



Chronic exposure to non-eruptive volcanic activity as cause of bronchiolar histomorphological alteration and inflammation in mice[☆]

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ABSTRACT

It is estimated that 10% of the worldwide population lives in the vicinity of an active volcano. However, volcanogenic air pollution studies are still outnumbered when compared with anthropogenic air pollution studies, representing an unknown risk to human populations inhabiting volcanic areas worldwide. This study was carried out in the Azorean archipelago of Portugal, in areas with active non-eruptive volcanism. The hydrothermal emissions within the volcanic complex of Furnas (São Miguel Island) are responsible for the emission of nearly 1000 tons of CO₂ per day, along with H₂S, the radio-active gas – radon, among others. Besides the gaseous emissions, metals (e.g., Hg, Cd, Al, Ni) and particulate matter are also released into the environment. We test the hypothesis that chronic exposure to volcanogenic air pollution alters the histomorphology of the bronchioles and terminal bronchioles, using the house mouse, *Mus musculus*, as bioindicator species. *Mus musculus* were live-captured at three different locations: two villages with active volcanism and a village without any type of volcanic activity (reference site). The histomorphology of the bronchioles (diameter, epithelium thickness, smooth muscle layer thickness, submucosa thickness and the histological evaluation of the peribronchiolar inflammation) and of the terminal bronchioles (epithelium thickness and classification) were evaluated. Mice chronically exposed to volcanogenic air pollution presented bronchioles with increased epithelial thickness, increased smooth muscle layer, increased submucosa thickness and increased peribronchiolar inflammation. Similarly, terminal bronchioles presented structural alterations consistent with bronchodysplasia. For the first time we demonstrate that chronic exposure to non-eruptive volcanically active environments causes inflammation and histomorphological alterations in mice lower airways consistent with asthma and chronic bronchitis. These results reveal that chronic exposure to non-eruptive volcanic activity represents a risk factor that can affect the health of the respiratory system of humans inhabiting hydrothermal areas.

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Abbreviations: BALT, bronchus-associated lymphoid tissue.

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1. Introduction

Air pollution is a worldwide phenomenon which is currently associated with the increasing respiratory health problems of human populations. According to WHO (2017), air pollution represents the single largest environmental risk to health globally. The association between exposure to air pollutants and respiratory issues is usually related to anthropogenic sources. Volcanically active areas are also recognized as relevant natural sources of several pollutants that affect both human health and ecosystems (with hazardous gases and heavy metals present in a daily basis (Amaral

and Rodrigues, 2011)), but the natural volcanogenic pollution is less studied than the former, representing an underestimated health risk to the 10% of worldwide human populations that inhabit these areas. This underestimation of the risk becomes aggravated when considering the chronic exposure to hydrothermal emissions since most of the carried studies are for acute exposure to air pollutants from eruptive volcanism (Longo and Yang, 2008; Tam et al., 2016). Several respiratory issues have already been linked to volcanogenic air pollution exposure, such as: acute bronchitis (Longo and Yang, 2008), bronchoconstriction and aggravated respiratory diseases (Hansell and Oppenheimer, 2004), reduced lung function (Tam et al., 2016) and also asthma (Horwell and Baxter, 2006). Hydrothermal emissions are a type of non-eruptive volcanism, which has been previously associated with chronic bronchitis (Amaral and Rodrigues, 2007), and increased prevalence of respiratory restrictions and obstructions (Linhares et al., 2017) in humans inhabiting volcanically active areas. Additionally, Camarinho et al. (2013, 2019), described an increase in lung injury, lung structural remodeling and overproduction of TNF- α in *Mus musculus* captured at Furnas village – S. Miguel Island.

The island of S. Miguel – Azores (Fig. 1) is considered volcanically active with presence of fumaroles and other phenomena that potentiates the emission of gases and other substances that daily affect the quality of the ecosystems and health of human populations (Amaral and Rodrigues, 2007; Rodrigues et al., 2012). This island belongs to the archipelago of Azores and has three major active central volcanoes (Sete Cidades, Fogo and Furnas). The volcanic complex of Furnas belongs to the eastern part of the island, where daily volcanic activity is marked by several hydrothermal manifestations which include active fumarolic fields, thermal and CO₂-rich springs and areas of soil diffuse degassing (Viveiros et al., 2010). These phenomena are responsible for the release of heavy metals, hazardous aerosols and gases such as the radioactive gas radon (²²²Rn), CO₂, hydrogen sulphide (H₂S), among others. Carbon dioxide is considered one of the most important and abundant gases released in this volcanic complex, corresponding to emissions of approximately 1000 t d⁻¹ (Viveiros et al., 2010). Also, CO₂ is one of the main soil gases that is released in a silent way, and is permanently diffused in degassing areas of volcanic and non-volcanic zones (Viveiros et al., 2016). Furthermore, CO₂ is

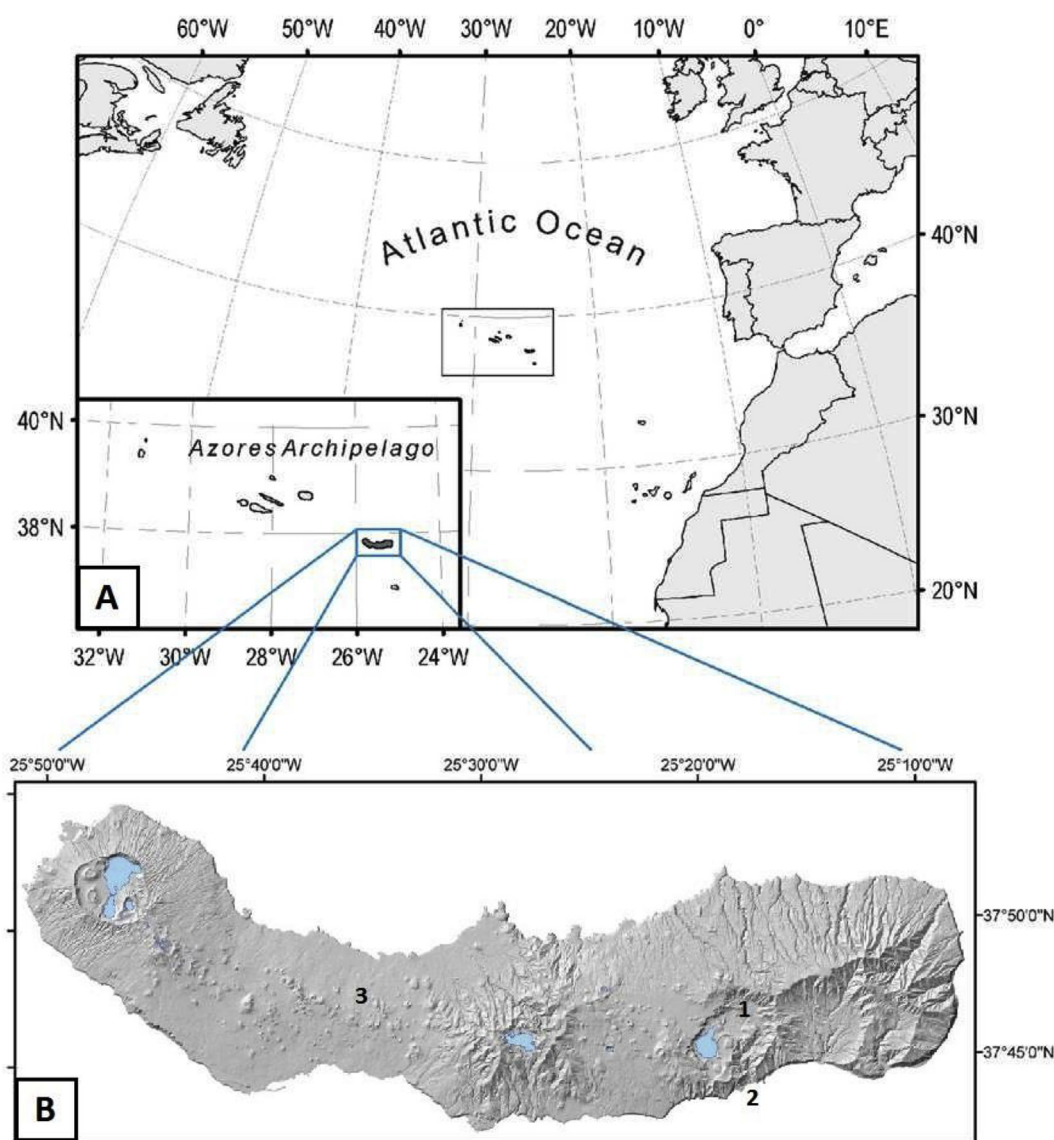


Fig. 1. (A) São Miguel island location in the Azores archipelago. (B) – Sampling areas of 1- Furnas Village, 2- Ribeira Quente Village, 3- Rabo de Peixe Village.

considered an inert asphyxiant gas with important bioregulatory properties and known activator of inflammatory processes (Norozián et al., 2011). The radioactive gas, radon, is also present and elevated values are found in these emissions (Silva et al., 2015). Radon is an inert gas and its exposure, and exposure to its decay products, are known to produce DNA damage (Linhares et al., 2017), DNA mutations (Jostes, 1996), and inflammation, resulting in immune system alterations (Nagarkatti et al., 1996). Similarly, exposure to other volcanic emissions, such as heavy metals and particulate matter, are accountable for immune system alterations (Larsen et al., 2016). Sulphur compounds, also present in the volcanic emissions, are responsible for oxidative stress and changes in tissues of respiratory organs (Meng et al., 2003). Sulphur dioxide is another main released gas that once emitted into the atmosphere affects the air and land environments, causing harmful effects on human health and ecosystems (Granieri et al., 2017). Other sulphur compounds can be responsible for irritant effects on the eyes, mucous membranes and skin, and can also depress pulmonary particle clearance, which is a major protective mechanism of lungs (Kikuchi, 2001; Komarnisky et al., 2003). In fact, gaseous mixtures of CO₂ and sulphur compounds have been frequently reported as the cause of lethal accidents of animals watering in small ponds near volcanoes (Carapezza et al., 2012). Pulmonary toxicity of heavy metals has been previously described in animals, causing inflammation, histopathological changes, oxidative stress and also airway irritation (Larsen et al., 2016). Other lesions and consequences of exposure to air pollutants also include chronic bronchitis (Hogg and Van Eeden, 2009), obstruction of the small conductive airways (Chung and Adcock, 2008), emphysematous destruction (Anciaes et al., 2011), hypertrophy of bronchial epithelium, peribronchial mononuclear cell infiltrate, increased bronchus-associated lymphoid tissue (BALT) and asthma (Guest and Sell, 2015). The bronchial tree is responsible for conducting the inhaled air to the alveoli. Any potential alterations of this conductive part can impair normal lung functions thus representing an aggravated risk to the respiratory mechanisms (Fishwick et al., 1997).

This study tests the hypothesis that chronic exposure to non-eruptive volcanogenic air pollution alters the histomorphology of the bronchioles and terminal bronchioles, and thus represents a health risk to humans inhabiting hydrothermal areas. To test this hypothesis, we used live-caught populations of the house mouse, *Mus musculus*, as surrogate species, in order to perform highly invasive tests that could never be performed in human populations.

2. Material and methods

Three separate groups of *Mus musculus* were live-captured at three different locations: two villages with active volcanism and a village without any type of volcanic activity (reference site). The selection of two volcanically active villages from the same volcanic complex is due to the different geographic setting of the terrain itself. Furnas village is located inside the volcanic crater, and having a low airflow, the levels of volcanogenic pollutants are elevated. Ribeira Quente village is located close to the seashore, on the external flank of the volcano, which provides an increased airflow that may dilute or disperse the air pollutants. Furnas village, with 1500 inhabitants, is built upon actively degassing grounds and other manifestations of active volcanism which include fumarolic fields and hydrothermal vents. Such phenomena are responsible for the natural human exposure to high levels of heavy metals and gases (Baxter et al., 1999; Viveiros et al., 2010). Ribeira Quente, with 800 inhabitants, has manifestations of active volcanism similar to Furnas village. The selected reference site was Rabo de Peixe village. This rural village, with 9000 inhabitants, does not present any type of volcanic manifestations since the 17th century (Carvalho, 1999),

and no apparent source of air pollutants.

According to some studies, field-captured specimens represent a more accurate measurement of pollution exposure and accumulation patterns, since that field conditions and laboratory environments may vary due to the differences in diet, behavior or mixtures of pollutants in the environment (Forsyth, 2001; Tersago et al., 2004). In this study, we used the house mouse, *Mus musculus*, as surrogate species since they share their habitat with humans (including the houses).

Fifty mice were captured at the three locations using live-catch mousetraps. The euthanization was performed insuring a painless death, by using isoflurane and followed by a necropsy with subsequent surgical extraction of the lungs. Right and left lungs were extracted en-bloc and later separated, and fixed in 4% buffered formaldehyde for routine histological processing. In this study, both males and females were captured and used. The sex-ratio ($\approx 50\%$) was similar between sites. The age of each mouse was determined through dry crystalline lens mass according to the methodology of Quéré and Vincent (1989).

The euthanization, necropsy and other procedures were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), directive 2010/63/EU and also the Portuguese law decree (DL 113/2013).

We made two sets of histological slides. Each set was made with one slide per mouse included in this study, and each slide consisted of sections with 4 μm thickness of both lungs.

2.1. Bronchiole histomorphometry and histopathological evaluation

One set of slides was histochemically stained with Picro-Sirius Red (Bancroft and Stevens, 1996) for collagen fibers proper identification and accurate measurements. The bronchiole analysis was performed only in cross sections of similar sizes. For each individual, a maximum of four bronchioles, of both lungs, were analyzed for the following histomorphometric measurements: bronchiolar epithelium thickness, bronchiolar diameter, bronchiolar smooth muscle thickness, and bronchiolar submucosa thickness. The bronchiolar submucosa thickness was measured from the basal lamina to the end of the fibers stained with Picro-Sirius Red. These measures were made with a 20 $\times$ magnification using a software of image analysis (Image Pro-Plus 5.0 by MediaCybernetics®) connected to a Leica® DM1000 microscope (Cambridge, UK). Simultaneously to this measurement, a peribronchiolar inflammatory evaluation, based on the methodology of Zeldin et al. (2001), was performed and scored 1 to 3: (1- no apparent or minimum inflammation, 2- moderate inflammation, 3-severe inflammation).

2.2. Terminal bronchiole histomorphometry and epithelium classification

The other set of histological slides was stained with the standard hematoxylin and eosin (H&E) protocol (Martoja et al., 1970), and the thickness of the terminal bronchiole epithelial cells was measured by conventional bright field microscopy. Measurements were made with a 40 $\times$ magnification using the same software of image analysis (Image Pro-Plus 5.0 by MediaCybernetics®) connected to a Leica® DM1000 microscope (Cambridge, UK). A maximum of four terminal bronchioles, of both lungs, were analyzed and a minimum of 15 epithelial measurements were performed for each individual. Based on the methodology described by Klopffleisch (2013), the epithelium of each terminal bronchiole was classified as: 1- simple epithelium, 2 – pseudostriated epithelium, 3 – bi-stratified epithelium.

2.3. Statistical analysis

All the data from the different *Mus musculus* were pooled by location of capture. Data regarding the classification of the epithelium of terminal bronchioles and peribronchiolar inflammation were compared using the non-parametric test Kruskal-Wallis, followed by Mann-Whitney U tests, to separate the different groups. The remaining analyzed parameters were compared by one-way ANOVA, followed by Tukey HSD tests to separate the different groups. Statistical analyses were made using IBM SPSS Statistics® V.25.0, and the level of statistical significance was set at $P < 0.05$.

3. Results

No statistical difference was found for the mice age (in days) between the studied groups (252.74 ± 22.34 , Furnas; 201.61 ± 10.04 , Ribeira Quente; 191.38 ± 16.00 , Rabo de Peixe; $F = 2902$; $df = 2$; $P = 0.064$; 1-way ANOVA). Similarly, no statistical differences were found in mice weight between the studied groups (13.52 ± 0.71 gr, Furnas; 13.86 ± 0.57 gr, Ribeira Quente; 14.04 ± 0.66 gr; Rabo de Peixe; $F = 0.117$; $df = 2$; $P = 0.890$; 1-way ANOVA).

3.1. Bronchiole histomorphometry and histopathological evaluation

No significant differences were observed for the bronchiolar diameter (151.89 ± 34.87 μm , Furnas; 130.47 ± 41.45 μm , Ribeira Quente; 132.12 ± 41.49 μm , Rabo de Peixe) between the studied sites ($F = 2.271$; $df = 2$; $P = 0.106$).

The epithelial thickness of the bronchioles differed significantly between the studied sites ($F = 116.732$; $df = 2$; $P < 0.01$; Fig. 3 – A; Fig. 2). Individuals from Furnas presented bronchioles with a significantly higher epithelial thickness (21.285 ± 0.844 μm) than those from either Ribeira Quente (16.826 ± 0.254 μm ; $P < 0.01$) or Rabo de Peixe (13.633 ± 0.181 μm ; $P < 0.01$). In addition, the bronchiole epithelial thickness of individuals from Ribeira Quente was significantly higher than that of the individuals from Rabo de Peixe ($P < 0.01$).

Significant differences were observed for the submucosa thickness, between the studied sites ($F = 106.713$; $df = 2$; $P < 0.01$; Fig. 3 – B; Fig. 2). Individuals from Furnas presented a thicker submucosa (54.578 ± 6.189 μm) than those from either Ribeira Quente (18.212 ± 11.111 μm ; $P < 0.01$) or Rabo de Peixe (10.455 ± 5.492 μm ; $P < 0.01$). Also, the submucosa thickness of individuals from Ribeira Quente was significantly higher than that of the individuals from Rabo de Peixe ($P < 0.05$).

Significant differences were observed for the bronchiolar smooth muscle thickness, between the studied sites ($F = 72.715$; $df = 2$; $P < 0.01$; Fig. 3 – C; Fig. 2). Individuals from Furnas presented bronchiolar smooth muscle thickness (16.055 ± 1.541 μm) significantly higher than those from either Ribeira Quente Village (6.886 ± 0.471 μm ; $P < 0.01$) or Rabo de Peixe (4.217 ± 0.367 μm ; $P < 0.01$). In addition, the bronchiolar smooth muscle thickness of individuals from Ribeira Quente was significantly higher when compared to the individuals from Rabo de Peixe ($P < 0.05$).

The peribronchiolar inflammatory evaluation was also significantly different between sites ($P < 0.05$; Fig. 3 – D; Fig. 2). The distribution of peribronchiolar inflammation ranked evaluation was significantly different between Furnas individuals (Mdn = 2; IQ = 1) and individuals from either Ribeira Quente (Mdn = 1; IQ = 0; $P < 0.01$) or Rabo de Peixe (Mdn = 1; IQ = 0; $P < 0.01$); (Mdn = Median; IQ = Interquartile Range). These results represent in the former a moderate inflammation rank, while on the other two groups most of the individuals presented no inflammation.

The epithelial thickness of the bronchioles presented significant positive correlations with the thickness of the bronchiolar submucosa ($r = 0.263$; $P < 0.01$) and with the bronchiolar smooth muscle thickness ($r = 0.290$; $P < 0.01$). Also, the peribronchiolar inflammation presented significant positive correlations with the bronchiolar epithelium thickness ($r_s = 0.398$; $P < 0.01$), with bronchiolar smooth muscle thickness ($r_s = 0.414$; $P < 0.01$) and with the thickness of the bronchiolar submucosa ($r_s = 0.436$; $P < 0.01$).

3.2. Terminal bronchiole histomorphometry and epithelium classification

The epithelial thickness (in μm) of the terminal bronchioles differed significantly between the studied sites ($F = 385.5$; $df = 2$; $P < 0.01$; Fig. 4 – A; B and C). Individuals from Furnas presented terminal bronchioles with a significantly higher epithelial thickness (15.607 ± 0.148 μm) when compared to individuals from either Ribeira Quente (13.72 ± 0.115 μm ; $P < 0.01$) or Rabo de Peixe (11.569 ± 0.064 μm ; $P < 0.01$). In addition, the terminal bronchiole epithelial thickness of individuals from Ribeira Quente was significantly higher than the observed in the individuals from Rabo de Peixe ($P < 0.01$).

The epithelial classification was also significantly different by the site ($P < 0.05$; Fig. 4 – A; B and D). The distribution of epithelial classification was significantly different between Furnas individuals (Mdn = 2; IQ = 1) and the individuals from either Ribeira Quente (Mdn = 1; IQ = 0; $P < 0.01$) or Rabo de Peixe (Mdn = 1; IQ = 0; $P < 0.01$). These results represent in the former a pseudostratified epithelium, while on the other two groups most of the individuals

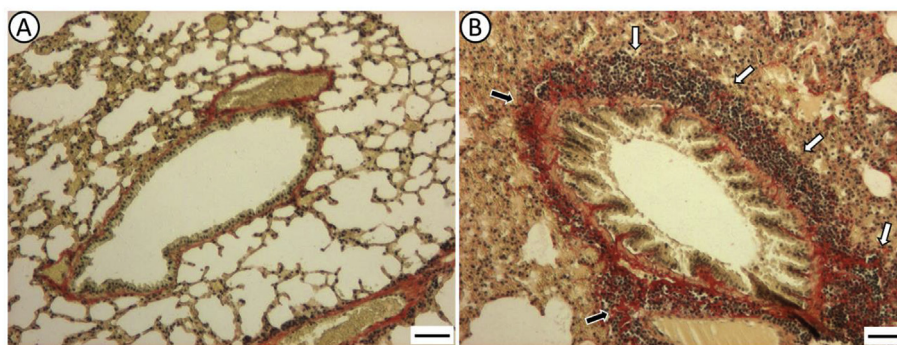


Fig. 2. Bronchioles histochemistry of modified Picro-Sirius Red stain for collagen fibers of mice captured at the reference site of Rabo de Peixe (A) and the volcanogenic air polluted Furnas village (B). Peribronchiolar inflammation represented by white arrows, fibrosis of the submucosa represented by black arrows; scale bar = 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

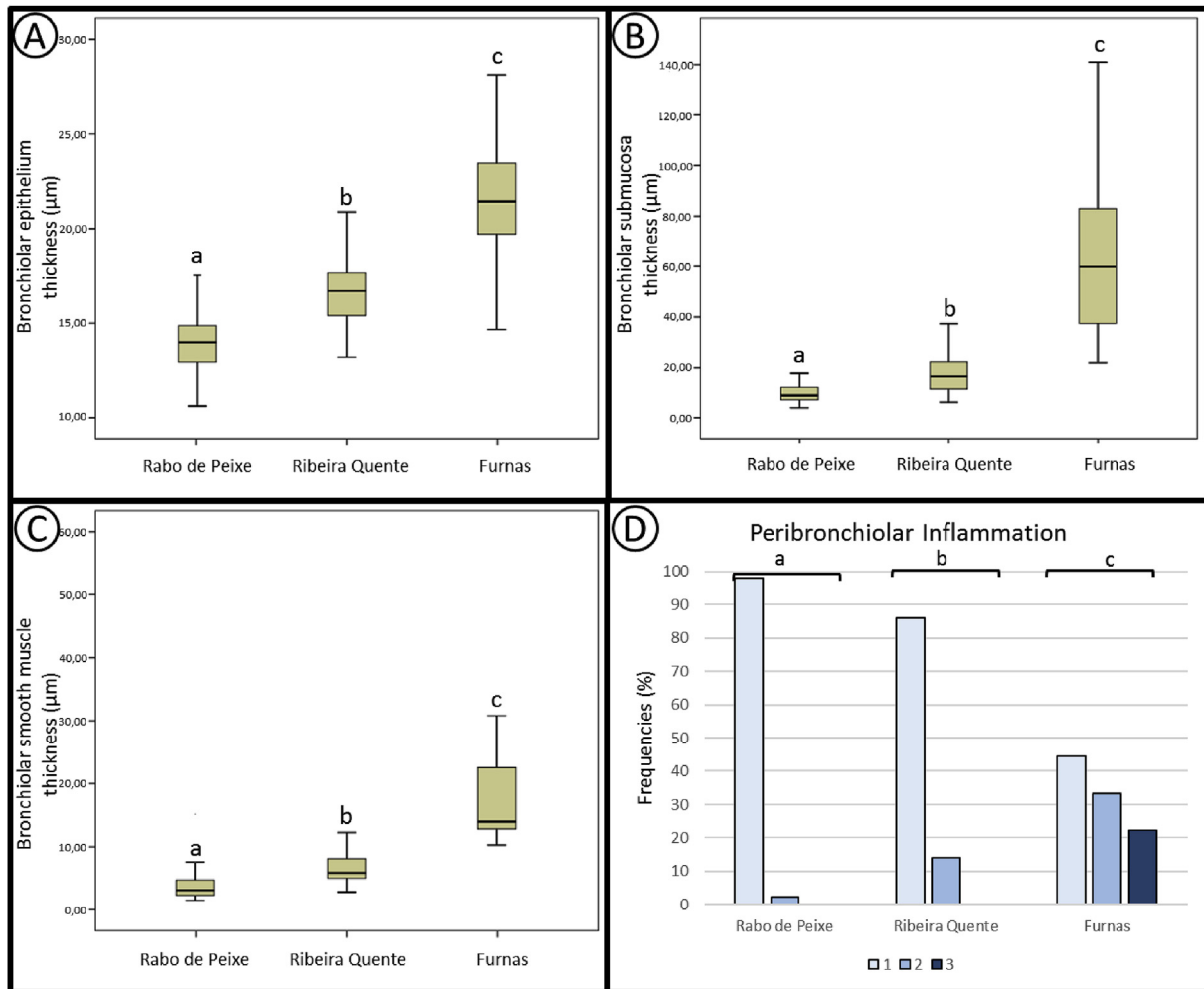


Fig. 3. Boxplot diagrams showing the distribution of bronchioles' epithelium thickness (A), submucosa thickness (B) and smooth muscle layer thickness (C). Locations with different letters are significantly different at $P = 0.05$ (Tukey-HSD tests). Histogram representing the frequency distribution of the peribronchiolar inflammation score (1–3) within each location (D), (1- no apparent or minimum inflammation, 2- moderate inflammation, 3-severe inflammation). Locations with different letters are significantly different at $P = 0.05$ (Man-Whitney test).

presented simple epithelium.

4. Discussion

Several studies in humans and animals have revealed a link between exposure to air pollution, lung anomalies and respiratory dysfunctions (Hiraiwa and Van Eeden, 2013; Saldiva et al., 1992), but the mechanisms accountable for these adverse respiratory effects are not entirely clear (Saunders et al., 2010). However, histological alterations present in asthma and chronic bronchitis (epithelial alterations, increased muscle layer and peribronchiolar inflammation (Jeffery, 1992)) are frequently linked to the development of lung pathologies like COPD (Fishwick et al., 1997) and emphysema (Chung and Adcock, 2008). The links between exposure to air pollution and adverse respiratory effects are frequently investigated for anthropogenic air pollution (Happo et al., 2013; Hu et al., 2015). Volcanogenic air pollution is still far less studied, even though active volcanoes are known to produce and release large amounts of contaminants that are considered harmful to humans (Amaral et al., 2008; Tam et al., 2016; Linhares et al., 2017) and animals (Camarinho et al., 2013). Furthermore, most of these studies concentrate on the alveolar part of the lung, focusing on the functional loss or structure damage, thus failing to consider the

bronchiolar part, its conductivity properties, and associated pathologies.

In this study, we focused on the histomorphological alterations and inflammation of the bronchioles and terminal bronchioles of mice chronically exposed to air pollutants from non-eruptive volcanic activity (hydrothermal activity). To our knowledge, this is the first study to evaluate and associate these biological parameters to this particular type of air pollution. Overall, our results show that the mice captured at the volcanically active sites have the most concerning values in all histomorphometrical data and evaluations.

Our results clearly show an increase in the epithelial thickness of the terminal bronchioles in mice chronically exposed to volcano-genic air pollutants, compared to the non-volcanic reference site. The bronchioles epithelial thickness was also increased, revealing that possibly all the upper airways are being affected by this kind of pollution. In fact, hypertrophy of the bronchiolar epithelium has been previously linked, not only to other several pollutants, namely anthropogenic air pollutants (Hogg and Van Eeden, 2009) and tobacco smoking (Lunghi et al., 2015), but also with other pathologies such as peribronchiolar inflammation and also increased BALF (Guest and Sell, 2015). Regarding the terminal bronchioles, our results also reveal significant differences in the frequency distribution of the classification of the epithelium on the mice captured

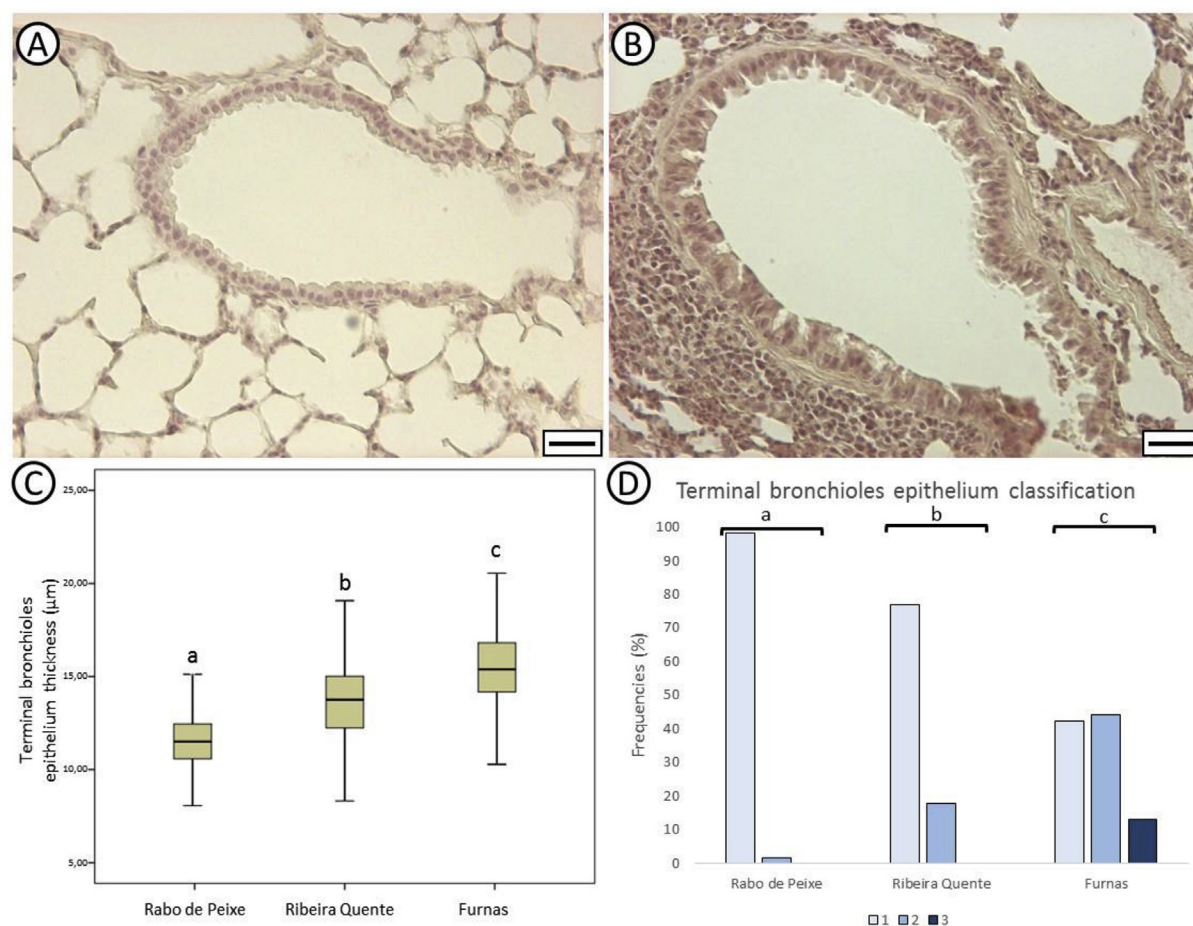


Fig. 4. Terminal bronchiole histology of mice captured at the reference site of Rabo de Peixe (A) and the volcanogenic air polluted Furnas village (B) - Hematoxylin & Eosin; scale bars = 25 μm. Boxplot diagrams showing the terminal bronchioles epithelium thickness (C). Locations with different letters are significantly different at $P = 0.05$ (Tukey- HSD test). Histogram representing the frequency distribution of the terminal bronchioles epithelium classification (1–3) within each location (D). Locations with different letters are significantly different at $P = 0.05$ (Man-Whitney test).

at Furnas village. These mice presented a pseudostratified epithelium, consistent with some studies that diagnose bronchodysplasia, and that it is associated with asthma and bronchitis (Dutau et al., 1994). These results are in agreement with the observed by Amaral and Rodrigues (2007), who showed that humans inhabiting hydrothermal areas have a higher risk of chronic bronchitis. Similarly, a study by Linhares et al. (2017) concluded that humans exposed to this kind of pollution presented an increased prevalence of respiratory defects and exacerbation of COPD.

Regarding the bronchioles, it was also possible to observe a significant increase in the smooth muscle layer, as well as a significant increase in the submucosal thickness. Together with the observed peribronchiolar inflammation, they provide a set of changes that precede the pathological changes of the bronchi, consistent with asthma, which also include the previously observed hypertrophy of the bronchiolar epithelium (Guest and Sell, 2015). In fact, in this study, there is a strong correlation between the peribronchiolar inflammation and the bronchiolar epithelium thickness, smooth muscle layer and submucosal thickness, enlightening the biorelevance of these combined parameters. Asthma is a pathology on which spasms occur in the bronchi, causing difficulty in breathing. It usually is the result of an allergic reaction or other forms of hypersensitivity leading to a reversible airway obstruction, airway hyperactivity and inflammation (Guest and Sell, 2015). Asthma has been associated with air pollution

exposure (Saunders et al., 2010) and tobacco smoking in humans (Dutau et al., 1994), among other factors. In all these studies, the bronchiolar lesions consistent with asthma are preceded by complex immune interactions and mechanisms which are not yet entirely clear (Guest and Sell, 2015). In fact, the repeated activation of these mechanisms often results in pathological features of chronic asthma: hyperplasia of smooth muscle, chronic peribronchiolar inflammation and thickening of the bronchial mucosa (Sell, 2001). Many of these biologically relevant alterations are present in the mice captured at the volcanically active villages of Furnas and Ribeira Quente, clearly revealing the hazardous potential of this specific type of pollution. However, the mice captured at Furnas village presented the most concerning values. This fact can be due to the geographical properties of the terrain. While Furnas village was built inside the volcanic crater, thus having low airflow and allowing the concentration of pollutants, Ribeira Quente is located on the external flank of the volcanic crater, near the sea, thus allowing increased air renewal that may help to dissipate the pollutants.

Asthma is considered a chronic obstructive lung disease alongside with chronic bronchitis (Mukhopadhyay et al., 2006). The main difference is that in chronic bronchitis the airway obstruction is not reversible, causing perpetual shortness of breath (Fishwick et al., 1997). Also, chronic bronchitis is commonly associated with pulmonary emphysema and considered a form of COPD (Chung and

Adcock, 2008). In fact, Camarinho et al. (2019) revealed an increase of pulmonary emphysema in mice chronically exposed to volcanogenic air pollution. Furthermore, Amaral and Rodrigues (2007) referred an increased incidence of human bronchitis in Furnas inhabitants and, more recently, Linhares et al. (2017) found an increased incidence of COPD in Furnas' inhabitants, further enhancing this study concerns regarding the bronchiolar alterations due to volcanogenic air pollution exposure in hydrothermal areas.

5. Conclusion

This study shows the hazardous potential of volcanogenic air pollution in hydrothermal areas to the bronchioles and terminal bronchioles of mice. We demonstrate for the first time that chronic exposure to non-eruptive active volcanism significantly induces structural alterations in the lower airways that are comparable with respiratory pathologies like asthma and chronic bronchitis. These results reveal that the inhabitants of volcanic hydrothermal areas should be regularly monitored for such pathologies in order to develop preventive measures of exposure and enhance public health policies.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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