteration, whose determination in biological fluids, tissues or exhaled air, evaluates the intensity of the exposure and the health risk (Amorim, 2003). Therefore, biomarkers aim to evaluate the exposure (absorbed amount or internal dose), the effect of chemical substances and the individual sensitivity (WHO, 1993). Moreover, they can be used independently of the source of exposure, either through the diet or the general environment. Biomarkers can essentially be of three types:

- Exposure biomarkers are those that can be used to confirm and evaluate individual or group exposure to a particular substance, establishing a connection between the external exposure and the quantification of the internal exposure.

- Effect biomarkers are those that include biochemical changes, physiological or any other type of change in tissues and fluids that can be used to document pre-clinic alterations or health adverse effects resulting from the exposure and absorption of a chemical substance. On this form, biomarkers linking between exposure and effects contribute to the definition of the relation dose-response.
- Sensitivity biomarkers are those that elucidate about the response degree that the exposure caused in the individuals (WHO, 1993).

2.5 – Localities environmental description

2.5.1 – Furnas village

Furnas village is located inside the Furnas volcano caldera (São Miguel island-Azores), where present-day volcanic activity is marked by several hydrothermal manifestations consisting of active fumarolic fields, thermal and CO_2 cold springs and soil diffuse degassing areas (Viveiros *et al*., 2008). The village has around 1500 inhabitants that are continuously exposed to gases of volcanic origin, particularly those from soil degassing. According to Baxter *et al*. (1999) one of the major soil diffuse degassing areas in the island of São Miguel extends below Furnas village, with some houses located in areas where the risk of asphyxia is high due to significant indoor soil CO_2 flux increases. On the other hand, indoor radon levels can be very high in Furnas village, as the field study by Silva *et al*. (2009) pointed out. In this study, ^{222}Rn measurements were performed in 301 station points at Furnas Caldera, and revealed values between 0 at 387 527,3 Bq m^{-3}, being the average value of 11305,9 Bq m^{-3}, which is many times more than the reference level (200 – 400 Bq m^{-3}) for indoor air in most countries (Amaral & Rodrigues, 2011).

2.5.2 – Ponta Delgada city

Ponta Delgada city is located in São Miguel Island-Azores, which is the largest and most populated island of the archipelago. This city with nearly 60 000 inhabitants is also the most populated city in the archipelago. Knowing that in Azores there is not a strong industrial sector, road traffic is the main responsible of the emissions of air pollutants of anthropogenic origin, namely CO_2 and VOCs.

2.5.3 – Rabo de Peixe village

Rabo de Peixe is a rural village also located in São Miguel Island-Azores. With nearly 7000 inhabitants, Rabo de Peixe village has no apparent pollution, nor does it present any type of volcanic manifestations.

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Chapter 3

Lung injury assessment, in mice, associated with chronic exposure to volcanogenic and anthropogenic air pollution

3.1 - Abstract

The aim of this work is to test the hypothesis that exposure to air pollutants of volcanogenic or anthropogenic origins increases lung injury. In order to realize this objective, three separate sets of a bioindicator species, *Mus musculus*, were caught in Furnas Village and Ponta Delgada city to access lung injury from exposure to volcanogenic and anthropogenic air pollutants, respectively, and Rabo de Peixe, a rural location chosen as control site. The mice were euthanized followed by a necropsy and the lungs were extracted, fixed and processed to histological evaluation. Multiple morphometric measurements and double-blinded rank evaluations were made to assess the extent of lung injury. Histopathological diagnosis was also made. The morphometric measurements of the histological sections were made in order to achieve the percentage of alveolar space, the alveolar perimeter and septal thickness.

The septal thickness was significantly augmented in individuals exposed to volcanogenic air pollutants, when compared with control. Double-blinded evaluations revealed a significant increase either in inflammatory status or amount of melano-macrophages centers, regarding the individuals exposed to both air pollution sources. Lesions consistent with non-small cell lung carcinoma were found in 40% of the retrieved individuals from the volcanogenic air polluted location. Chronic exposure to pollutants of volcanogenic or anthropogenic origin is associated to lung injury, since increases the inflammation status, the amount of melano-macrophages centers and alveolar septal thickness resulting in defective lung functionality. Volcanogenic environmental factors could have genotoxic effects that could contribute to explain the higher incidence rate of lung neoplasia.

3.2 - Resumo

O objectivo deste trabalho é testar a hipótese de que a exposição a poluição do ar, quer de origem vulcânica, quer de origem antropogénica, aumenta o dano pulmonar. De forma a cumprir o objectivo, três conjuntos de uma espécie bioindicadora, *Mus musculus*, foram capturados vivos na Vila das Furnas e na cidade de Ponta Delgada, para avaliação de dano pulmonar derivado da exposição a poluição do ar de origem vulcânica e origem antropogénica, respectivamente, e Rabo de Peixe, uma localidade rural escolhida como controlo. Os ratos foram eutanizados e de seguida foi feita uma necrópsia com extracção dos pulmões, que foram depois fixados e processados para análise histológica. Vários parâmetros morfométricos foram obtidos (percentagem de espaço alveolar, perímetro alveolar e espessura dos septos), assim como avaliações qualitativas através do teste duplo-cego (grau de inflamação e abundância de centros pigmentares em melano-macrófagos), e diagnóstico histopatológico.

A espessura dos septos foi significativamente superior nos indivíduos expostos a poluição vulcanogénica quando comparado com o controlo. As avaliações duplamente cegas revelaram um aumento significativo quer nos valores da inflamação quer na abundância de centros presentes nos melano-macrófagos dos grupos expostos face ao grupo não exposto. Foram encontradas lesões consistentes com o diagnóstico de carcinoma de células não-pequenas em 40% dos indivíduos capturados no local de exposição a poluição volcanogénica. A exposição crónica a poluentes aéreos, quer de origem vulcânica quer antropogénica, está associada a danos pulmonares, uma vez que aumenta os índices de inflamação, a quantidade de centros nos melano-macrófagos, assim como a espessura dos septos, resultando num défice na função

respiratória. O ambiente vulcanicamente activo poderá ter efeitos genotóxicos que possam estar na origem da elevada taxa de carcinomas do pulmão encontrada no grupo de indivíduos provenientes das Furnas.

3.3 - Introduction:

The adverse health effects of air pollution have been noted since the middle ages, particularly in relation to the smoke produced from burning coal. The contribution of vehicular exhausts to atmosphere pollution increased greatly during the 20th century, although its importance was not recognized for several decades (Gregory & Burr, 2011). It was only after the severe air pollution episodes that occurred between 1900 and 1965 in northern Europe and North America, which resulted in thousands of deaths, that it became evident that the current, relatively lower, air pollution levels had adverse, short- and long-term health effects including an increase in mortality (Katsouyanni *et al*., 2011).

Air pollution has now been associated with the increasing respiratory health problems in human populations. World Health Organization (WHO) members point out 800 000 cases of cancer caused by direct exposure to pollutants and also a great increase in other pathologies, such as asthma, bronchitis, coronary diseases, low weight birth and congenital defects (Cohen *et al*., 2003). The Portuguese environmental state report for 2009, produced by the Portuguese environmental agency (Vilão *et al*., 2010), mentions that Portugal is already aware of the climate change problem, and that it demands a strong political intervention in the creation of countermeasures to the presented values of environmental pollution. Also announced, are the most important compounds for the evaluation and management of air quality:

SO_2, NO_2, Pb, CO_2, CO, Benzene, O_3, Ar, Cd, Ni, Hg and particulates PM_{10} and $PM_{2.5}$. Particulate matter is recognized as one of the most noxious air pollutants to human health. Among the effects due to inhalation of particulate matter, the following are highlighted: asthma, lung cancer, several cardiovascular diseases and premature death (WHO, 1993). Also, accordingly to the Portuguese environmental state report in 2008 the energy industries (23%), other industries (23%) and road traffic (22%) were the sectors that gave the major contribution for the emission of air pollutants (Vilão *et al.*, 2010).

These numbers are widely related with anthropogenic pollution, which is the main responsible and with major impacts in the environmental quality, and thus with relevant impacts in the general health of human populations. For the time being, volcanogenic pollution has been widely ignored as a source of pollutants impacting on health and environment quality, despite the fact that in nature, levels of certain compounds are much greater than expect and, thus, with elevated toxicity to humans and other living organisms [e.g. yearly, volcanoes are responsible for the release of almost 150 million tons of CO_2 into the atmosphere (Amaral & Rodrigues, 2011)]. The island of S. Miguel (Azores – Portugal) has a constant volcanic activity and major production and emission of gases to the atmosphere (some radioactive like radon), and other substances, that daily affect the environment quality and human population's health. According to Viveiros *et al.* (2010), in Azores, the Furnas volcano – Furnas caldera and Ribeira Quente (S. Miguel Island) - emits about 1000 tons a day of CO_2 from soil degassing. Volcanic gaseous emissions include CO, CO_2, SO_2, HCl, HF, H_2S and ^{222}Rn. Radon is a chemically inert radioactive gas of natural origin, produced by the disintegration of uranium and radium located in earth's crust. Radon inhalation is the

main source of exposure to radioactivity for most people throughout the world (Catelenois *et al.,* 2006), and reports have proven a significant increase in lung cancer risk after domestic radon exposure (Darby *et al.,* 2005; Krewsky *et al.,* 2005; Lubin, 2003). A field work of Silva *et al*. (2009) for ^{222}Rn measurements was performed in 301 station points at Furnas Caldera, and revealed values between 0 at 387 527,3 Bq m^3, being the average value of 11305,9 Bq m^3, which is many times more than the reference level (200 – 400 Bq m^{-3}) for indoor air in most countries (Amaral & Rodrigues, 2011).

When discussing either natural or anthropogenic pollution, the CO_2 concentrations are the main concerning values, since CO_2 has gained the most attention of all the greenhouse gases because it has the highest radiative force (Mandlebaum & Nriagu, 2011). CO_2 is considered an inert asphyxiant gas, which means it replaces oxygen, but it does not cause direct toxic effects over biological tissues. However, some recent studies have suggested that CO_2 may be a molecular species with significant bioregulatory properties, by altering the neutrophils activity and behavior (Coakley *et al.,* 2002; Norozian *et al.,* 2011). Neutrophils are the dominant cells in the acute inflammatory response and are a critical component of the host's reaction to invading microorganisms (Walker & Willenze, 1980). Although central to the inflammatory response, neutrophils are implicated in the pathogenesis of some respiratory diseases, such as emphysema (Janoff, 1985). Furthermore, infiltration of neutrophils in the airways is also proven to increase in severe asthma (Nakagome & Nagata, 2011). The neutrophils activation is also known to increase the levels of several cytokines which then leads to the migration of other leukocytes, including lymphocytes (Megiovanni *et al*., 2006). Therefore, it is possible that a constant

activation of neutrophils leads to mobilization of lymphocytes and stimulate the appearance of macrophages (Akahoshi *et al*., 2003). Not only neutrophils behavior can be modulated by air pollution, through CO_2 concentrations, but also, there are other cell types which respond to other air pollutants, like particulate matter. Melano-macrophages are macrophages that are usually packed, and form clusters, and enlarge after active phagocytosis of heterogeneous materials (Agius & Agbede, 1984; Agius, 1985). In fact, the appearance of these types of macrophage can vary accordingly to physiological conditions within the same species, varying from age (Kranz & Gercken, 1987), pathological and inflammatory conditions (Volgenbein *et al*., 1987) to immunological processes (Fulop & McMillan, 1984) and environmental changes (Fournie *et al.,* 2001).

In order to assess the effects of exposure to air pollutants of anthropogenic and volcanogenic origins in the extent of lung injury, a bioindicator species (*Mus musculus* L.) was used as model for this study. This specie has already been considered a good bioindicator (Timm, 1994) by sharing the same houses as humans; being in constant and close contact with soil, not only by living in holes made in the soil, but also by ingesting it, and due to the fact that mice population is usually large.

Lung injury extent was assessed by studying the following histological morphometric parameters: percentage of alveolar space, alveolar perimeter and septal thickness. Also considered were the inflammatory status and quantity of melano-macrophages centers within the lung tissue.

3.4 - Material and Methods

Three separate sets of *Mus musculus* were caught alive in the designated areas of Furnas village, Ponta Delgada city and Rabo de Peixe village.

Furnas village, our designated place for exposure to volcanogenic air pollutants, is a rural location built upon actively degassing ground inside a volcanic crater, where fumarolic fields and hydrothermal vents are current manifestations of volcanism and are the cause for ongoing natural exposure to high levels of heavy metals and gases (Baxter *et al*., 1999; Cruz, 2003; Ferreira *et al*., 2005). Ponta Delgada city is an urban location with nearly 60 000 inhabitants and is the most populated city in the Azores archipelago in which road traffic is the main responsible of the emissions of air pollutants of anthropogenic origin like CO_2 and VOCs.

Rabo de Peixe village, like Furnas village, is also a rural location, though it does not present any type of volcanic manifestations since the seventieth century (Carvalho, 1999). This village has no apparent source of pollutants.

The group exposed to volcanogenic pollution corresponded to 10 adult mice from several households in Furnas village. The group exposed to anthropogenic pollution corresponded to 10 adult mice caught in the northeastern part of Ponta Delgada city. The control group corresponded to 4 adult mice caught in the southern area of Rabo de Peixe village.

Mice capture was performed using live-catch mousetraps in which the mouse would be housed no more time then the necessary to be euthanized. The euthanization process was made with chloroform, followed by a necropsy with the extraction of the lungs. Right and left lungs were then fixed in 4% buffered

formaldehyde followed by a traditional histological processing, using ethanol as a dehydrator agent, xylene as clearing agent and paraffin wax as an embedding material.

The histological slides were then prepared and consisted of several (10 minimum) 5-µm thick sections, of each lung per mouse. The slides were then stained with hematoxylin and eosin in the standard manner (Martoja & Martoja Pierson, 1970).

Lung injury: histological morphometric evaluation

The alveolar septal thickness was measured at 100 locations for each test subject (50 in each lung); measurements were made between two adjacent alveoli with detected epithelial and endothelial layers at a 40x magnification.

The alveoli perimeter was measured at 20 (10 per lung) randomly chosen microscopic fields' locations for each test subject. A similar procedure was used to assess the percentage of space occupied by the alveoli within the microscopic field. These measures were made with a 10x magnification, within a microscopic field area of 307200 μm^2, and using an image analyzer and a microscope (Leica DM1000, Cambridge, UK). For the alveoli perimeter and percentage of alveolar space within each observed microscopic field, the segments with less than 75 µm were not accounted due to setup characteristics of the image analyzer software. For all morphometric measures all the areas with neoplasia (when existent) weren't taken into account as they were later diagnosed.

For each morphometric parameter, averages of the left and right lungs were considered for each subject. The number of tested subjects was 10, 10 and 4 for volcanogenic-polluted, anthropogenic-polluted and control sites, respectively.

Lung injury: inflammatory status and extent of melano-macrophages centers

The status of interstitial inflammation (extent of hypercellularity, neutrophils infiltration and lymphocytes reactivity (modified of MacCarrick *et al*., 2010) was assessed by double-blinded rank evaluations. The slides were coded and randomly assigned to two investigators who were not aware of group assignment. They were scored from 1 – 4 accordingly to the percentage of lung tissue with interstitial inflammation, as follow: 1 – 0 to 5%; 2 – 6 to 20%; 3 – 21 to 50% and 4 - >50%. The extent of melano-macrophage centers was assessed in a new set of slides with 3-µm thickness, which were then deparaffinized and mounted with DPX. No stain was used to ensure a clear view of the centers with no dye pigments interference. The slides were coded and randomly assigned to two investigators that were not aware of group assignment. They were scored from 1 – 4 defined as follows: 1 – no apparent melano-macrophages centers; 2 – few small, scattered melano-macrophages centers; 3 – some and focal melano-macrophages centers; 4 – extensive and multi-focal melano-macrophages centers.

The inflammatory status and extent of melano-macrophage centers was observed in 18, 20 and 8 microscopic fields of the left and right lungs (2 per mouse) of subjects from volcanogenic-polluted (n=10), anthropogenic-polluted (n=10) and control (n=4) sites, respectively.

Pathologies diagnosis

Pathologies diagnosis was performed using multiple parameters such as: Pleomorphism anisokaryosis, nucleolar reactivity, lymphocyte infiltration and capsulation (when existent). Primarily, lung cancer can be classified through simple

differentiated morphology in small-cell lung carcinoma and non-small cell lung carcinoma (Popper, 2011). Small-cell lung carcinoma is defined as “a malignant epithelial tumor consisting of small cells with scant cytoplasm, ill-defined cell borders, finely granular nuclear chromatin, and absent or inconspicuous nucleoli” (Van Meerbeeck *et al*., 2011). As small-cell lung carcinoma incidence is around 25% and majorly in smokers, non-small cell lung cancer incidence is around 75 % of the cancer diagnosis (Kenfield *et al*., 2008) and is the leading cause of cancer death worldwide (Parkin, 2000). Non-small cell carcinoma can then be subdivided by more specific conditions into: adenocarcinoma, squamous-cell carcinoma, large-cell carcinoma and poorly differentiated variants (Goldstraw *et al*., 2011). Squamous-cell carcinoma arises from the uncontrolled multiplication of malignant cells derived from epithelium and can show characteristics of squamous cell differentiation such as keratin or desmossomes. Adenocarcinoma is a type of cancer that originates from the epithelium of a glandular tissue, and most of them resemble the tissue of which were originated. Large cell carcinoma is a heterogeneous group of undifferentiated malignant neoplasms originating from transformed epithelial cells in the lung. Whenever it not possible to assure a correct diagnosis, the designation non-small cell lung carcinoma – NOS (Not Otherwise Specified), can be used (Goldstraw *et al*., 2011).

Statistical analysis

In this study the factors analyzed were the sources of air pollution considering Furnas as source of volcanic air pollution, Ponta Delgada as anthropogenic pollution source and Rabo de Peixe as control site.

Changes in the lungs alveolar septal thickness, alveolar perimeter and percentage of alveolar space were compared by two-way ANOVA using pollution source sites (volcanogenic, anthropogenic and control site) and lung side (left and right lungs) as main factors. When ANOVA showed significant differences ($P< 0.05$) between data sets, paired comparisons of each mean were made using Tukey HSD tests. Data regarding the extent of interstitial inflammation and of melano-macrophages centers were compared for pollution source sites and control site using the non-parametric test Kruskal-Wallis. Where statistical differences existed for distribution function between data sets ($P< 0.05$), Mann-Whitney U tests were used to separate the differing groups. To test the association between the studied variables, Pearson's correlations were done for alveolar septal thickness, percentage of alveolar space and alveoli perimeter, while Spearman's rank correlations were performed between these variables and the extent of interstitial inflammation and the extent of melano-macrophages centers. All statistical analyses were made using SPSS 15.0 (SPSS Inc., Chicago, USA).

3.5 - Results

Histological morphometric evaluation

The percentage of alveolar space (mean±SE) was not significantly affected by the site (F=1,474; df=2; P=0,241; 2-way ANOVA), the lung side (F=0,019; df=1; P=0,890; 2-way ANOVA) or by the interaction between these factors (F=0,335; df=2; P=0,718; 2-way ANOVA). Although no significant differences were observed, the percentage of alveolar space in both polluted sites was slightly lower (43,96±2,47 % and

42,47±2,41%, for volcanogenic and anthropogenic polluted sites, respectively) than in the control site (49,90±2,40%) (Fig. 1).

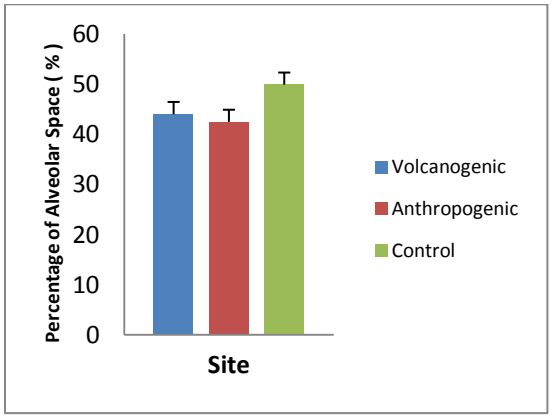

Fig.1 –Percentage (mean±SE) of lung alveolar space of mice captured in volcanogenic (Furnas village) and anthoprogenic (Ponta Delgada city) air polluted sites, and in the control site (Rabo de Peixe village).

Similarly, the alveolar perimeter (mean±SE) was not significantly affected by the site (F=2,737; df=2; *P*=0,077; 2-way ANOVA), the lung side (F=0,013; df=1; *P*=0,910; 2-way ANOVA) or by the interaction between these factors (F=0,109; df=2; *P*=0,817; 2-way ANOVA). Although no significant differences were observed, the alveolar perimeter followed the same tendency observed for the percentage of alveolar space: in both polluted sites, the alveolar perimeter was slightly lower (38678±1864 µm and 42698±2074 µm, for volcanogenic and anthropogenic polluted sites, respectively) than in the control site (46621±1154 µm (Fig. 2).

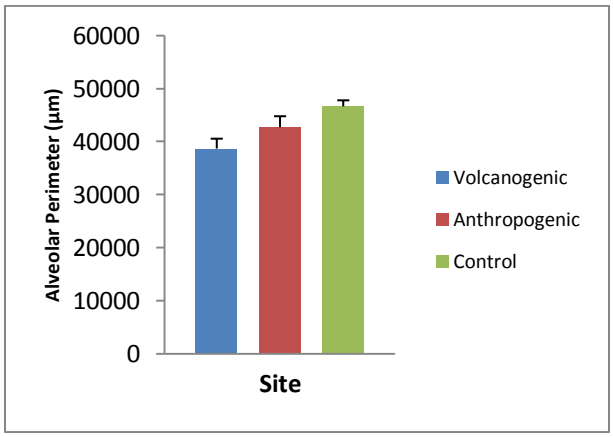

Fig.2 –Lung alveolar perimeter (mean±SE) of mice captured in volcanogenic (Furnas village) and anthoprogenic (Ponta Delgada city) air polluted sites, and in the control site (Rabo de Peixe village).

On the other hand, the septal thickness was significantly affected by the site (F=4.464; df=2; *P*=0,018; 2-way ANOVA), while neither the lung side (F=0,096; df=1; *P*=0,758; 2-way ANOVA) nor the interaction between these factors (F=0,026; df=2; *P*=0,975; 2-way ANOVA) affected significantly the septal thickness.

The septal thickness measured in individuals exposed to volcanogenic air pollution (6,68±0,68 µm) was significantly higher (*P*=0.015, Tukey-HSD test) than in the control group (3,51±0,09 µm). Although no significant differences were observed between the anthropogenic polluted site and the control group (*P*=0.255, Tukey-HSD test), the septal thickness of the former was higher in average (5,22±0,56 µm), suggesting that septal thickness is increased in mice exposed to air polluted sites (Fig.3).

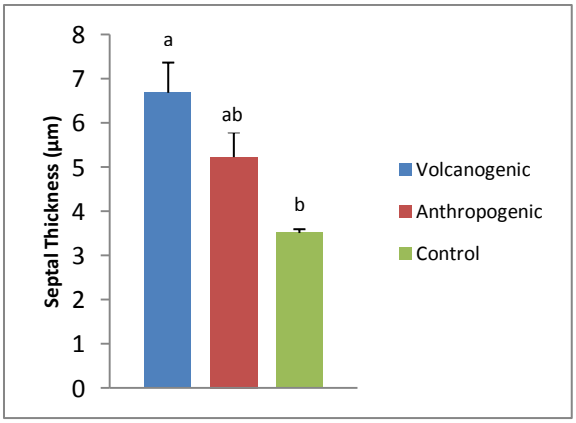

Fig.3 –Lung septal thickness (mean±SE) of mice captured in volcanogenic (Furnas village) and anthoprogenic (Ponta Delgada city) air polluted sites, and in the control site (Rabo de Peixe village). Bars with different letters are significantly different at *P*< 0.05(Tukey-HSD tests).

Inflammatory status

Significant differences were observed for the distribution of the inflammatory histological scores between the studied sites (H=11,372; df=2; *P*=0.003, Kruskal-Wallis test). Individuals from either volcanogenic (U=24; *P*=0,006; Mann-Whitney test) or anthropogenic (U=19; *P*=0,001; Mann-Whitney test) air polluted sites had significantly higher scores for inflammatory status, when compared to control site (Table 1, Fig. 5).

Table 1 – Inflammatory status of lung tissue of mice exposed to volcanogenic (Furnas village) and anthropogenic (Ponta Delgada city) sources of air pollution and from the control site (Rabo de Peixe village).

Inflammatory status	**Volcanogenic**	**Anthropogenic**	**Control**
Score (median)*	**4[a]**	**3[a]**	**2[b]**

* Medians with different letters show significant different scores at *P*< 0.05 (Mann-Whitney tests).

Although not significant, the inflammatory status of mice from the volcanogenic polluted site was scored higher (median=4) than in the anthropogenic polluted site (median=3).

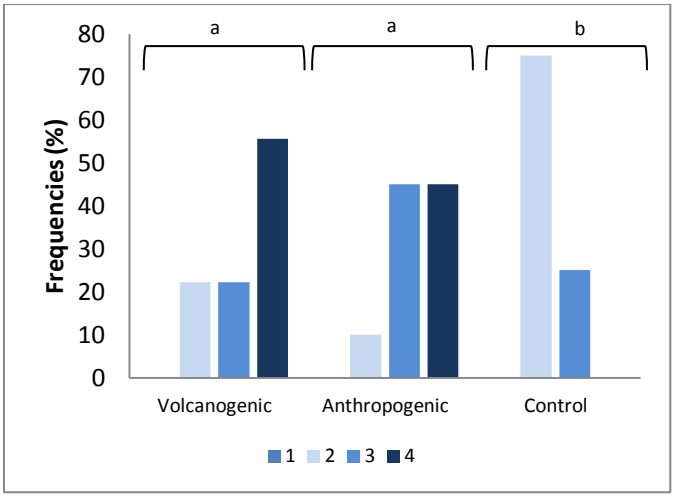

Fig.4– Histological scores of inflammatory status in mice exposed to volcanogenic (Furnas village) and anthropogenic (Ponta Delgada city) sources of air pollution and from the control site (Rabo de Peixe village); the frequency (bars) of histological scores (1 to 4) within each site is represented in terms of percentage (%); Sites with different letters are significantly different in overall score at $P< 0.05$ (Mann-Whitney tests).

Extent of melano-macrophages centers

Similarly to the observed for inflammatory status, the distribution of histological scores for melano-macrophages centers differed significantly between the studied sites (H=10,876; df=2; *P*=0.004, Kruskal-Wallis test). Individuals from either volcanogenic (U=20,5; *P*=0,003; Mann-Whitney test) or anthropogenic (U=34; *P*=0,018; Mann-Whitney test) air polluted sites had a significantly increased number of melano-macrophages centers in the lung tissue, when compared to control site (Fig. 5). Once again, the histological score for melano-macrophages centers of mice from the volcanogenic polluted site was higher (median=3) than in the anthropogenic polluted site (median=2), although not significantly different.

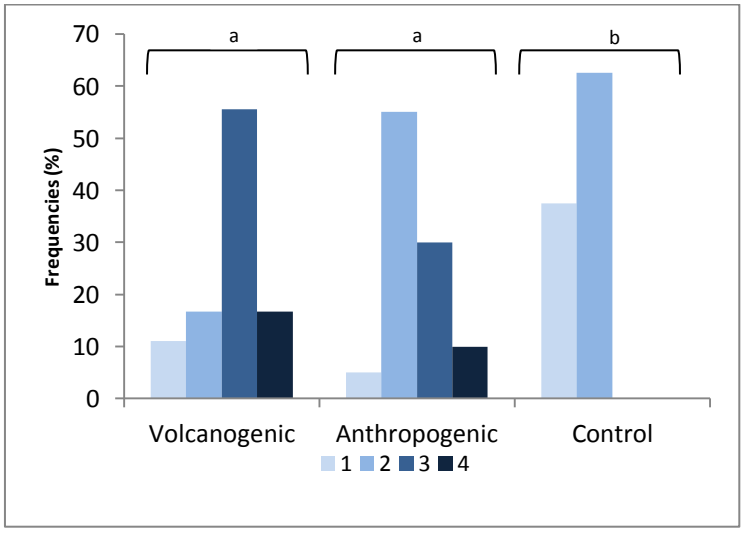

Fig.5– Histological scores of melano-macrophages centers in mice exposed to volcanogenic (Furnas village) and anthropogenic (Ponta Delgada city) sources of air pollution and from the control site (Rabo de Peixe village); the frequency (bars) of histological scores (1 to 4) within each site is represented in terms of percentage (%); Sites with different letters are significantly different in overall score at $P< 0.05$ (Mann-Whitney tests).

Association between the studied variables

Significant negative correlations between septal thickness and the percentage of alveolar space (R^2=-0,653; P<0,001; Pearson's correlation) and the alveolar perimeter (R^2=-0,767; P<0,001; Pearson's correlation) were observed. On the other hand, the alveolar perimeter was positively correlated to the percentage of alveolar space (R^2=0,768; P<0,001; Pearson's correlation).

Spearman's rank correlations between these variables and the inflammatory status and the extent of melano-macrophages centers, revealed that there was a significant and positive relationship between the inflammatory status and thickness of alveolar septa (R^2=0,780; P<0,001), and a significantly negative correlation between inflammatory status and the variables percentage of alveolar space (R^2=-0,726; P<0,001) and alveolar perimeter (R^2=-0,705; P<0,001)). The extent of melano-macrophages centers was positively correlated to septal thickness (R^2=0,299; P=0,043, Spearman's Rank Correlations).

Pathology Diagnosis

It was possible to diagnose a total of four individuals with neoplasia of the lung. All the types of neoplasias found are consistent with non-small cell lung carcinoma-NOS (Fig. 6).

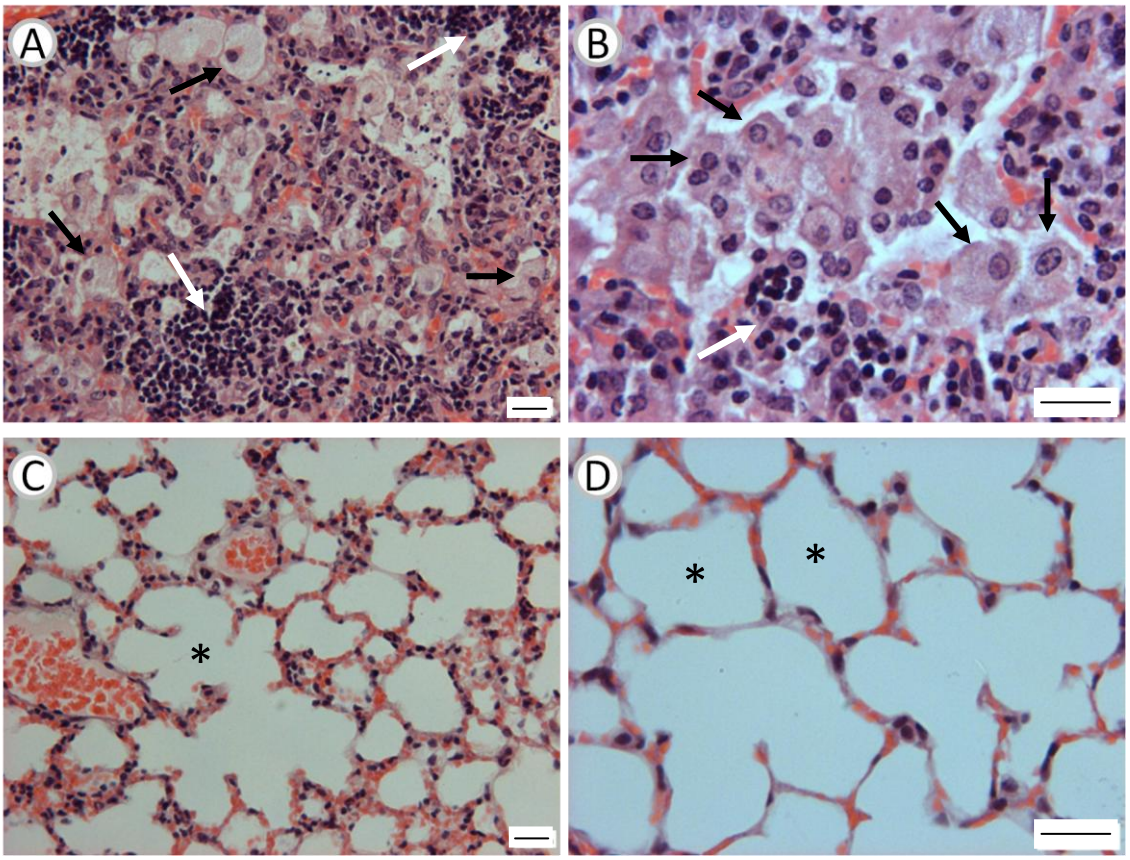

Fig. 6 – Photomicrographies of lung histological sections. (A), (B) Non-small cell lung carcinoma of lung sections in mice exposed to volcanogenic air pollution (Furnas village); (C), (D) Normal histology of lung sections in mice caught in the non-exposed site (Rabo de Peixe). Scale Bar = 20 µm; black arrows – non-small malignant cells; white arrows - lymphocite infiltration; asterisks – alveolar space.

3.6 - Discussion

Globally, our results are consistent with the hypothesis that chronic exposure to air pollution of volcanogenic or anthropogenic origin increases lung injury (Fig. 1 - 6, Table 1).

Despite the fact that no significant differences were observed in the average values of the percentage of alveolar space and of the alveolar perimeter, a tendency could be stated of a general decrease of such values from individuals exposed to pollutants of volcanogenic origin and those from the control group. When analyzing

the percentage of alveolar space we observed that this histological morphometric parameter was slightly lower in mice exposed to volcanogenic or anthropogenic pollution than in mice from the control site. Such decrease may be enough to result in a lower breathing efficacy, since the data gathered represents the efficiency of gas exchange (O_2 and CO_2, namely) in the lung. Similarly, the same case-scenario could be established when analyzing the morphometric data from alveolar perimeter, since individuals exposed to air pollutants (volcanogenic or anthropogenic) have a lesser contact surface available for the gas exchange when compared with the control individuals (even though such difference was not significant). These results are consistent with the findings of other authors which suggest that air pollutants affect the breathing efficacy (Steinvil *et al*., 2009; Cakmak *et al*., 2011).

On the other hand, average septal thickness was significantly higher in mice exposed to volcanogenic air pollution than in the control group. Moreover, though no significant differences were observed between the anthropogenic polluted site and the control group, the septal thickness of the former was higher in average, suggesting that septal thickness is increased in mice exposed to air polluted sites. Although most authors consider septal thickness increase as a result of neutrophils activation associated with acute exposure to increased CO_2 concentrations (Norozian *et al*., 2011; Coakley *et al*., 2002; Yuliang *et al*., 2008), some consider it to be a consequence of the increase in other cell types such as melano-macrophages that phagocyte undetermined particles (Agius & Roberts, 2003) and form clusters (Agius & Agbede, 1984) in the interstitial space. Actually, our results showed a positive correlation between the amount of melano-macrophages centers and septal thickness, and thus support the melano-macrophages increase hypothesis. However, there was also a

significant positive correlation between inflammatory status and septal thickness, leading to believe that both analyzed parameters (inflammatory status and extent of melano-macrophages centers) may have contributed to the general increase of septal thickness in individuals exposed to both sources of air pollution. Furthermore, we can assume that melano-macrophages centers are increased in air polluted locations by the increase of PM_{10} and $PM_{2.5}$, since these cells phagocyte undetermined particulates (Agius & Agbede, 1984; Agius, 1985) and tend to develop within maturing chronic inflammatory lesions (Agius & Roberts, 2003), which corroborates with the previous explanation for the increase of septal thickness. Moreover, inflammatory status proved also to be strong and negatively correlated to the percentage of alveolar space and alveolar perimeter, and thus effectively contributing to a decrease in the lung functionality. Findings of Nozorian *et al*. (2011) also stated an increase in septal thickness due to pro-inflammatory status of neutrophils.

In a general overview, results showed that the individuals exposed to volcanogenic air pollution had the worst case scenario in all the morphometric measurements, inflammatory status and extent of melano-macrophages centers. Such fact is consistent with the environmental characteristics of sites with active volcanism, where individuals are not only are exposed to SO_2, NO_2, Pb, CO_2, PM_{10} and $PM_{2.5}$, (Vilão *et al*., 2010), which are typical of anthropogenic pollutants, but are also exposed to other natural gaseous pollutants, like HCL, HF, H_2S and ^{222}Rn (Viveiros *et al*., 2010) and to a great amount of heavy metals, such as Al, As, Cd, Cu, Pb, Mg, Hg, Rb, (Amaral & Rodrigues, 2011). In fact, it is possible that the four cases of non-small cell lung cancer-NOS found in the individuals caught in the volcanogenic air polluted site are somehow associated with the radon exposure values frequently registered in that

area. Several authors report a significant lung cancer risk after domestic radon exposure (Darby *et al.,* 2005; Krewsky *et al.,* 2005; Lubin, 2003) while some even consider low levels of exposure to be carcinogenic, when that exposure is chronic (Darby *et al*., 2001). Considering that, in Furnas Village, our selected location for volcanogenic air pollution assessment, average radon values of 11305,9Bq m^{-3} (Silva *et al*., 2009), [which is many times more than the reference level (200 – 400 Bq m^{-3}) for indoor air in most countries (Amaral & Rodrigues, 2011)], it is not unexpected to have an higher incidence of lung carcinomas in the mice collected at that location. In fact, Amaral & Rodrigues (2006) found an elevated cancer incidence in human population of Furnas village when compared to the one of Sta. Maria Island (an island without active volcanism), especially of lip, oral cavity and pharynx cancer, and also breast cancer, that could be partially explained by the chronic exposure to environmental factors of volcanogenic origin. The non-small cell lung carcinoma-NOS diagnosis, although acceptable (Goldstraw *et al*., 2011), could not be more specific due to the lack of immunohistochesmestry stainings, which could allow a better and more accurate diagnosis (Popper, 2011).

Results showed that chronic exposure to pollutants of volcanogenic or anthropogenic origin is associated to lung injury, since increases the inflammation status, the amount of melano-macrophages centers and alveolar septal thickness resulting in defective lung functionality. Moreover, since some of the volcanogenic pollutants are considered to be carcinogenic, their genotoxic effects may have contributed for the higher incidence rate of lung neoplasia in the mice captured in Furnas village.

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Chapter 4

Final Remarks

4.1 – Main conclusions

The use of the bioindicator species *Mus musculus* provided useful information relatively to the air pollution effects on the lung tissues. Through the use of the morphometric data of percentage of alveolar space, alveolar perimeter, septal thickness and double-blinded rank evaluations of inflammatory status and quantity of melano-macrophages centers, it was possible to assume that exposure to either volcanogenic, or anthropogenic air pollutants can cause lung injury.

The septal thickness was significantly higher in mice exposed to volcanogenic air pollution than in the control group. Moreover, though no significant differences were observed between the anthropogenic polluted site and the control group, the septal thickness of the former was higher in average, suggesting that septal thickness is increased in mice exposed to air polluted sites. Although most authors consider septal thickness increase as a result of neutrophils activation associated with acute exposure to increased CO_2 concentrations, some consider it to be a consequence of the increase in other cell types such as melano-macrophages that phagocyte undetermined particles. Actually, our results showed a significant positive correlation between the amount of melano-macrophages centers and septal thickness, and thus support the melano-macrophages increase hypothesis. However, there was also a significant positive correlation between inflammatory status and septal thickness, leading to believe that both analyzed parameters may have contributed to the general increase of septal thickness in individuals exposed to both sources of air pollution. Moreover, inflammatory status proved also to be strong and negatively correlated to the percentage of alveolar space and alveolar perimeter, and thus effectively contributing to a decrease in the lung functionality.

It is possible that the four cases of non-small cell lung cancer-NOS found in the individuals caught in the volcanogenic air polluted site are somehow associated with the radon exposure values frequently registered in that area, as well as other volcanogenic compounds, as their genotoxic effects may have contributed for the higher incidence rate of lung neoplasia in the mice captured in Furnas village. Previous epidemiologic studies developed by Public Health and Ecotoxicology Research Group (PHERG) also found a higher incidence of cancer in human population living in Furnas village.

Results showed that chronic exposure to pollutants of volcanogenic or anthropogenic origin is associated to lung injury, since increases the inflammatory status, the amount of melano-macrophages centers and alveolar septal thickness, reduces alveolar perimeter and percentage of alveolar space, resulting in defective lung functionality.