Michele Galhardoni Padovan

Carbonaceous particulate matter in the alveolar macrophage and lung surface tissue compartments of residents from São Paulo, Brazil

Thesis presented to the

Faculty of Medicine, University of São Paulo

Department of Pathology

Advisor: Raymundo Soares de Azevedo Neto

São Paulo

2017

Michele Galhardoni Padovan

Material particulado de carbono nos compartimentos de tecidos de macrófagos alveolares e de superfície pulmonar de residentes de São Paulo, Brasil

Tese apresentada à Faculdade de Medicina

da Universidade de São Paulo para

obtenção do título de Doutor em Ciências

Programa de Patologia

Orientador: Prof. Dr.Raymundo Soares de Azevedo Neto

São Paulo

2017

Dados Internacionais de Catalogação na Publicação (CIP)

Preparada pela Biblioteca da
Faculdade de Medicina da Universidade de São Paulo

Padovan, Michele Galhardoni
Material particulado de carbono nos compartimentos de tecido de macrófagos alveolares e de superfície pulmonar de residentes de São Paulo, Brasil / Michele Galhardoni Padovan. -- São Paulo, 2017.
Tese(doutorado)--Faculdade de Medicina da Universidade de São Paulo. Programa de Patologia.
Orientador: Raymundo Soares de Azevedo Neto.

Descritores: 1.Poluição do ar 2. Macrófagos alveolares 3. Poluição por fumaça de tabaco 4.Material particulado 5.Fuligem 6.Antracose

USP/FM/DBD-036/17

Acknowledgments

Thank you,

To the **Department of Pathology**, Professor Luiz Fernando Ferraz da Silva, Professor Thais Mauad, Professor Paulo Hilario do Nascimento Saldiva and all the research team, for valuable contribution and availability.

A special thank you to my supervisor, Prof. Dr.**Raymundo Soares de Azevedo Neto** who adopted me after the project has started, for the confidence, encouragement, patience and support.

To the members of my qualification exam - Prof. Dr. Fabiola Bernardi, Prof Dr. Lucia Garcia and Prof. Dr Fatima Mitiko Tengan.

Big thank you to my dearest friends from FMUSP for all encouragement, for sharing moments of daily life and for the restless laughter: Cristiane Centrone, Rodrigo Pessoa, Clara Felix, Renata Moscolini, and Sabri.

To the amazing people from The Department of Pathology and Experimental Air Pollution Laboratory, Prof. Regiani Carvalho de Oliveira, Profa. Dolores Helena Rodriguez Ferreira, Prof .Dr. **Alfésio Luís Ferreira Braga**, Mariana Veras e Luis Fernando Amato.

To Prof. Dr. Carlos Augusto Gonçalves Pasqualucci, to give us permission to work along with São Paulo Autopsy Centre - **SVOC**, and all the amazing staff out there,

technicians and pathologists for the help with collecting samples, official reports and all the hard working routine, which they do under admirable ethics and respect .

To Prof. Dr. **Nelson Gouveia** and **Mateus Habermann** for the huge contribution on the traffic methods, giving their valuable time geocoding all my population data.

To the staff of the Museum at the Faculty of Medicine for all support. Ana, Carlos and Reginaldo. Special thank you to the member of the secretary of the Department of Pathology, Thiago Rezende for all help and intermediation whenever I needed. To the people who, directly or indirectly, contributed to the development of this work, and with my scientific and intellectual development, my sincere gratitude.

A huge thanks for Professor **Jonathan Grigg**, to receive me in his laboratory at the Blizard Institute, Queen Mary University of London, where I spent a year learning his techniques. It was an honour. Thank you to all his amazing team who became friends, Kate Harris, Gioia Mosler, Reetika Suri, Lisa Miyashita, Abigail Whitehouse, Norrice Liu and Lizette Ahenda. All members of staff always supportive. Shirley, Jaya and Julia. And friends I made there, Shirleny Cardoso, Hemanth and Kaveh.

To CAPES for the scholarship awarded. To my dearest friends Danielle Coelho, Rafaela Marins, Thaisa Imamura, Rafinha Silva, who, from near or far, are extremely important to my life and always give me all the support I need!

To a very special person, **Adam Waite**, who is completing a year since he came into my life, and I do not want to let him go anymore! Thank you for the restless support and

patience, hours shared by my side working, training, or simply being silent with me – I love you.

To my family, all of them! Especially my dear cousins Gilberto, Benazir, Thiago and Viviane, for friendship and companionship. To my uncle Gilberto to always be there.

A special thanks to my mother **Vera**, who I admire so much and is the basis of my life, for all the support, affection and unconditional love. I have being learning so much from you my whole life, and words are not enough to thank you. To my father **Luiz**, and my step brothers, that even living far are in my heart.

And finally, I want to thank **God** – for life, and to make all the impossible things possible to me.

“If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward.”

Martin Luther King Jr.

This thesis has been done according to the following standards at the time of this publication:

References: Adapted from International Committee of Medical Journals Editors (Vancouver).

University of São Paulo. Faculty of Medicine. Division of Library and Documentation. Guide presentation of dissertations, theses and monographs. Prepared by Anneliese Carneiro da Cunha, Maria Julia L. A. Freddi, Maria F. Crestana Marinalva de Souza Aragon, Suely Campos Cardoso, Valeria Vilhena. 3rd ed. São Paulo: Library and Documentation Division; 2011.

Abbreviations of journal titles according to List of Journals Indexed in Index Medicus.

Table of Contents

List of Figures

List of Tables

List of Abbreviations, Acronyms and Symbols

AM: alveolar macrophage

SVOC: São Paulo Autopsy Centre

IQR: interquartile range

μm^2: square micrometer

μm^3: cubic micrometer

vs: versus

$\mu m/m^3$: micrometer per cubic meter

P= p value

MA: macrófagos alveolares

Km^2: square kilometres

WHO: World Health Organization

PM: particulate matter

e.g.: exempli gratia

nm: nanometer

CS: cigarette smoke

CSE: cigarette smoke exposure

UK: United Kingdom

%: percentage

CETESB: São Paulo Environmental Agency

μm: micrometer

LSurC: Lung surface carbon

AMC: Alveolar macrophage carbon

FMUSP: Faculty of Medicine of University of São Paulo

HC-FMUSP: Hospital das Clínicas, Faculty of Medicine of University of São Paulo

CAP Pesq: Research Ethics Committee of the Faculty of Medicine, University of São Paulo

cm^2: square centimetre

H&E: haematoxylin and eosin

LM: lung macrophage

h: hours

m: meters

DWTD: distance-weighted traffic density

CET: São Paulo Municipal Traffic Engineering Company

TDi: traffic density

i: each region (local streets)

∑: sum

SLR: segments of local roads

T: traffic for each local street segment

≥: greater than or equal to

AMDL: annual mean daily traffic

VPH: vehicles per hour

L: length of the street segment

Ai: area of the census tract

D: distance

Y: weighting factor

Π: Pi value

exp: exponential function

<: less than

Rs: Spearman rank test

NS: non-significant

EPA: Environmental Protection Agency

PMF: progressive massive fibrosis

>: greater than

Summary

Carbonaceous Particulate Matter in the Alveolar Macrophage and Lung Surface Tissue Compartments of Residents from Sao Paulo, Brazil

Rationale

Smokers inhale large amounts of carbonaceous particulate matter, which may contribute to pulmonary and systemic adverse effects. It is clear that alveolar macrophages (AM) play a critically important role in the recognition and processing of any inhaled foreign material and are the predominant cells that process and remove inhaled particulate matter from the lung. There is also long-term surface deposition of carbon seen in the lungs of smokers at post-mortem. At present the distribution and retention of cigarette smoke-derived particulate matter when the person is also exposed to high levels of background air pollution is unclear. Therefore we sought to assess both AM carbon loading and lung surface deposition in a population exposed to high background air pollution (São Paulo) in both smokers and non-smokers.

Methods

A cohort of 72 *post-mortem* subjects was obtained from São Paulo Autopsy Centre (SVOC). Images of lung surfaces were obtained under standard conditions and small fragments of lung tissue were collected for macrophage analysis using smear technique. The total surface black carbon was analysed using Image J (National Institute of Health,

MD, USA), blinded to smoking status. Internal AM carbon uptake was measured using Image Pro Plus (The Proven Solution, Media Cybernetics Inc., USA). Ethical approval was obtained. Mean macrophage black carbon in both smokers and non-smokers was analysed using Mann Whitney and expressed as median (IQR).

Results

Smokers have a significantly higher level of mean macrophage black carbon (103.4 (IQR 29.44 to 226.3) vs. 9.27 (IQR 3.1 to 85.13) μm^2, P<0.001)103.4μm^2. There was no significant difference between the mean area of surface deposition of carbon in the lungs of smokers and non-smokers 6.74 cm^2 (IQR 3.47 to 10.02) versus 5.20cm^2 (IQR 2.29 to 7.54) P=NS.

Conclusion

AM carbon content is clearly much higher in the smokers than the non-smokers. However the lung surface analysis showed no significant difference. This could indicate that in an area of high air pollution the main contributing factor to long term lung carbon deposition is pollution exposure with limited effects from cigarette smoke exposure. AM black carbon still appears significantly influenced by cigarette smoke exposure.

Resumo

Material particulado de carbono nos compartimentos de tecidos de macrófagos alveolares e de superfície pulmonar de residentes de São Paulo, Brasil

Introdução

Os fumantes inalam grandes quantidades de partículas de carbono, o que pode contribuir para efeitos adversos pulmonares e sistêmicos. É sabido que os macrófagos alveolares (MA) desempenham um papel extremamente importante no reconhecimento e processamento de qualquer material estranho inalado e são as células predominantes que processam e removem partículas inaladas. Existe também a deposição superficial a longo prazo do carbono observado nos pulmões de fumantes em autópsias. Atualmente, a distribuição e retenção de partículas de fumo derivadas de cigarros quando a pessoa também está exposta a níveis elevados de poluição do ar ainda não é clara. Portanto, procurou-se avaliar a carga de carbono nos MA e a deposição de superfície pulmonar em uma população exposta a alta poluição atmosférica (São Paulo), tanto em fumantes como não-fumantes.

Métodos

Uma coorte de 72 sujeitos *post mortem* foi obtida do Serviço de Verificação de Óbitos da Capital da Universidade de São Paulo (SVOC). As imagens das superfícies pulmonares foram obtidas sob condições padrão e pequenos fragmentos de tecido

pulmonar foram coletados para análise de macrófagos usando a técnica de esfregaço. A superfície total de negro de carbono foi analisada utilizando o programa Imagem J (National Institute of Health, MD, EUA), teste cego ao fumo. A absorção interna de carbono nos MA foi medida utilizando o programa Image Pro Plus (The Proven Solution, Media Cybernetics Inc., EUA). A aprovação ética foi obtida. A média de negro de carbono de macrófagos tanto em fumantes como em não-fumantes foi analisada utilizando teste de Mann Whitney e expressa como intervalo interquartil (IQR).

Resultados

Os fumantes têm um nível significativamente mais elevado de negro de carbono nos macrófagos alveolares (103.4 (IQR 29.44 to 226.3) vs. 9.27 (IQR 3.1 to 85.13) μm^2, P<0.001)103.4μm^2. Não houve diferença significativa entre a área média de deposição superficial de carbono nos pulmões de fumantes e não fumantes de 6, 74 cm2 (IQR 3, 47 a 10, 02) versus 5, 20 cm2 (IQR 2, 29 a 7, 54) P=NS.

Conclusão

O teor de carbono nos MA é claramente muito maior nos fumantes do que os não-fumantes. No entanto, a análise da superfície pulmonar não mostrou diferença significativa. Isso pode indicar que, em uma área de alta poluição do ar, o principal fator que contribui para a deposição de carbono no pulmão a longo prazo é a exposição à poluição com efeitos limitados da exposição à fumaça de cigarro. O preto de carbono

nos MA ainda aparece significativamente influenciado pela exposição à fumaça de cigarro.

1. Introduction

1.1 Background

In 2012 the Global Burden of Disease study stated that outdoor air pollution was the ninth leading cause and indoor air the fourth leading cause of morbidity and mortality worldwide; estimating that over 3.5million deaths worldwide each year can be attributed to indoor and outdoor pollution (Lim et al, 2012). Air pollution is associated with a myriad of health problems including respiratory diseases such as emphysema, bronchitis and asthma, impaired lung development in children, premature births and low birth weight, lung cancer and heart disease.

São Paulo has a population that exceeds 11.5 million people living in an area of 1,509 km^2. This information about the large population of São Paulo points to a significant potential to explore the role of several urban conditions in the conduction of effective studies on health effects and urbanity. The use of autopsies to explore urban health is innovative since it stems from a multivariate population from a big city like São Paulo. More than 80% of people living in urban areas that monitor air pollution are exposed to air quality levels that exceed the World Health Organization (WHO) limits, and São Paulo is one of the top highest polluted cities in world (WHO, 2016).

Smokers inhale large amounts of carbonaceous particulate matter, which may contribute to pulmonary and systemic adverse effects (Pinkerton et al., 2000). It is clear that alveolar macrophages play a critically important role in the recognition and processing of any inhaled foreign material and are the predominant cells that process and remove inhaled particulate matter from the lung (Hiraiwa and van Eeden, 2013). Most particles

inhaled are readily removed by mucociliary clearance aided by macrophage phagocytosis.

To date, little is known about the amount of PM retained in lung tissue and exposure to fossil fuel- and CS-derived PM. This is, in part, because measuring the amount of carbonaceous PM in lung tissue is very difficult to achieve in vivo. By contrast, assessment of the amount of carbon in airway macrophages (AM) is straightforward since AM may be sampled non-invasively by sputum induction. Analysis of AM obtained by induced sputum is a practical way of quantifying natural exposure of the lower airway to carbonaceous particles from the burning of biomass fuel. Previously, researches have reported a weak association between modelled exposure to fossil-fuel derived PM at the home address and airway macrophage carbon loading in children (Kulkarni et al., 2005), and recently Belli et al (Belli et al., 2016) reported that both smoking and exposure to environmental $PM_{2.5}$ is associated with accumulation of carbonaceous PM in airway macrophages.

Tobacco smoking is linked to a long and growing (Barnes, 2014; Carter et al., 2015) list of fatal illnesses (e.g., emphysema, cancer, and stroke) and is the major preventable cause of human death. Cigarette smoking for many years caused damages in the lungs and leads to emphysema. There are thousands of chemicals in cigarette smoke and many of them have been linked to the development of lung cancer, although it has been difficult to point those that are responsible for smoking-related emphysema. Moreover, cigarette smoke also contains large numbers of small particles and relatively little is known about the role played by these particles in smoking-related disease (You et al, 2015).

Recently, there has been renewed interest in study the causes of lung blackening and particles deposition in lung surface. Previously, little was known about the composition of the substance that causes this blackening, or its significance in the development of emphysema. Now, by studying lung tissue taken from smokers with emphysema, You et al. have shown that this black substance is made of nano-sized particles of a material called carbon black (which is also known as elemental carbon). These nanoparticles are produced by the incomplete combustion of the cigarettes, but the effect of carbon black in smokers and non-smokers is less known.

1.2 Rationale

Retention of inhaled carbonaceous particulate matter (PM) in the lung is associated with a wide range of adverse health effects. The association between accumulation of carbonaceous PM in the lung and chronic lung injury was first described over 100 years ago in miners (Arnold C A, 2015). By contrast, evidence for the long-term adverse effects of environmental carbonaceous PM, mainly from fossil-fuel combustion in urban areas, has emerged recently. For example, a 2016 report by the Royal College of Physicians (UK), concluded that long-term exposure to carbonaceous fossil-fuel derived PM less than 10 micrometres in aerodynamic diameter (PM_{10}) is associated with a wide range of long-term effects including reduced lung function growth in children, accelerated lung function decline in adults, lung cancer, and new onset asthma (Every Breath We Take). In adults, an additional source of carbonaceous PM exposure is cigarette smoke (CS) (Geber et al., 2015).

Assessment of the amount of carbon in airway macrophages (AM) is straightforward since AM may be sampled non-invasively by sputum induction. Using induced sputum

in children, we found a weak association between modelled exposures to fossil-fuel derived PM at the home address and AM carbon loading (Kulkarni et al., 2005), and more recently, Belli et al (Belli et al., 2016) reported that both smoking and exposure to environmental $PM_{2.5}$ is associated with accumulation of carbonaceous PM in AM. By contrast, little is known about the amount of PM retained in lung tissue after exposure to fossil fuel- and CS-derived PM, in part because measuring carbonaceous PM in lung tissue is difficult to do in vivo. Although one of the hallmarks of long-term smoking is considered to be blackening of the lung tissue surface (Tour et al., 2015), no studies to date have compared surface carbonaceous PM loading in smokers and non-smoking adults. However, recently, You et al (2015) performed high resolution transmission electron microscopy of the residual black material after complete proteolytic digestion of human emphysematous lung from smokers and found 20–50nm spheroids aggregates compatible with carbon black, and found a signature for nanoparticulate carbon black in dendritic cells from the same lungs. You et al (2015) also found that exposure of mice to CS, increased black staining both at the lung surface and within dendritic cells. However, this study did not compare lungs from non-smokers, and whether smoking is the major determinant of lung tissue carbon in adults living in areas of high environmental PM therefore remains unclear.

Existing studies have only looked at the association between environmental exposure on the same day or during the previous few days before death and the severity of illness. To avoid variables and confounding bias in the air pollution data, residential addresses were successfully geocoded to better demonstrate the estimation of exposure across the city. Additionally, exposure to traffic related air pollution was based on annual means of traffic counts that might not reflect seasonal, monthly or either temporal variations in

vehicle traffic data. This premise confirm the effectiveness of this study accessing PM exposure with accurate spatial variability, once air pollution levels can vary considerably across different areas of the city and how close subjects are from busy roads. Traffic density was weighted for the distance from the busy road to the home address using Gaussian distribution to approximate the decay of PM emissions into the surrounding streets previously described by Pearson (Pearson et al., 2000).

To date, little is known about the amount of PM retained in lung tissue and exposure to fossil fuel- and CS-derived PM. This is, in part, because measuring the amount of carbonaceous PM in lung tissue is very difficult to achieve in vivo. By contrast, carbon PM loading in the airway macrophages is relatively straightforward using non-invasive sampling by sputum induction. Indeed in children, we have reported a weak association between modelled exposure to fossil-fuel derived PM at the home address and airway macrophage carbon loading (Kulkarni et al., 2005), and recently Belli et al (Belli et al., 2016) reported that both smoking and exposure to environmental $PM_{2.5}$ is associated with accumulation of carbonaceous PM in airway macrophages.

1.3 Exposure Assessment

1.3.1 Particulate Matter

Ambient particulate matter (PM) is formed by a mixture of solid and liquid suspended particles, with three portions based on their aerodynamic diameter: coarse, fine and ultrafine (Lippmann et al., 1980). Short and long-term exposure to particulate matter causes respiratory and cardiovascular disease, atherosclerosis, adverse birth outcomes, impacts on children's development of the brain and nervous system, diabetes, and can result in death. PM is also linked to respiratory infections and asthma in young children.

Retention of inhaled carbonaceous particulate matter (PM) in the lung is associated with a wide range of adverse health effects. While the association between the inhalation of non-combustion derived carbon PM by miners and chronic lung injury was first described over 100 years (Arnold et al, 2015), evidence for the long-term adverse effects of environmental carbonaceous PM (mainly from fossil-fuel combustion in urban areas) has emerged more recently. For example, a 2016 report by the Royal College of Physicians (UK) in reviewing the recent evidence, concluded that long-term exposure to carbonaceous fossil-fuel derived PM less than 10 micrometres in aerodynamic diameter (PM_{10}) is associated with a wide range of effects including reduced lung function growth in children, accelerated lung function decline in adults, lung cancer, and new onset asthma according to the report from Royal College of Physicians, Every Breath We Take (2016). In adults, fossil-fuel combustion is not the only source of carbonaceous PM since cigarette smoke (CS) contains high concentrations of carbonaceous PM (Gerber et al., 2015).

1.3.2 Health Effects Associated with Particles

Air pollution was positively associated with mortality due to lung cancer and cardiopulmonary disease but not with mortality from all other causes (Dockery et al., 1993). Human exposure to PM has been linked to a number of different adverse health effects, increased numbers of emergency room visits, hospital admissions, and increased mortality where a large scale of cases in the literature has been demonstrating the association between carbonaceous particles in the air and daily mortality and morbidity. Previous studies reported that the level of PM_{10} is associated with the rate of death from all causes and from cardiovascular and respiratory illnesses. Associations of a range of different respiratory health effects have been found, such as reduction in lung function. Additionally there is evidence for severe effects on the cardiovascular system, such as increases in myocardial infarction (World Health Organisation Europe 2004)

Short-term Effects	Long-term Effects
• Lung inflammatory reactions	• Increase in lower respiratory symptoms
• Respiratory symptoms (including Asthma episodes2)	• Reduction in lung function in children
• Adverse effects on the cardiovascular system	• Increase in chronic obstructive pulmonary disease
• Increase in medication usage	• Reduction in lung function in adults
• Increase in hospital admissions	• Reduction in life expectancy, owing mainly to cardiopulmonary mortality and lung cancer
• Increase in mortality	• Increase in lung cancer1

Table 1: Effects of increased particle exposure. Main source: World Health Organisation Europe (2004), 1 (International Agency for Cancer Research 2013), 2 Delfino et al. (1998; 2002)

Health effects associated with PM have been studied on the last years trying to predict short and long-term PM exposure. One main objective of this study is to determine a model which can predict long-term individual exposure. Long-term health effects are therefore of particular interest to this study (see summary in Table 1 above). A number of studies have shown an association between daily changes in air pollution levels and adverse health effects to the cardiovascular and respiratory system (Peters et al. 2001; Maynard et al. 2007; Törnqvist et al. 2007; Wellenius et al. 2012; Pope III & Dockery 2006).

In a specific study leaded in Massachusetts researches found an estimated increase in the relative rate of death from all causes in 0.51% for each increase in the PM_{10} level of 10ug per cubic meter and the estimated increase in the relative rate of death from cardiovascular and respiratory causes of 0.68% for each increase in the PM_{10} level of 10ug per cubic meter (Samet et al 2000). Another study by Schwartz, Dockery, & Neas (1996) calculated from data collected for the Harvard six cities study showed that an increase of 10μg/m3 in $PM_{2.5}$ was associated with a 1.5% increase in total daily mortality whilst a study conducted by the World Health Organisation Europe (2013) reviewed dose-response relationships for PM_{10} from the literature and derived a relative risk for mortality of 1.026 for each 10μg/m3 increase for an adult population.

The evidence that air pollution may promote permanent obstructive defects received support from population-based studies (Abbey et al., 1998), histologic studies in humans (Souza et al., 1998), and panel studies in children (Kulkarni et al., 2006). Moreover, evidence of particle trapping into the lungs was observed in individuals living in areas with high pollution concentrations (Saieg et al., 2011).

Other important aspect is analysing different lung compartments in terms of carbonaceous deposition and how cigarette smoking influence the carbon intake in lung tissues. Smoking has been described of one of the major sources of confounding. Studies in human and animal models describes the acute effects of cigarette smoking as a potential marker of inflammation and oxidative stress and increased epithelial permeability in chronic smokers than non-smokers (van der Vaart et al, 2004).

Many epidemiological studies have been conducted in order to understand the relationships between particulate matter and health effects (Brunekreef & Holgate 2002; World Health Organisation Europe 2004; Pope III & Dockery 2006). Traditionally those health assessment studies focus either on short-term (e.g. hours, days or weeks) or long-term (e.g. years) exposure. Short-term associations between pollution levels and acute health outcomes are traditionally assessed with time-series studies (Bell et al. 2004).

The major cause of death of the subjects on this present study was cardiovascular disease related. The Harvard six cities study provided crucial evidence in establishing the relationship between increased particle exposure and mortality (Dockery et al. 1993) by comparing PM_{10} concentrations measured at central sites in six cities in the USA with daily mortality. Exposure to air pollution is associated with increased cardiovascular morbidity and deaths from myocardial ischemia, arrhythmia, and heart failure (Mills et al, 2008). Air pollution is strongly linked with heart (cardiovascular) disease and increases the risk of mortality. Several studies indicate that particulate matter can make existing heart conditions worse and can cause cardiovascular events, including heart attacks and strokes, among vulnerable people.

The relationship of long-term health in a given population or population group to air pollution is usually assessed by looking at geographical differences in cohort studies (World Health Organisation Europe 2004). Cohort studies in this context refer to studies which compare differences in pollution concentrations at an individual's homes to differences in health between individuals of a population cohort. The World Health Organization estimates that a quarter of the world's population is exposed to unhealthy concentrations of air pollutants. The American Heart Association recently issued a scientific statement highlighting the increased cardiovascular risk associated with exposure to air pollution and emphasized the importance of establishing a mechanistic link to explain these epidemiological observations. Changes in the mortality rates associated with short-term changes in PM have been observed in studies across the U.S, Europe and other parts of the world. The elevated risk of death associated with air pollution is primarily caused by elevated respiratory and cardiovascular mortality (EPA 1996; Pope et al., 1999).

These studies have proven to be useful tools to assess the health risk of a population. But being more critical, most time-series studies neglect geographical variation and cohort studies neglect variation over time. In addition, temporal variability of some people's exposure during their daily routine may be poorly reflected in the short-term variability at a nearby monitoring site. More refined models for personal exposure have therefore been developed.

1.3.3 Road and Traffic Emissions

Air pollution is a serious problem in the world's major cities owing to the combustion of fossil fuels such as diesel oil. In particular, there has been recent interest in the

consistent association between increased levels of air pollution and cardiovascular morbidity and mortality.

Particularly in the city of Sao Paulo, the automotive fleet shows up as the most significant contributor to emissions of pollutants into the atmosphere. There are also fixed sources of pollution, represented by two thousand large industries. São Paulo Environmental Agency (CETESB) maintains an ongoing effort to manage and expand its monitoring network and to better characterize the emissions inventory of pollutants.

In the literature, studies suggested that urban dwellers have particle deposition and retention in the lungs, which are associated with areas of bronchiolar fibrosis and mucus hyperplasia (Souza et al., 1998; Churg et al., 2003). The rapid urbanization associated with the industrial revolution increased traffic exposure and as a result an increase in tobacco smoking (which will be more detailed on the section below), the widespread use of internal combustion engines, and the introduction of new industrial sources of air pollution. Several important epidemiologic studies have associated urban air pollution with adverse health effects (de Kok et al., 2006), specifically respiratory morbidity and mortality (Schuwartz J., 1995; Dockery et al., 1993).

1.3.4 Cigarette Smoking Exposure

In contrast to the white or pink appearance of normal lungs, the lungs of heavy smokers are typically dark brown or black (Churg et al., 2005).

In the past few years, little was known about the composition of the substance that causes this blackening, or its significance in the development of emphysema. Now, by studying lung tissue taken from smokers with emphysema, You et al. have shown that this black substance is made of nano-sized particles of a material called carbon black

(which is also known as elemental carbon), these nanoparticles are produced by the incomplete combustion of the cigarettes. Researchers have previously demonstrated vascular dysfunction in cigarette smokers.

Another thing to consider is that the particle deposition in the lungs depends on its size. The size of particles in the smoke inhaled directly from a cigarette has been studied in a variety of systems. Some studies shown that the mass median aerodynamic diameter of particles is 0.3 to 0.4 μm (Martonen 1992; Bernstein 2004). Particles of this size penetrate to and are deposited in the deep lung. Large particles (e.g., pollen and road dust) are removed in the upper airway, largely by impaction (USDHHS 1984). Small particles, with a mean aerodynamic diameter less than about 2.5 μm, reach the lungs, where they deposit in airways and alveoli by impaction, sedimentation, or diffusion. About 60 percent of the particles inhaled in cigarette smoke are deposited. Although these particles are subject to handling by the mucociliary apparatus and alveolar macrophages, removal is not complete because of their very high numbers in the lungs of long-term smokers, which show evidence of a substantial burden of retained particles (Cohen et al. 1979; USDHHS 1984).

1.3.5 Health Effects Associated With CSE

One of the indicators of long-term smoking is the blackening of the lung tissue that persists even if the person stops smoking. Tobacco smoking is linked to a long and growing (Barnes, 2014; Carter et al., 2015) list of fatal illnesses (e.g., emphysema, cancer, and stroke) chronic inflammatory diseases (e.g. chronic bronchitis, and chronic obstructive pulmonary disease (COPD)) (USDHHS 1984) and is the major preventable cause of human death.

Despite public awareness of the harmful effects of smoking, in many large developing countries the prevalence of smoking is growing (Eriksen et al., 2014). You et al. also confirmed that the nanoparticles of carbon black can cause emphysema in mice, and because the nanoparticles cannot be cleared, they are released into the lung when cells die, which perpetuates lung inflammation and damage (You et al, 2015).

Tobacco smoke contains many noxious chemicals (e.g., carbon monoxide, sulfur, nitrogen dioxide, nitric oxide, and methane), aromatics (e.g., benzene, toluene, and xylene) and chlorinated (e.g., methyl chloride, chloroethene, and chloroform) volatile organic compounds, as well as particulate matter (Wang et al., 2012; Perfetti and Rodgman, 2013; Salvi, 2014). One or more of these agents is thought to underlie the carcinogenic potential of smoke, involving at least eight different cancers; accordingly, the role of volatile carcinogens found in smoke has been studied extensively (Pope et al., 2011). Far less is known about the pathogenic effects of particulate matter that is suspended in smoke and which includes nanoparticulate carbon. Histopathological analysis of the lungs of heavy smokers invariably reveals dark-staining anthracotic pigment often attributed to poorly soluble material found in tobacco smoke (Mitchev et al., 2002). Anthracotic pigment is also found in the lymph nodes of smokers (Churg et al., 2005). Anthracotic pigment can also appear in the upper lungs as a dense masses of fibrous tissue, more than 1cm in diameter causing a progressive massive fibrosis, or black lung disease, although more common from miners smoke (Black Lung in Appalachia, 2016).

There is strong evidences in the literature that cigarette smoking exposure is associated with mortality risks. At the Harvard Six Cities Study, researchers previously reported the adverse health effects of air pollution and fine particles in smokers. Mortality rates

were more strongly associated with cigarette smoking. After adjusting for smoking and other risk factors, they observed statistically significant ad robust associations between air pollution and mortality (Dockery et al., 1993).

1.4 Synopsis of the study design

Data for this study were collected on São Paulo Autopsy Centre from January to August in the year 2014, under the supervision of the responsible pathologist. The present research uses a retrospective cohort study to access carbon in both lung surface and lung macrophages aiming to indicate significant correlation between particulate matter and carbon deposition in lungs of smokers and non-smokers living in a high polluted city. Understanding the link between air pollution and carbon deposition in lungs, we will provide new insights to health effects associated with air pollution and cigarette smoke exposure. This is the first study to undertake a linear analysis of carbon loading over lung surface carbon and airway macrophage carbon and its effects under long-term residents of São Paulo.

Although, few limitations were faced due confounding bias such as lack of information accessing subject's occupational history and other sources of exposure such indoor exposure and passive smoking.

1.4.1 Structure outline

Chapter 1 gives an introduction on the literature on exposure assessment to particulate air pollution, health effects associated with PM, and retention of inhaled carbonaceous particulate matter in the lung.

Chapter 3 will describe the methods implemented to assess carbonaceous particles intake through different lung compartments in long-term residents of São Paulo. For the first time, a study used image analysis to measure the amount of carbonaceous PM at the lung surface of long-term residents of São Paulo, and reproducing a successfully implemented method, we were able to assess environmental PM also in the lung macrophages. Estimating carbon intake in the lungs in smokers and non-smokers subjects living in a large urban area the present study aimed to find the major determinant of long-term air pollution health effects, and stablish a causal association between smoke and air pollution exposure.

After a meticulous literature research, and based on evidences that autopsies are a very rich source of material, we defined the target population of post-mortem long-term residents of São Paulo metropolitan area. The analysis of carbon was divided in lung surface carbon (LSurC) through digital imaging and alveolar macrophage carbon (AMC) obtained from a small lung fragment and stained to future microscopical analysis. To determine environmental and ambient exposure we evaluate smoking status, measured particulate matter exposure and exposure to local traffic.

Another important part of this study will be found in section 3.8 and 4.6, where we evaluated the distribution of particles in the lung using an additional histological sample.

Finally, Chapter 4 summarises results from this thesis and reflects about on the implications of the findings in the wider context of particle intake and environmental exposure assessment, which will be discussed further on Chapter 5 as well as the limitations on Chapter 6.

2. Aims and Objectives

In this study, we sought to identify the determinants of lung surface carbon (LSurC) and alveolar macrophage carbon (AMC) in long-term residents of São Paulo, with high levels of cigarette smoking and exposure to environmental PM using measured PM_{10} or markers of exposure to traffic at the home address.

The present study aims to prove an association between carbonaceous particulate matter and long-term health effects in the two different compartments (LSurC and AMC), and analyse the pathways between smokers and non-smokers.

3. Methods

3.1 Study population

The School of Medicine of the University of Sao Paulo (FMUSP) seeks to make the best possible use of the great potential associated to the 14,000 autopsies performed annually at the São Paulo Autopsy Centre (SVOC - Serviço de Verificação de Óbitos da Capital da Universidade de São Paulo). Because of autopsies seemed to be a very rich source of material, we decided to use lung samples from this specific population. Autopsy is the only reliable way to validate findings acquired using medical imaging techniques, a field experiencing increase in spatial, contrast and functional resolution to deal with medical diagnosis. The post mortem lungs were obtained from São Paulo Autopsy Centre from January to August in the year 2014. Collection data was standardised as upper right lungs, with no chronic respiratory disease at the macroscopic examination. There were 72 necropsies where 23 were males, 49 females and their age ranged from 37 to 99 years (median age 66), further divided by smokers and non-smokers groups.

Smoking history was determined from a questionnaire completed by relatives.

3.1.1 Target Cohort and Recruitment

Target cohort was initially 100 adult subjects, smokers and non-smokers. They were recruited through the São Paulo Autopsy Centre (SVOC), at Hospital das Clínicas, Faculty of Medicine of University of São Paulo (HC-FMUSP).

3.1.2 Subject selection

Inclusion criteria

- Adults aged over 18 (eighteen) years old, of both genders living within the city limits of São Paulo for at least 10 years;
- Absence of lung chronic disease at the gross examination;
- Family willing to give written informed consent.

Exclusion criteria

- Subjects were seeing by the responsible pathologist of each shift. Subjects showing any macroscopic alterations at the time of examination, due to chronic or inflammatory pulmonary diseases (such as emboli, bronchitis, pneumonia, tuberculosis, emphysema), or pulmonary impairment due to systemic diseases (as bronchiectasis, lymphangitis carcinomatosis, collagen diseases or infectious diseases).

3.1.3 Gaining Consent

The initial approach to the families was made by a member of the Autopsy Centre. They were then informed in order to obtain written in order to use official data from death reports and described lung tissue samples in this study.

The study was approved by the Research Ethics Committee of the Faculty of Medicine, University of São Paulo (Research Protocol CAP Pesq.11621; 05/11/2013; certificate showing on the attachments).

3.2 Analysis of carbon

3.2.1 Lung surface carbon

To access lung surface carbon (LSurC), a digital image was obtained from the whole surface of the right upper lobe from each specimen. Images were taken using a Nikon digital camera (Nikon D-3300) in a professional light box with blue background, as showing in Figure 1. A 7 x 7 cm cropped image from the right upper lobe was then obtained from the area with the least surface indentations. Lung surface carbon was analysed using Image J (National Institute of Health, MD, USA), with the operator blinded to the information about the deceased. By comparing to the original colour digital image, an operator adjusted the "threshold" command in Image J, to best capture black areas. Lung surface carbon was expressed as cm^2 black carbon /49 cm^2 lung surface (Figure 2)

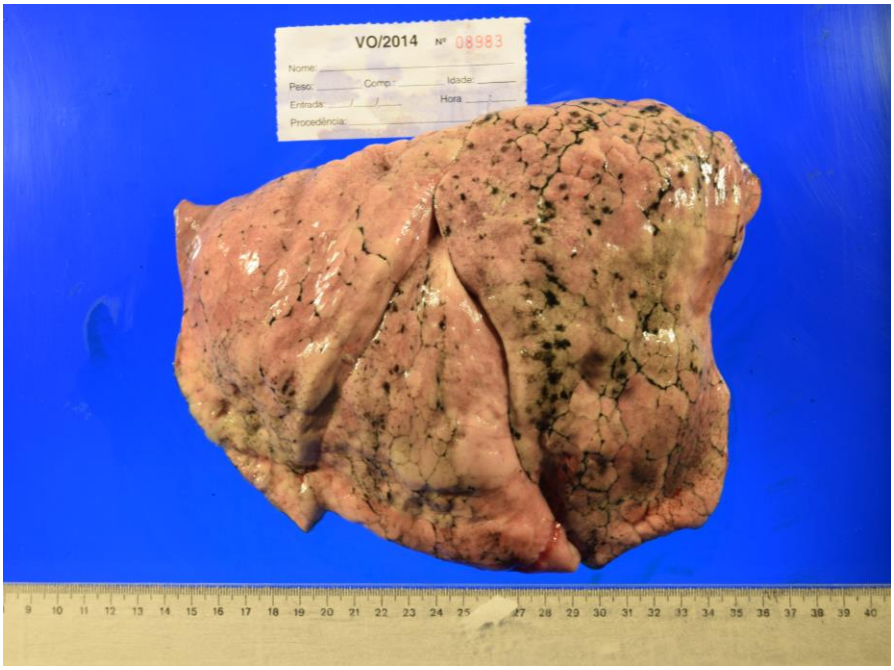

Figure 1: Upper right lung collected at SVOC after the Pathologist evaluation. Digital picture taken using a Nikon digital camera (Nikon D-3300) in a professional light box with blue background. For all lung specimens we used same camera distance and same light.

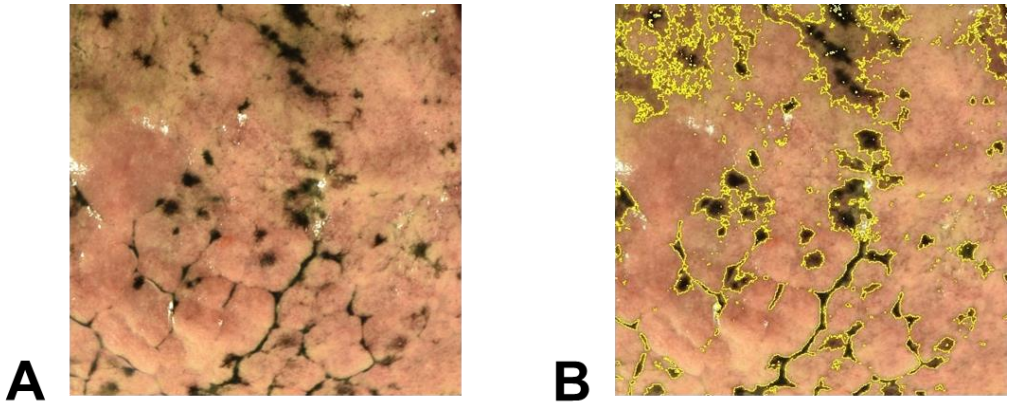

Figure 2: A: Image crop of lung surface carbon (LSurC) specimen measuring 7 x 7 cm. B: The same image with carbonaceous areas identified using the threshold command (yellow borders) from Image J software adjusted to maximise identification of black carbon and minimise identification of areas without carbon. The area of lung surface carbon is 9.37 cm^2 black carbon PM area/49cm^2 lung surface.

3.2.2 Alveolar macrophage carbon

Alveolar macrophages were obtained by first performing a 2 cm cut at right angles to the lung surface, then pressing a microscope slide onto the cut face from smear and fixed with alcohol (70% concentration) until the slides be processed. Slides were processed and stained at the Cytology laboratory at the Faculty of Medicine (FMUSP) using stain technique with haematoxylin and eosin (H&E) previously described by Weigert's Resorcin-Fuchsin (Weigert C, 1898).

After staining process, images of all AM were obtained in a predetermined scan of each slide using Pannoramic 250 slide scanner (3DHISTECH Ltd., Hungary) (Figure 3)

Carbon uptake by AM was indefinable as black areas within the AM cytoplasm (Figure 4) and was quantified using image analysis as previously reported by Kulkarni et al: digital colour images of 50 randomly chosen AM per subject with an intact cell wall were obtained using a Pannoramic Viewer (3DHISTECH Ltd., Hungary) slide scanner, with 50x magnification. Kulkarni and colleagues had previously ascertained that 50 cells produced a reliable estimate of the median surface area of carbon.

Briefly, this study selected digital images of 50 AM per lung specimen obtained using Pannoramic Viewer (3DHISTECH Ltd., Hungary) slide scanner, previously described above and showed at the representative picture below (Figure 4).

The images were analysed for AMC using the software Image Pro Plus (The Proven Solution, Media Cybernetics Inc., USA).

Previously, the software was calibrated using a range of the obtained macrophage images (Figure 5). A macro was recorded in order to precisely catch only the black

carbon area inside the macrophages. For this, a range of pictures samples was selected to record a sensitive macro for these measurements. Based on the shades and colours of a small sample of the pictures previously taken, the threshold command was adjusted to obtain the "best fit" for carbon that was visible on the colour image. A very sensitive macro was successfully recorded in order to the software do not select nonblack areas (e.g. darker areas as the nucleus or haemoglobins).

The median area of carbon per AM per subject (the primary measure), and the percentage of AM containing one or more area of carbon per subject were calculated from 50 cells.

To determine AMC, the nucleus of each AM was cut using the software and a line drawn surrounding the cytoplasm. By comparing to the original colour digital image, an operator adjusted the "threshold" command in Image J, to best capture black areas in the cytoplasm as done for LSurC (above). Airway macrophage carbon was expressed as mean area carbon (μm^2) per cell per specimen.

Figure 3: Pannoramic 250 slide scanner (3DHISTECH Ltd., Hungary) used to scan the cytology slides, using a magnification of 40x Zeiss Plan-Apochromat objective. Scanner A is a 250 slides capacity and scanner B 150 slides capacity/time scan.

a. b. c. d.

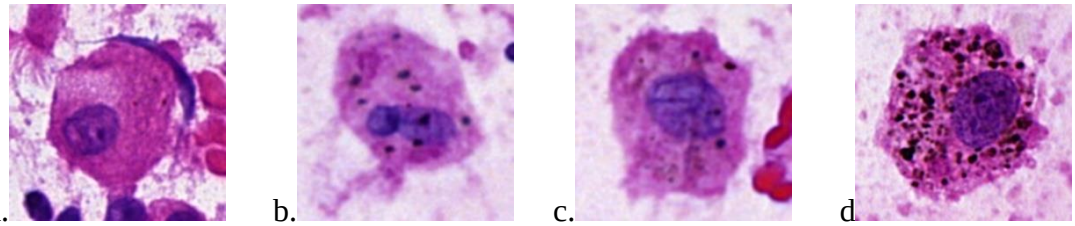

Figure 4: Digital images of lung macrophages obtained by pressing a microscope slide to the cut surface of the right upper lobe. Cells were stained by haematoxylin and imaged under oil immersion (x100 magnification). There is marked heterogeneity of lung macrophage loading; (a) no black carbon, and (b, c, d) increased levels of phagocytosed carbon.

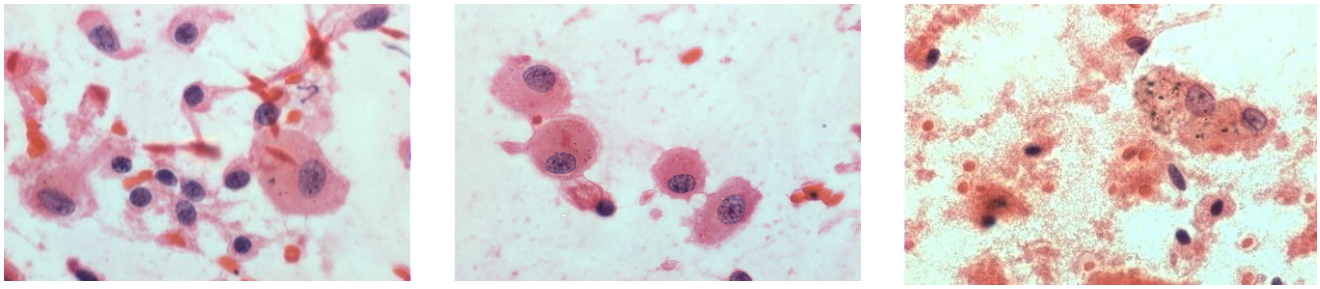

Figure 5: Software calibration using a range of the obtained macrophage images. Calibration was based on the colour tones of the images and calibrated to be sensitive to the black intensity relative to the black carbon spots on the cytoplasm.

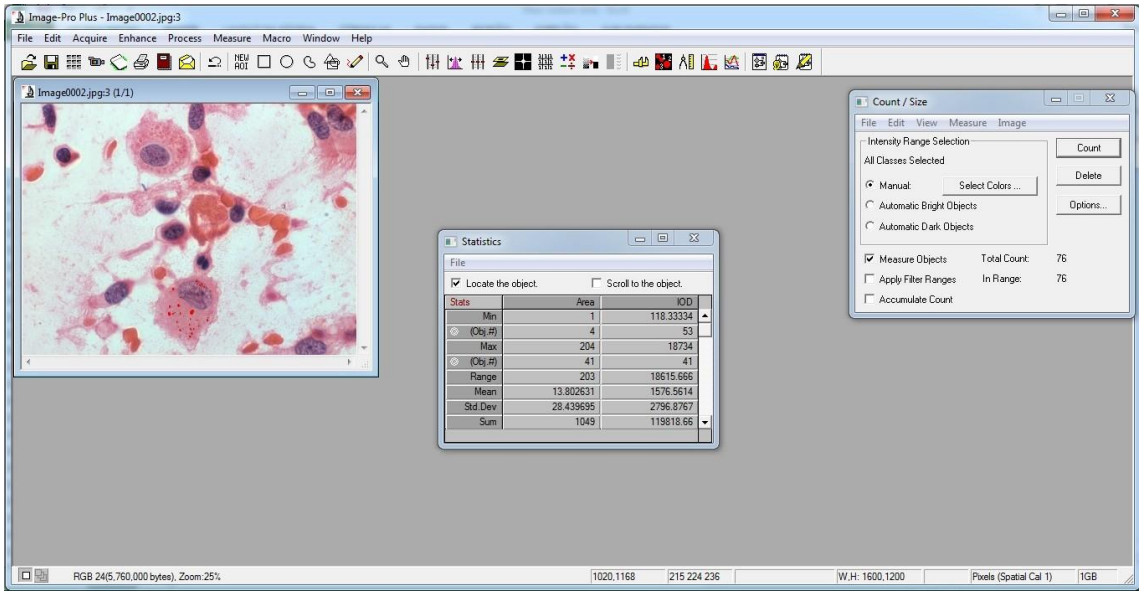

Figure 6: Screen shot of analysed AMC using the software Image Pro Plus (The Proven Solution, Media Cybernetics Inc., USA). Picture shows how the software command captures the black areas on the macrophages.

3.3 Cigarette smoking

Smoking status was determined from the questionnaire of relatives used by the São Paulo Autopsy Centre (SVOC). In this questionnaire, relatives are asked if the subjects were current smokers or non-smokers. Personal information and cause of death was obtained from the official medical records.

3.4 Measured environmental PM exposure

We obtained measurements of environmental PM_{10} using data from monitoring stations across São Paulo metropolitan area, which was supplied by the São Paulo Environmental Agency (CETESB, 2010). Monitoring stations are situated throughout the São Paulo metropolitan area, and the whole population therefore lives within 10 km of a station. For each subject, PM_{10} measurements was obtained from the nearest monitoring station based on their home addresses. Data was recorded 24 hours before death, and mean annual exposures at the home address for 2013, 2012, and 2011 respectively. For PM_{10} model units were expressed by micrograms per cubic meter ($\mu g/m^3$).

3.5 Exposure to local traffic

Road and traffic emissions near to the home address was assessed using a method previous described by Habermann and Gouveia (Habermann and Gouveia, 2014). Exposure to local traffic was determined using the distance from home to heavy traffic roads (m), and the distance-weighted traffic density (m).

3.6 Traffic Methods

Using distance from home to heavy traffic roads, distance-weighted traffic density (DWTD) and levels of particulate matter 10 as indicators, it is assumed that the dispersion of emissions produced by vehicles on roads approximates to a Gaussian (normal) distribution, and that 96% of the pollutants spreads within a distance of up to 500 feet (150 m) from the centre of the road as the model developed and applied by Pearson et al. (2000).

Most dispersion models use a combination of pollution, meteorological and topographical data. The major advantage of dispersion models compared to most other modelling techniques of ambient air pollution concentrations is that they not only include spatial, but also temporal variations, which makes them some of the most widely applied models for air pollution predictions (Hoek, Beelen, et al. 2008; Baklanov et al. 2007; Daly & Zannetti 2007).

3.6.1 Road and traffic data

Data from 2007 on the road system, such as traffic counts and simulation on the main streets and roads (thoroughfares, cross streets, and rapid transit) were provided by the São Paulo Municipal Traffic Engineering Company (CET), including tables, the street grid, and the number of vehicles per hour. For stretches that lacked traffic statistics (5.8% of the total), the study assigned a mean value based on the respective street's overall classification.

Local street traffic was estimated by the CET in 926 regions demarcated by the public traffic and transportation-planning department (origin-destination zones). The sum of

traffic in each of these regions was divided by the total length of all the local streets contained in them. The traffic density (*TDi*) (vehicles per meter of local streets) was then obtained for each region (*i*). This measure was then multiplied by length of the segments of local roads (*SLR*), in meters, contained in the respective regions, thus obtaining the traffic for each local street segment (*T*).

$$T = \left(\frac{\sum_i traffic}{\sum_i length} \right) \times SLR \qquad (1)$$

3.7 Evaluation of the exposure:

3.7.1 Distance from roads with heavy traffic flows

We defined roads with heavy traffic flow those with vehicle traffic volumes greater than the 95th percentile (≥1,876 vehicles/hour) of the distribution. This included most of the rapid transit, arterial and collector roads. The shortest distance between the subject's home address and these roads were then estimated.

3.7.2 Traffic density by census tract

This indirect indicator of exposure to pollutants generated by motor vehicle traffic was constructed for each census tract. Since the census tracts vary considerably in size, many of them represent only one block or building each, and therefore do not contain any stretch of street and could have a null traffic value. This question was solved using the same approach as Gunier et al. (2003) and Reynolds et al. (2002) , where the limits

of each tract were extended with a 200 m surrounding buffer. This 200 m distance also represents the dispersal of air pollutants from a street (Chacraborty et al. 1999, Reynolds et al. 2002, Gunier et al. 2003). Traffic density consists of multiplying the vehicle volume by the length of the street segments, followed by dividing by the area of the census tract (with the 200 m buffer).

$$TDi = \frac{\sum_i AMDL \times L}{A_i} \qquad (2)$$

Where *TDi* is the traffic density in each census tract +200 (*i*), AMDT is the annual mean daily traffic (vehicles/hour - VPH), *L* is the length of the street segment (km), and *A* is the area of the census tract (in km²) with the 200 m buffer. The unit is expressed as VPH/km². For the purposes of this study, census tracts + 200 m are referred to generically as tracts.

We then computed exposure to traffic according the census tract where the subject homes addresses were contained.

3.7.3 Distance-weighted traffic density

In the DWTD indicator, it is assumed that the dispersion of emissions produced by vehicles on roads approximates to a Gaussian (normal) distribution and that 96% of the pollutants spreads within a distance of up to 500 feet (150 m) from the center of the road as the model developed and applied by Pearson et al. (2000).

For each subject studied, the shortest distances to roads within a radius of 750 feet (228.6 m) around the subject's residential address was calculated. For each distance (D), the value Y was calculated as a weighting factor for vehicle flows obtained for each road within the area.

$$Y = \left(\frac{1}{0.4\sqrt{2\pi}} \right) \times \exp\left[\left(\frac{(-0.5)\left(\frac{D}{500} \right)^2}{(0.4)^2} \right) \right] \qquad (3)$$

The Y was used to weigh the products of the traffic intensities of all road segments within the buffer. The weighted values were summed for each subject to obtain the DWTD.

3.8 Histopathology

To assess the distribution of carbon in the lung, an additional right lung specimen was randomly selected at the Autopsy Centre after the pathologist's final evaluation. The lung was infused with 10% neutral buffered formalin with a non-specific pressure and fixed for 48 hr. A set of transverse sections was performed after that in order to observe the deep layers and the carbon intake. Sections were stained with haematoxylin and imaged with a Nikon D-3300 camera.

3.9 Statistical analysis

Data are summarized as median (IQR). Comparisons between groups of smokers and non-smokers in both LSurC and AMC were done by Mann Whitney test. Correlations were done by Spearman rank test. A P value <0.005 was considered significant. Statistical analysis was done using SPSS version 24 for Windows software (SPSS Inc., Chicago, IL, USA).

4. Results

4.1 Demographics and Cause of Deaths

LSurC and AMC was assessed in all 72 specimens analysed. From the entire population, 18 subjects were formal smokers and 54 were non-smokers, with median age of 66 years (Table 1).

Cause of death was assessed through formal questionnaire provided by São Paulo Autopsy Centre. At the Smokers group, we observed that 72% of the causes were cardiovascular diseases, 6% classified as neurological diseases and 22% from other causes, whereas at the non-smokers group 85% of the causes of death were cardiovascular diseases, 4% neurological cause and 11% caused by any other reason.

Our demographic data showed an interesting but not surprising statistic about the main causes of death between the subjects on the present study. According to the recent report submitted to the Royal College of Physicians, exposure to air pollution is associated with increased cardiovascular morbidity and deaths from myocardial ischemia, arrhythmia, and heart failure. Also, fine particulate matter derived from the combustion of fossil fuels is thought to be the most potent component of the air pollution composition. Particulate matter upregulates systemic proinflammatory and oxidative pathways, either through direct translocation into the circulation or via secondary pulmonary-derived mediators. The physicians also found out that exposure to particulate matter has the potential to impair vascular reactivity, accelerate atherogenesis, and precipitate acute adverse thrombotic events, which supports the higher number of deaths by cardiovascular causes. In patients with coronary heart

disease, exposure to combustion-derived particulate can exacerbate exercise-induced myocardial ischemia.

Characteristic	Smoker n = 18	Non-Smoker n = 54
Age at death (in years/average)	**58 (39-84)**	**69 (37-99)**
Gender (n= %)		
Female	**10 (55%)**	**39 (72%)**
Male	**08 (45%)**	**15 (28%)**
Cause of Death (n= %)		
Cardiorespiratory	**13 (72%)**	**46 (85%)**
Neurological	**01 (6%)**	**02 (4%)**
Other	**04 (22%)**	**06 (11%)**

Table 2: Demographic table, smoker and non-smoker groups, showing age at death in each group; gender; main cause of death; PM_{10}, particulate matter with aerodynamic diameter ≤ 10 μm (μg/m3) for the years 2011, 2012, 2013 and 24 hour before death. Data are presented as mean (standard deviation) for continuous variables, or n (%) for categorical variable.

4.2 Lung Surface Carbon

There was no association between age and LSurC (Rs= -0.018; P= NS), and there was no difference in LSurC between males and females (4.24cm^2 (1.9 to 8.45) vs. 6.18cm^2 (4.69 to 9.95) P=NS). There was no difference in LSurC between smokers and non-smokers 6.74 cm^2 (3.47 to 10.02) versus 5.20cm^2 (2.29 to 7.54) (Figure 7). There was no association between LSurC and exposure to environmental PM at the nearest monitoring station to the home address (Table 6). There was no difference in LSurC and distance from road, either when expressed as a continuous variable (m), or categorised by <150 m and $\geq$ 150m (4.26cm^2 (1.69 to 6.77) vs. 6.11cm^2 (3.5 to 9.55) P=0.058).

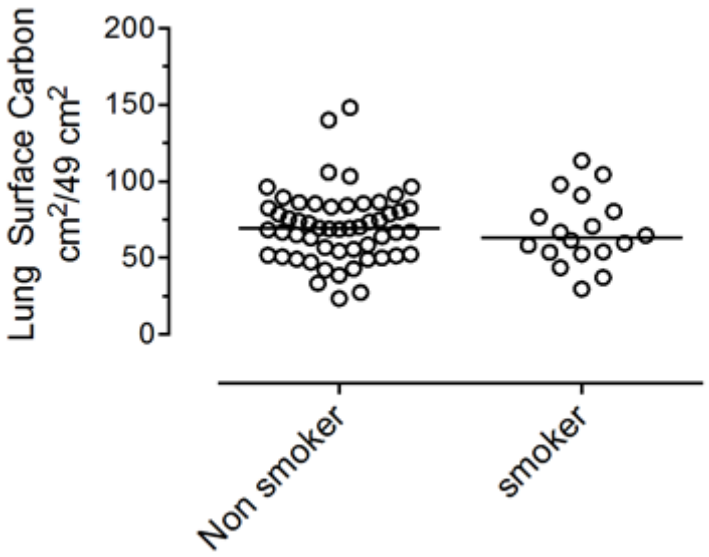

Figure 7: Dot plot of lung surface carbon in smokers and non-smokers. Lung surface carbon was assessed using image analyses and expressed as cm^2/49 cm^2 lung surface. There is significant difference between groups by Mann Whitney test. Bar represents median.

There was a marked heterogeneity of LSurC between individuals. In some specimens, the surface was predominately pink with the fissures relatively free of carbonaceous PM, in other specimens, large amounts of carbonaceous PM had accumulated both in fissures with and across whole lung surface (Figure 8).

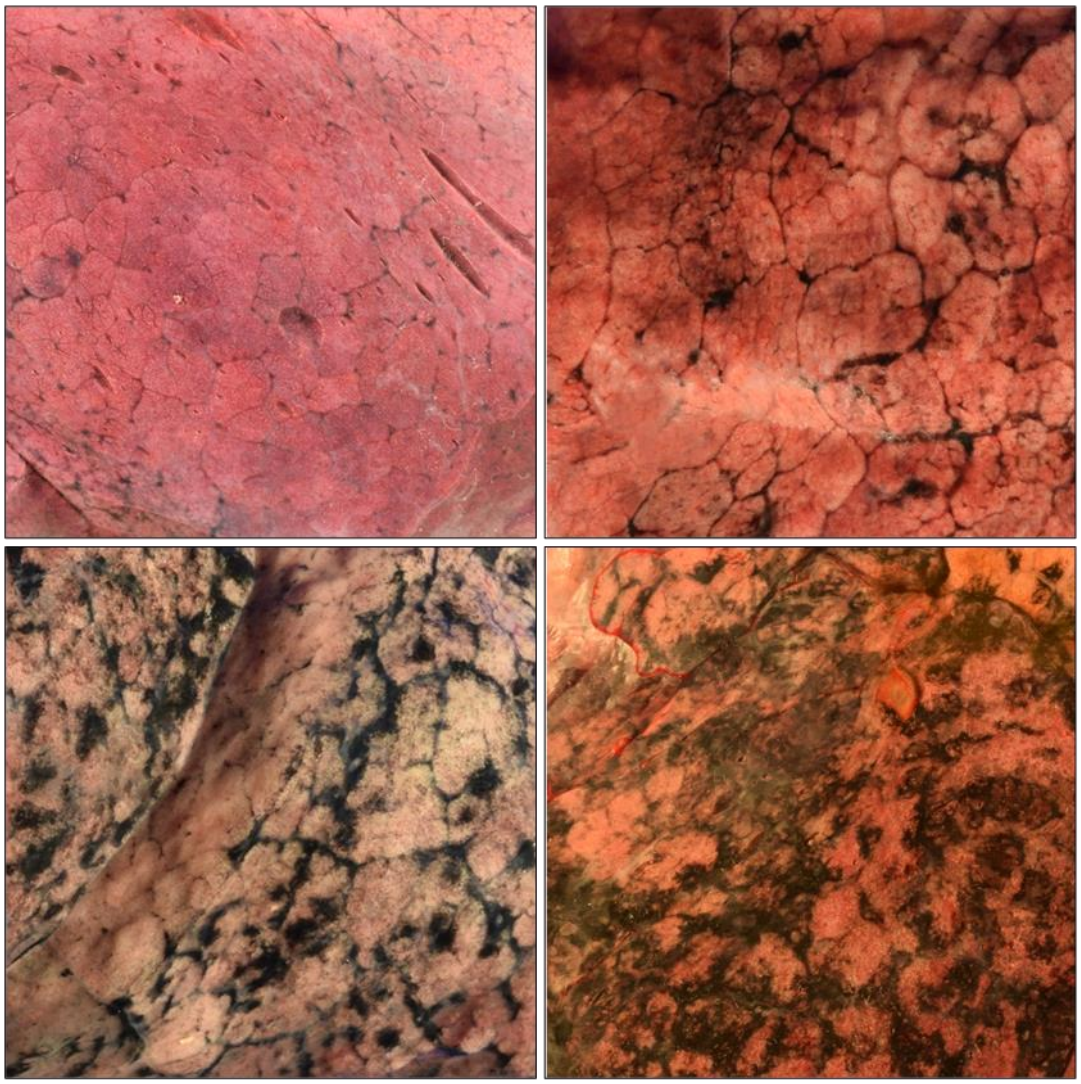

Figure 8: Image of the lung surface. Standard crops using Image J software. In clockwise order: 1. Non-smoker, female, 85 years old; 2. Smoker, male, 70 years old; 3. Non-smoker subject, female, 76 years old; 4. Smoker, female, 47 years old.

4.3 Alveolar Macrophage Carbon

Alveolar macrophage carbon, showed marked heterogeneity within and between specimens. There was no association between age and AMC (Rs= -0.197; P= NS), and there was no difference in AMC between males and females (26.66 (5.5-122.29) vs 28.5 (3.20-99.8) P=NS).

Smokers had increased AMC compared with non-smokers (103.4 (29.44-226.3) vs. 9.27 (3.1-85.13) μm^2, P< 0.001, Figure 9). Overall, there was no association between AMC and any markers of exposure to environmental air pollution. (Table 6).

There was no difference in AMC and distance from road, either when expressed as a continuous variable (m), or categorised by <150 m and $\geq$ 150m 33.39 μm^2 (5.05 to 107.8) vs. 16.27 μm^2 (3.13 to 103.5) P=0.26).

Furthermore, there was no association between AMC and estimates of environmental PM pollution when the analysis was limited to non-smokers (Table 6).

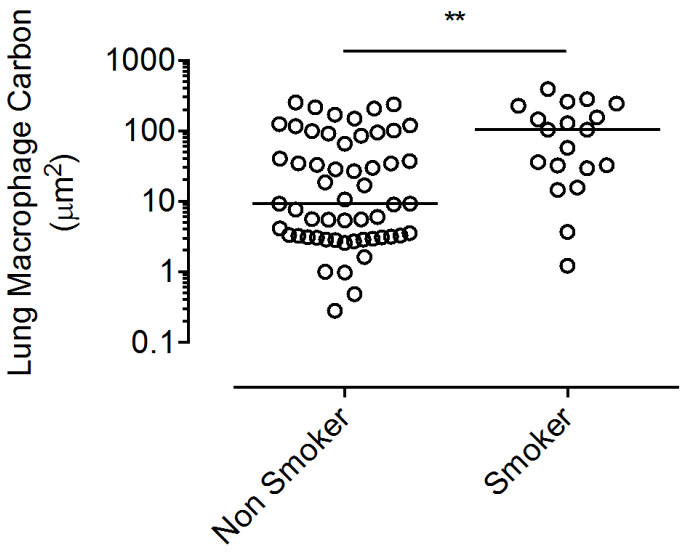

Figure 9: Dot plot of alveolar macrophage carbon in smokers and non-smokers. Airway macrophage carbon was assessed using image analyses and expressed as μm^2. There is significant difference between groups by Mann Whitney test. (103.4 (29.44 to 226.3) vs. 9.27 (3.1 to 85.13) μm^2, $P<0.001$). Bar represents median.

Subject	LSurC	AMC
01	No data	29.44
02	1.59	226.28
03	6.02	242.92
04	28.85	390.35
05	1	260
06	5.1	103.71
07	3.62	154.24
08	9.07	57.66
09	5.28	281.48
10	13.81	103.42
11	7.23	3.66
12	8.58	1.22
13	9.27	32.38
14	9.83	14.44
15	2.94	32.52
16	10.58	15.66
17	6.26	145.3
18	3.03	35.9

Table 3: Full results of smokers subjects group for Lung surface carbon (LSurC) and alveolar macrophage carbon (AMC). Data are expressed as μm^2.

Subject	LSurC	AMC
2737	0.7	10.57
2781	0.42	9
3722	No data	3.05
3990	5.68	236.82
4000	1.01	85.13
4417	3.46	16.87
7553	8.72	65.62
7554	3.54	251.48
7555	17.49	3.26
7559	2.18	149.12
7588	10.58	4.12
7806	6.15	169.1
7819	10.61	37.15
7824	8.55	100.5
7826	7.25	34.25
7827	5.06	116.59
8108	6.1	207.75
8551	0.75	34.8
8553	0.03	215.5
8648	2.33	119.92
8652	3.55	99.1
8653	3.46	5.6
8658	0.94	1.62
8661	5.7	7.62
8663	12.95	5.48
8698	7.17	29.76
8719	2.77	0.97

8722	4.24	124.43
8732	4.05	5.97
8735	8.36	9.27
8799	5.57	2.87
8804	4.28	3.53
8808	0.26	2.82
8811	1.85	3.25
8813	10.24	2.7
8815	1.68	91.14
8817	16.3	2.87
8818	0.19	9.31
8885	6.34	26.66
8902	1.95	0.28
8908	11.66	32.88
8940	3.27	5.55
8957	10.31	1
8975	6.33	40.2
8977	7.26	3.33
8980	5.58	5.4
8982	2.32	3.08
8983	9.37	3.1
8985	11.01	2.95
8988	5.35	0.48
9060	0.11	28.5
9061	4.91	18.5
9063	5.94	3.16
9064	3.09	2.58

Table 4: Full results of non-smokers subjects group for Lung surface carbon (LSurC) and alveolar macrophage carbon (AMC). Data are expressed as μm^2.

4.4 Measured PM Exposure

PM10 measurements were represented by annual mean through the years 2012 to 2014. Air quality levels recommended by the WHO are showing on table 5. The official report of PM10 from CETESB is showing on tables 7 and 8, where the maximum annual mean reached 50 μg/m3 and the minimum 27 μg/m3 with average annual mean exposure of 34μg/m3 in both groups, smokers and non-smokers. Correlations between exposure to environmental PM10 and pre-mortem exposure between LSurC and AMC were done by Spearman Rank test, where all correlations are non-significant, and we can see the correlations represented on table 6.

	Averaging times	**Air quality guidelines**
PM_{10}	**1 year**	**2 μg/m^3**
	24 hours	**50 μg/m^3**

Table 5: Air quality levels that are recommended by the WHO and that all countries should aim for. For particulate matter, average levels are recommended over 1 year and for 24 hours, because both short- and long-term effects occur. It is thought that no guideline will ever provide complete protection, but health effects can be reduced.

4.5 Road and Traffic Exposure

	24 hour before death	Annual mean 2013	Annual mean 2012	Annual mean 2011	DWTD vehicles/hour	Home to major road (150m)
LSurC	P=0.077	P=0.06	P=0.49	P=0.563	P=0.376	P=0.16
	Rs= -0.201	Rs= -0.21	Rs= 0.078	Rs= 0.066	Rs= 0.10	Rs= 0.15
AMC	P=0.454	P=0.066	P=0.009	P=0.572	P=0.092	P=0.655
	Rs= -0.088	Rs= -0.215	Rs= -0.303	Rs= -0.067	Rs= -0.197	Rs=0.053

Table 6: Correlations done by Spearman Rank test. All correlations are non-significant. Table representing correlations between exposure to air pollution and pre-mortem exposure values between LSurC and AMC. PM_{10}, particulate matter with aerodynamic diameter ≤ 10 μm (μg/m3).

Subject	PM 2011	PM 2012	PM 2013	year 14	dt census tract	DWTD	dist.perc 95%
01	38	35	33	33	1218.113356	113.764917	1733.127293
02	36	32	29	31	16.33315693	2.71826101	4081.19818
03	36	32	29	31	1247.314374	43.7286417	508.289333
04	36	32	29	31	4442.238252	39.3969092	191.280446
05	36	32	29	31	4564.603681	120.829373	370.71624
06	36	36	32	34	27.95710148	8.31565438	342.304286
07	33	34	33	37	2748.23784	160.912301	228.000082
08	38	35	31	30	122.9226124	232.525082	2014.929598
09	33	34	33	37	2483.939215	738.202823	1139.998239
10	38	34	29	33	0	0	12407.87725
11	50	34	43	35	5556.437002	1184.0446	116.79234
12	33	34	28	27	3056.190825	1616.79371	586.114789
13	33	34	33	37	11911.0207	1239.94836	323.57195
14	38	44	40	41	11709.19058	185.729003	321.785096
15	38	35	31	30	1738.14127	14.3254185	291.443414
16	33	34	33	37	476.3942907	473.024034	493.460913
17	33	34	33	37	1057.580685	17.4296139	694.946417
18	33	34	33	37	27545.86186	347.315307	133.248886

Table 7: Full results of Smokers subjects group. In this table we presented the results for PM exposure represented in mean and the traffic exposure results for density by census tract (m), distance-weighted traffic density (in vehicles/hour) and distance from roads with heavy traffic flows (dist. perc 95%; 150m).

Subject	PM 2011	PM 2012	PM 2013	PM 2014	dt census tract	DWTD	dist perc 95%
19	33	34	33	37	417.19667	31.902546	900.180999
20	33	34	33	37	1927.2101	66.926784	574.836206
21	38	44	40	41	54217.84	307.23542	419.457679
22	38	34	29	33	6941.4141	305.47061	316.654364
23	33	34	33	37	264.57256	46.839034	2409.829895
24	38	35	30	29	1467.622	468.04402	261.03288
25	36	32	29	31	670.54906	68.08986	703.129122
26	36	32	29	31	5791.0775	1137.2145	601.104465
27	33	34	33	37	820.17571	156.11067	192.899033
28	36	32	29	31	4364.7762	432.28354	3047.739395
29	33	34	28	27	24983.783	1120.7029	187.135381
30	38	35	31	30	8012.7161	207.54166	144.402894
31	38	35	31	30	10453.321	268.69932	174.067176
32	39	36	33	40	7203.6609	582.50161	103.163568
33	38	34	29	33	2896.2637	496.36727	548.702711
34	33	34	33	37	3459.3393	9.9534528	778.002254
35	39	36	33	40	11961.59	42.287725	220.933752
36	39	36	33	40	529.06544	14.475292	496.51336
37	33	34	33	37	3593.4593	910.80063	1377.037191
38	36	32	29	31	54.220405	16.227002	446.159755
39	36	36	32	34	662.29307	442.82122	557.737363
40	38	44	40	41	35.654851	12.119176	1852.552685
41	39	36	33	40	4491.5642	355.34097	128.971068
42	33	34	33	37	356.69114	578.09978	10655.69596
43	33	34	28	27	2522.7504	419.24157	574.124275

44	33	34	28	27	31703.729	1160.432	111.342181
45	36	32	29	31	6939.4851	383.2052	1709.764827
46	33	34	33	33	348.43565	84.450663	1891.924254
47	36	36	32	34	0	0	2260.083106
48	36	36	32	34	2377.7246	1190.8131	352.857339
49	33	34	33	37	645.18344	10.713737	1283.473748
50	36	33	32	33	456.97197	12.08723	725.620095
51	33	34	33	37	914.22295	13.259389	805.33413
52	39	36	33	40	1495.0677	2062.2728	49.703972
53	38	34	29	33	9028.6488	628.85179	468.776588
54	33	34	33	37	3810.1155	19.14178	189.177402
55	36	32	29	31	3005.8103	856.16282	543.199669
56	32	33	32	37	1249.7841	1020.6238	114.388035
57	39	36	33	40	15457.026	2366.8204	7.039245
58	39	36	33	40	1603.4047	511.60753	719.081207
59	36	36	32	34	511.58629	1167.4326	398.19598
60	38	35	33	33	0	0	988.096095
61	33	34	33	37	1336.8815	33.636092	582.740091
62	33	34	33	37	531.18069	271.98547	950.834747
63	38	34	29	33	7.0201754	1.5086765	610.444609
64	37	34	28	27	2121.4108	468.62684	543.228599
65	38	35	31	30	1894.5368	31.966746	399.889248
66	39	36	33	40	410.57036	37.841663	293.266366
67	39	36	33	40	15992.254	1279.8031	119.34043
68	38	35	30	29	23430.629	4362.5181	42.713184
69	36	32	29	31	14.972393	6.735237	447.094924
70	36	36	32	34	9272.3053	1574.6586	47.772368
71	33	34	28	27	5563.3247	156.78749	181.943003
72	33	34	33	37	1960.9006	1302.6436	2297.56582

Table 8: Full results of non-smokers subjects group. In this table we presented the results for PM exposure represented in mean and the traffic exposure results for density by census tract (m), distance-weighted traffic density (in vehicles/hour) and distance from roads with heavy traffic flows (distance percentile 95%; 150m).

4.6 Additional Histopathology Analysis

Histological analysis of a lung specimen showed the presence of carbonaceous PM both in AM, and under lung surface and deep within the lung tissue compartment. (Figures 10 A and B).

The lung randomly selected for histological analysis was from a smoker. Carbonaceous PM was present in AM, in tissue at the lung surface, and in non-surface lung tissue (Figure 11).

As we could observe on the picture (Figure 11), the black pigments in lung surface may be intracellular or extracellular. The anthracotic spots in the lung surface presented a rounded or irregular shape, ranging from hardly distinguishable (<1 mm in diameter) to >50 mm (Figure 10 A and B) (Mitchev et al., 2002). According to the study conducted by Mitchev and colleagues, the highest prevalence of black spots are throughout the pleura. This indicates that the process is very common at least in urban dwellers.

Based on these evidences, we could conclude that black spots are present in the lung surface (LSur) of the vast majority of the urban population and they are more common in the elderly people.

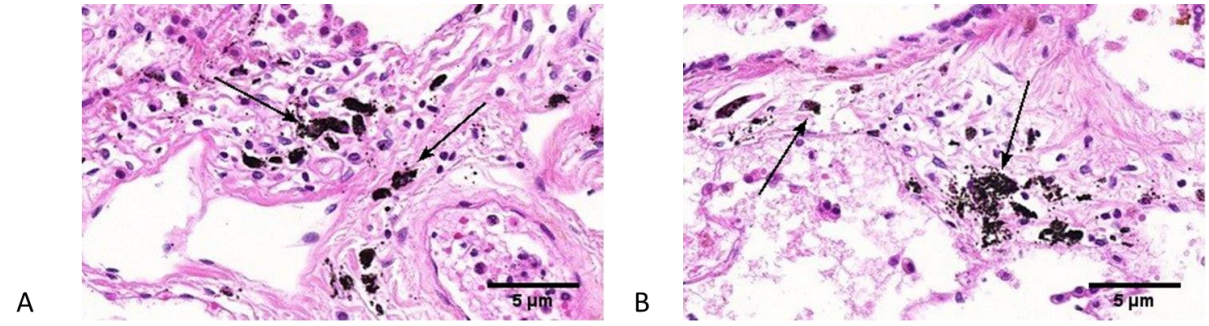

Figure 10: Lung section from a male smoker aged 59 showing; (A) carbon in alveolar macrophages (arrow), and (B) carbon in lung tissue (arrow) (Original magnification 20x)

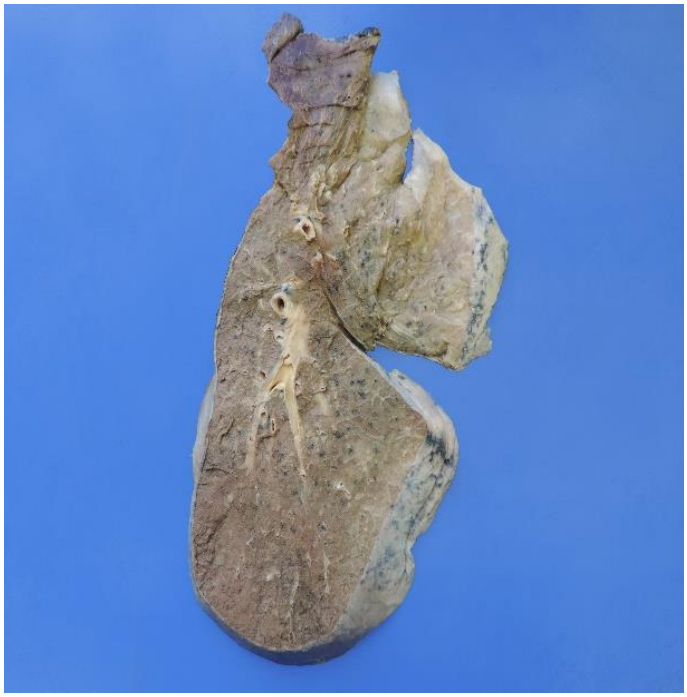

Figure 11: Additional hystological analysis. Right upper lung, randomly selected. The presented subject was a smoker, male, 59 years old. We can observe accumulation of anthracotic pigment deposition along the lung tissue.

5. Discussion

In this study we, for the first time, used image analysis to measure the amount of carbonaceous PM on the lung surface of long-term residents of São Paulo. Since Saxena and colleagues (Saxena et al, 2011) reported high levels of extracted elemental carbon deposits in autopsied lungs of smokers, we hypothesised that smoking is the major determinant of carbonaceous PM at both the lung surface and in lung macrophages. But, despite significant LSurC in many lung specimens, we found no difference in LSurC between smokers and non-smokers. By contrast, and compatible with previous reports (You et al, 2015), we found smoking was associated with AMC compared with non-smokers.

Since we found no difference in LSurC between smokers and non-smokers, our hypothesis that smoking is the major determinant of black staining at the lung surface is not supported. However, our AM findings provide clear evidence that smoking does increase the carbon PM burden. Why this is not reflected by increased translocation of PM to the lung surface is to date unclear. A putative explanation is that in residents of São Paulo, the contribution of smoking is overwhelmed by chronic inhalation of very high levels of ambient PM (Kulkarni et al., 2006; CETESB, 2009; Habermann et al., 2014).

Indeed, previous studies suggest that environmental PM is a major driver of accumulation of carbonaceous PM within lung tissue. For example, Saxena *et al* (Saxena et al., 2011) reported extracted elemental carbon PM from the lungs of non-smokers, and Pinkerton *et al* (Pinkerton et al., 2000), reported carbonaceous PM in lymphatics in the subpleural interlobular septa of non-smoking Californian residents.

Furthermore, animal models suggest an association between LSurC and inhaled dose of environmental PM. First, in cats exposed to 20h diesel exhaust PM for 28 days, Pepelko *et al* (Pepelko et al., 1980) reported increased charcoal grey staining of the lung surface compared control animals exposed to air. Second, in rats, Kato *et al* (Kato et al., 2003) reported that exposure for 24 to 60 weeks to urban roadside filtered air, resulted in a dose-dependent increase in carbonaceous staining of the lung surface. Third, in rats with repeated inhalation of biodiesel PM for 13 weeks**,** Finch *et al* (Finch et al., 2002) reported that increased inhaled dose resulted in increased grey discoloration of the lung surface. Although our study is the first to quantify the amount of carbon at the lung surface of human smokers and non-smokers, evidence of a major role of environmental PM on black staining is provided by the 1971 study of Pratt and Kilburn (Pratt PC and Kilburn KH, 1971), who in an analysis of 250 non-emphysematous adults lungs, concluded that pigmentation may be an indicator of exposure to environmental PM.

Although we found that smoking is not a major determinant of LSurC, and that the most likely source of LSurC is therefore environmental PM air, we were not able to establish a causal association. Specifically, we found no association between LSurC and markers of environmental PM - either mean annual PM_{10} at the nearest monitoring station to the home address, or distance-weighted traffic density, or distance of home to the nearest heavily used road. Although these exposure variables are reported to be associated with adverse health effects in urban populations (Jacobs et al., 2011; Bunn et al., 2001; Bai et al., 2015; Pearson et al., 2000), we speculate that either these markers do not accurately reflect exposure to environmental PM or there was misclassification with the exposure data. Indeed, a detailed address history was not routinely recorded by the standard

autopsy questionnaire completed by relatives (which was used by us to obtain exposure data).

By contrast, increased AMC found in smokers strongly suggests that relative-reported smoking status is accurate, and is compatible with a previous study (Niewoehner et al., 1974).

The role of accumulation of carbonaceous PM at the lung surface in mediating lung injury was not determined by the present study. However, pathological abnormalities in the lung are found in areas adjacent to carbon PM aggregates. For example, Pinkerton *et al* (Pinkerton et al., 2000) reported an association between retained tissue carbon and fibrosis in adjacent respiratory bronchioles. Although we did not assess fibrosis, we did confirm in a randomly selected lung (which by chance was from a smoker), that LSurC results from significant accumulation of interstitial carbon at the pleural surface. The effect of leaching of toxins from retained PM on inflammatory signalling pathways should therefore be a focus of future studies.

Another important thing to be considered, is the mechanism of deposition and pulmonary clearance of inhaled particles. According to Stuart (Stuart, 1976), macrophage removal is accepted to be a major clearance mechanism within the pulmonary region of the lung. At this study, Stuart found that macrophage migration is thought to occur either up the tracheobronchial tree or perhaps via regional lymph nodes. In addition, particle bearing macrophages may migrate to subpleural and paraseptal positions, to perivascular sites, or to peribronchial positions where long-term storage in the lung may occur, as discussed in another study by Green (Green GM, 1973).

5.1 Particulate matter and health effects

The adverse health effects of air pollution on cardiovascular health have been established in a series of major epidemiologic and observational studies (Dockery at al., 1993; Hoek G, 2002)

Supporting the findings that in large urban areas, air pollution emissions are the major determinant of an increase in morbidity and daily mortality risk, Mills and colleagues demonstrated for the first time that inhalation of diesel exhaust, a common urban air pollutant, can impair vascular function in humans. Using a double-blind, randomized, cross-over study design, they have assessed the effects of diesel exhaust inhalation on two important and complementary aspects of vascular function: the regulation of vascular tone and endogenous fibrinolysis. After that, Mills et al, were able to demonstrate that both are impaired and plausibly related to the well-documented adverse cardiovascular effects of air pollution. These important findings provide a plausible mechanism that links air pollution to the pathogenesis of atherothrombosis and acute myocardial infarction (Mills et al, 2007)

There is consistent evidence that the levels of particulate matter in the air are associated with the risk of death from all causes and from cardiovascular and respiratory illnesses. Likewise our demographic data, a study done by Samet and colleague sin 20 of the largest cities and metropolitan areas in the United States from 1987 to 1994, researches finding that the association between PM_{10} levels and the risk of death was strongest for cardiovascular and respiratory causes of death is consistent with the hypothesis that persons made frail by advanced heart and lung disease are more susceptible to the adverse effects of air pollution. They find that the estimated increase in the relative rate

of death from all causes was 0.51 percent (95 percent posterior interval, 0.07 to 0.93 percent) for each increase in the PM_{10} level of 10μg per cubic meter (Samet et al., 2000).

Other findings from several epidemiologic studies of the longer-term effects of air pollution on the risk of death suggest that exposure to air pollution may do more than simply shorten life by a few days (Pope et al., 1995), as it was demonstrated in the Harvard Six Cities Study that the residents in the cities with the lowest PM levels had a survival rate roughly 2 years longer than those individuals living in cities with the highest PM levels (Dockery et al., 1993), while these studies have only examined the association between air pollution exposures on the same day of death of in the last few days our study correlated the last 4 years of exposure assessment of each subject.

Despite particulate air pollution was being previously described as a predictor of mortality (Pope et al., 1995), our study found no association between PM_{10} and alveolar macrophage or between PM_{10} and lung surface tissue compartment.

Although we cannot identify the exact mechanisms involved in short and long-term carbon deposition in lung tissues, our data suggest that carbonaceous deposition in the AM may be a factor in long-term PM-related outcomes. Previous described data suggested that difference in the composition of particles is associated with intercompartmental clearance. In the London archival autopsies in lung tissues from the smog episode found that intercompartmental differences in PM content should largely be related to the duration of retention in the lung (Hunt et al, 2003). Not less important, an epidemiologic study accessing the carbon content of airway macrophages in children as a marker of individual exposure to particulate matter derived from fossil fuel, sought

direct evidence of this association.

In addition to that, a study conducted in mice determined that the mechanisms of distribution and retention of carbonaceous deposition could be affected by agglomeration and inflammation caused by inhalation of particles (Ryman-Rasmussen et al, 2009).

On the other hand another study observed an estimation between air pollution and cigarette smoke risk factor showed that mortality risk when the data were separated by smoking status and gender, the risk was as high for never-smokers as it was for ever-smokers and as high for woman as it was for men (Pope et al., 1995).

There is limited evidence to support the effectiveness of lung surface and alveolar macrophage black carbon area to predict PM exposure in smokers and non-smokers populations.

5.2 Histopathology and Prevalence of the Black Spots

As we could observe on figures 8 and 11, the black spots deposits on the lung compartment may be intracellular or extracellular. Particle deposits are often a heterogeneous mixture of types and sizes and this suggests that the variation in the particle burden might provoke a different biologic response on the lung tissue (Mitchev et al., 2002). The anthracotic spots in the lung surface presented a rounded or irregular shape, confluent or not, ranging from hardly distinguishable (<1 mm in diameter) to >50 mm (Figure 10 A and B).

Based on the data obtained in this study, we could conclude that black spots are present in the lung surface (LSur) of the vast majority of the urban population and they are more common in men and in the elderly population.

In the Black Lungs study in Appalachia, we could observe a marked deposition of particles in lung surface, where a section of lung showed the ravages of progressive massive fibrosis (PMF). In that study, researchers represented the disease characterizing by a large, and dense masses of fibrous tissue that often appear in the upper lungs more than 1 cm in diameter, which often appear in the upper lungs. The lung itself often appears blackened, and they could observe a presence of fibrosis which impairs the ability of the lungs to bring oxygen to the blood, which leaves sufferers chronically short of breath and may result in death. The lung section itself published in their article showed the black deposition due to the slow build-up of coal dust particles over the years. Aside that, on a histological sample, highly reactive particles of coal mine dust can infiltrate the deepest reaches of the lung, and these inhaled particles create a chronic inflammatory response that damages the lung and ultimately scars the lungs (EHP – Appalachia, 2016). In some studies, cigarette smoke is also an evidence of black pigmentation in lungs. In contrast to the white or pink appearance of normal lungs, the lungs of heavy smokers are typically dark brown or black (Churg et al., 2005). In the study conducted from You and colleagues, they found that the same anthracotic pigmentation observed in the lung cells from smokers, were not present in the same cells isolated from non-smokers, suggesting lacked the pigment (You et al., 2015).

Therefore, the lack of information about the occupation of the subjects does not allow further interpretation of the work related determinants of black spots, and it will be discussed in more details on the section below.

5.3 Limitations

5.3.1 General limitations

We recognize that there are some limitations to this work.

For example, on the data collection, we have initially reached the target population but some samples had to be reject after further microscopically analysis. In some cases due to severe embolism or further confirmed diagnosis of pneumonia the quality of the slide was compromised. In other cases we had to reject the case subject after poor processing of the slides technique during the cytology staining process or during the scanning process when the quality of the images or quality of the slides compromised the final results on the image analysis.

We also find some limitations collecting demographic data and subject history with the relatives. The lack of a specialised nurse or professional interviewer exclusively to our research team at the São Paulo Autopsy Centre (SVOC), limited us on the possibility of applying a more detailed questionnaire. In this case, missing bias in exposure history was the main limitation in the study population.

Confounding bias such as indoor exposure or pack of cigarettes per year could not be accessed.

An exploration of how much “history” is needed in terms of interpretation and predictive power is also required: it may be that in this domain, only the past n years of observation are required.

5.3.2 Method Limitations

The main limitation in the present study were accessing lung macrophage carbon.

We have performed our analysis using a different software analysis (Image pro Plus, The Proven Solution, Media Cybernetics Inc., USA) than the original method developed by Kulkarni et al, 2006 (Image J, National Institute of Health, MD, USA).

In the method performed by Kulkarni et al. (Kulkarni et al., 2006), digital colour images of 50 randomly chosen AM per subject with an intact cell wall were obtained using a light microscope, at 1000x (times) magnification under oil immersion. They had previously ascertained that 50 cells produced a reliable estimate of the median surface area of carbon. An image of a stage micrometre graticule was obtained at the same time using the same magnification. Analysis for cytoplasmic carbon was performed blinded. The analysis steps are illustrated in Figure 12 A and B, showing below. Each AM image was initially processed doing the following: first, the nucleus was removed from the image since the stained nucleus was identified as a large aggregate by the image analysis software. Second, Scion image software (Scion image, Scion, USA) was used to calculate carbonaceous particle area. The individual areas of carbon were added together to produce the total two-dimensional surface area of carbon for each AM. The median area of carbon per AM per subject (the primary measure), and the percentage of AM containing one or more area of carbon per subject were calculated from 50 cells.

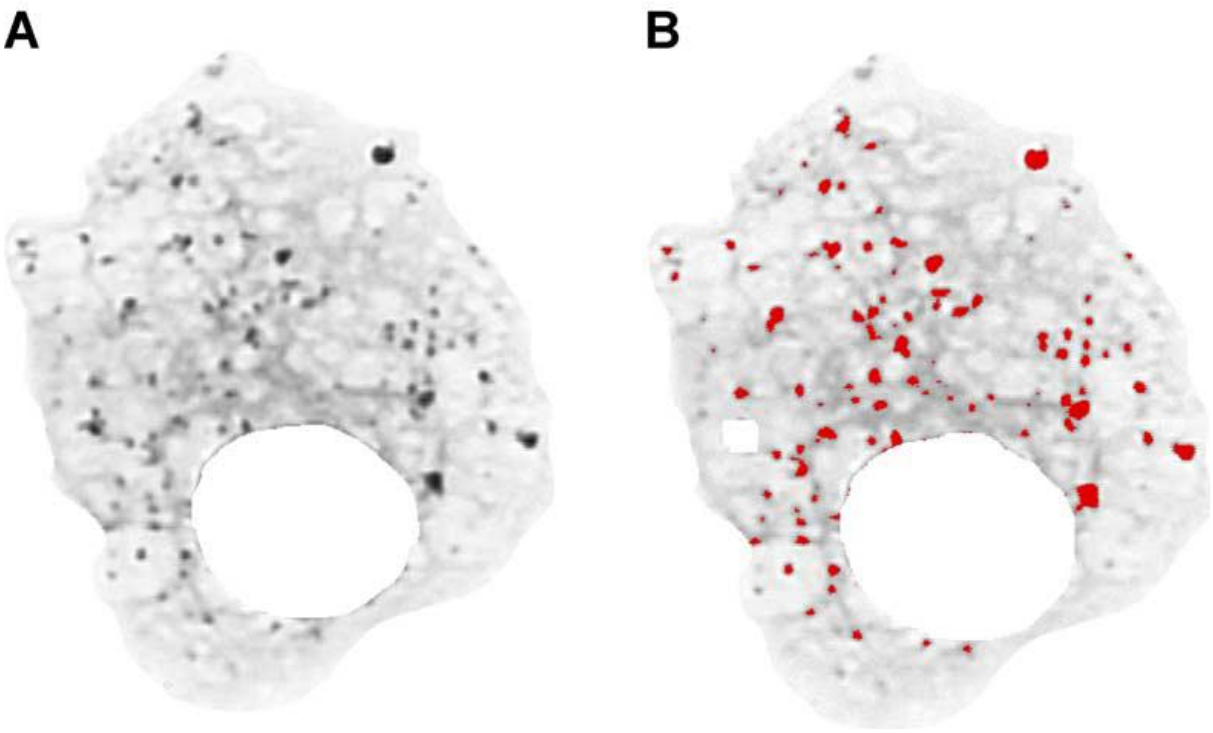

Figure 12: Represents image analysis of alveolar macrophages (AM) for cytoplasmic carbon using Image J (Kulkarni et al., 2006). (A) Digital image of AM obtained under oil immersion light microscopy (x1000). The black material in the cytoplasm is phagocytosed carbon. The AM nucleus has been cut using the image analysis programme. (B) Areas of carbon particles detected by the Scion image analysis software.

Following the same principles and following the method applied in Kulkarni's research, we performed our AM carbon (Figure 13) analysis with the method described on the Methods section above (section 3.2.2). Briefly, we selected digital images of 50 LM per lung specimen obtained using Pannoramic Viewer (3DHISTECH Ltd., Hungary) slide scanner, previously described above and showed at the representative picture (Figure 7), after that the images were analysed for AMC using the software Image Pro Plus Software (The Proven Solution, Media Cybernetics Inc., USA) scaling was calibrated using a sample of images. The intensity command was adjusted to obtain the best fit for carbon that was visible on the colour image. If the software selected nonblack areas

(usually areas of intense blue staining), these were automatically excluded from the analysis.

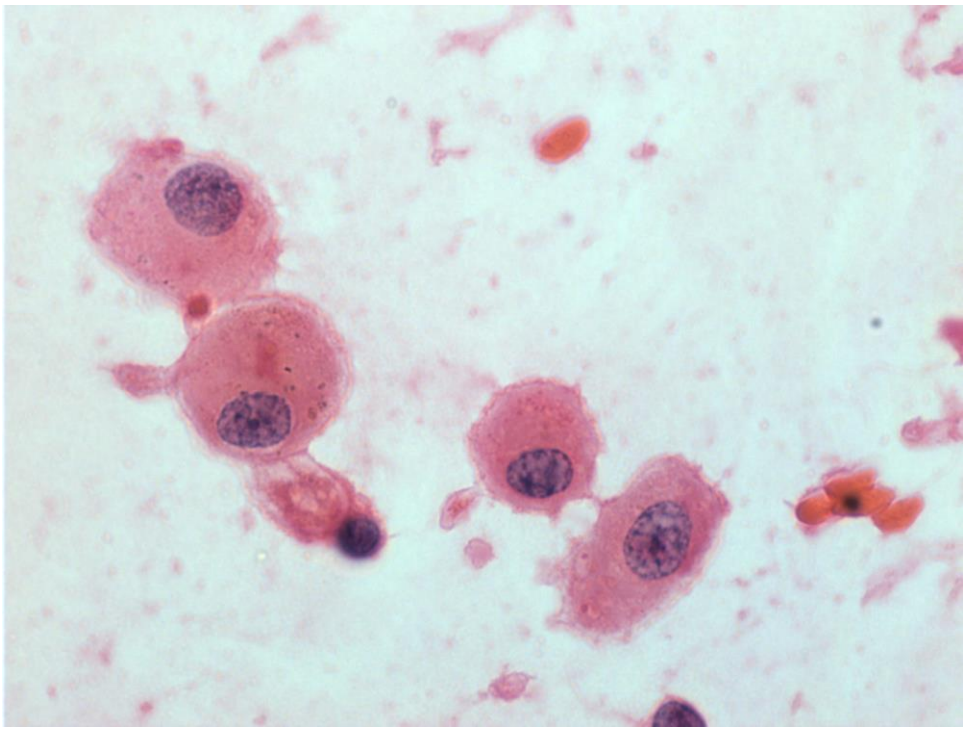

Figure 13: Image analysis accessing alveolar macrophages (AM) for cytoplasmic carbon using Image Pro Plus. Digital image of AM obtained using Pannoramic Viewer (x100). The black material in the cytoplasm is phagocytosed carbon.

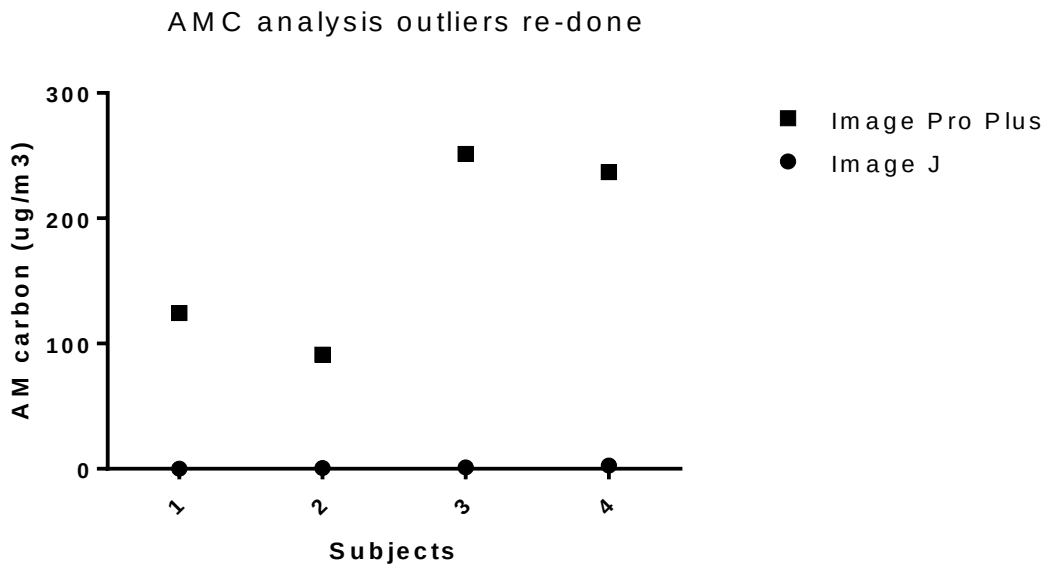

Figure 14: A Spearman correlation was performed in order to compare the discrepancy between the two methods in 4 (subjects) who presented high AMC results, (R= 0.600; P= NS).

Even reproducing the principles of the original method (Kulkarni et al., 2006) our study had few outliers on AMC final results. That can be explained by the fact the different software have different sensitivity calibration to capture black intensity on the macrophage images. Another explanation is the fact that the data was analysed by two different operators. That could lead to select different cells and give different results.

At Image Pro Plus method, the threshold command from the macro pictures used to calibrate the program is quite variable, what mean it can picks up only the very dark or more intense black areas and not areas with soft dark pigment, which certainly has accumulation of particle deposition but perhaps not as much as the most marked ones. Since you used a "yes or no" threshold command and not a black intensity calculation, that is mean colour density or integrated optical density, a possible uncertainty is

whether this cannot interfere on the final results. In theory, the clearer AMC that have been picked up at the threshold command and the ones presenting more darker areas should both have no difference on the final data, but perhaps, if evaluated the intensity of the colour, and maybe they can influence on the discrepancy at the final results that we could observe on the outliers.

This is a possible explanation for the lack of differentiation between the groups of smokers and non-smokers. And also could explain the differences between final AM areas, where the difference could not be the area occupied by black pigment, but the intensity of black areas captured.

Based on the evidences, we concluded that the most appropriated method is the method defined by Kulkarni and colleagues, using Image J as main tool.

5.4 Future Applications and Outlook

A large body of research has been undertaken which falls in the category of potential applications of large-scale epidemiological studies. These studies include both cohort and time-series studies that would be too large for the collection of individual scale information on time-activities and more detailed personal information that should be collected using validated questionnaires.

For long-term exposure a large range of health effects was for example studied by previous studies, including cerebrovascular events, and natural-cause mortality (Stafoggia et al. 2014; Beelen, Raaschou-Nielsen, et al. 2014).

Large studies have as well been undertaken for short-term health effects, such as for ischemic stroke and mortality (Maynard et al. 2007; Wellenius et al. 2012; Pope III &

Dockery 2006). Long-term exposure is to some extent the average of repeated exposures during a daily routine, and we should also take in consideration that for some health effects, caused by wholelife exposure can be important. It would be very difficult to reconstruct time-activity profiles of a population for such extended time periods, especially in a retrospective study using post-mortem individuals where we face some obstacles that can lead to missing data and bias interviewing relatives.

6. Conclusion

This study found that image analysis quantifies accumulation of PM carbon at the lung surface. We disproved our hypothesis that smoking is a major determinant of LSurC.

Although the most likely source of LSurC is environmental PM, we could not identify an association with markers of exposure.

In summary, we have shown that a combination of quantitative analysis of carbon on the lung surface (LSurC), histology, and assessment of carbon on alveolar macrophages (AMC) is a valid approach for evaluating the effects of airborne particles on the human lung to summarise and bring together the main areas covered in the writing, in a retrospective way, on the attempt to determine long-term effects of air pollution in the human lungs.

Attachments

Hospital das Clínicas da FMUSP
Comissão de Ética para Análise de Projetos de Pesquisa - CAPPesq

PROJETO DE PESQUISA

Título: A UTILIZAÇÃO DA AUTÓPSIA NA INVESTIGAÇÃO DE DOENÇAS HUMANAS E A SUA RELAÇÃO COM O MEIO AMBIENTE
Pesquisador Responsável: Paulo Hilário Nascimento Saldiva **Versão: 1**
Pesquisador Executante: Luis Fernando Amato Lourenço **CAAE:** 17261814.8.0000.0068
Co-autores: Regina Maura de Miranda, Paulo Afonso de André, Thais Mauad, Luiz Fernando Ferraz da Silva, Carlos Augusto Pasqualucci, Elaine Maria Frade Costa, Edson Amaro Junior, João Eduardo Ferreira, Lígia Vizeu Barrozo, Julio da Motta Singer, Michele Galhardoni Padovan.
Finalidade Acadêmica: Doutorado
Instituição: HCFMUSP
Departamento: PATOLOGIA

PARECER CONSUBSTANCIADO DO CEP

Registro on-line: 11621
Número do Parecer: 537.195
Data da Relatoria: 19/02/14
Apresentação do Projeto: Adequada.
Objetivo da Pesquisa: A utilização da autópsia na investigação de doenças humanas e a sua relação com o meio ambiente.
Avaliação dos Riscos e Benefícios: Positiva.
Comentários e Considerações sobre a Pesquisa: Projeto relevante.
Considerações sobre os Termos de apresentação obrigatória: Os termos apresentados estão de acordo.
Recomendações: Nenhuma.
Conclusões ou Pendências e Lista de Inadequações: Nenhuma.
Situação do Parecer: Aprovado.
Necessita Apreciação da CONEP: Não.
Considerações Finais a critério do CEP: Em conformidade com a Resolução CNS nº 466/12 – cabe ao pesquisador: **a)** desenvolver o projeto conforme delineado; **b)** elaborar e apresentar relatórios parciais e final; **c)**apresentar dados solicitados pelo CEP, a qualquer momento; **d)** manter em arquivo sob sua guarda, por 5 anos da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo CEP; **e)** encaminhar os resultados para publicação, com os devidos créditos aos pesquisadores associados e ao pessoal técnico participante do projeto; f) justificar perante ao CEP interrupção do projeto ou a não publicação dos resultados.

São Paulo, 19 de Fevereiro de 2014

PROF. DR. ALFREDO JOSÉ MANSUR
Coordenador
Comissão de Ética para Análise de Projetos de Pesquisa - CAPPesq

Rua Dr. Ovídio Pires de Campos, 225 - Prédio da Administração - 5º andar
CEP 05403-010 - São Paulo - SP.
55 11 2661-7585 - 55 11 2661-6442 ramais: 16, 17, 18 | marcia.carvalho@hc.fm.usp.br

Attachment A. Official report of the Research Ethics Committee of the Faculty of Medicine, University of São Paulo signed and approved

References

A Scourge Returns Black Lung in Appalachia. https://ehp.niehs.nih.gov/124-A13/

Abbey DE, Burchette RJ, Knutsen SF, McDonnell WF, Lebowitz MD, Enright PL. Longterm particulate and other air pollutants and lung function in nonsmokers. Am J Respir Crit Care Med. 158(1):289-98. 1998.

Arnold C. A scourge returns. Black lung in Appalachia. Environ Health Perspect; 124:A13–8. 2015.

Bai Y, Brugha RE, Jacobs L, Grigg J, nawrot T, Nemery B. Carbon loading in airway macrophages as a biomarker for individual exposure to particulate matter air pollution – A critical review. Environ Int; (74): 32-41. 2015.

Barnes PJ; 372s:631-640. Cellular and molecular mechanisms of chronic obstructive pulmonary disease. Clinics in Chest Medicine 35:71–86. doi: 10.1016/j.ccm.2013.10.004, 2014.

Belli JA, Bose S, Aggarwal N, DaSilva C, Thapa S, Grammer L, Paulin LM, Hansel NN. Indoor particulate matter exposure is associated with increased black carbon content in airway macrophages of former smokers with COPD. Environ Research; 150: 398-402. 2016.

Bernstein JA, Alexis N, Barnes C, Bernstein IL, Bernstein JA, Nel A, Peden D, Diaz-Sanchez D, Tarlo SM, Williams PB. Health effects of air pollution. J Allergy Clin Immunol. 2004.

Brunekreef & Holgate. Air pollution and health. Lancet, 2002.

Bunn HJ, Dinsdale D, Smith T, Grigg J. Ultrafine particles in alveolar macrophages from normal children. Thorax 2001; 56 (12): 932-4.

Carter BD, Abnet CC, Feskanich D, Freedman ND, Hartge P, Lewis CE, Ockene JK, Prentice RL, Speizer FE, Thun MJ, Jacobs EJ. Smoking and mortality—beyond established causes. New England Journal of Medicine; 372s:631–640. 2015

CETESB (São Paulo Environmental Agency) (2010) Report of air quality in the State of Sao Paulo 2009 [in Portuguese]. Sao Paulo: CETESB. 290 p.

Chacraborty J, Schweitzer LA, Forkenbrock DJ. Using GIS to assess the Environmental Justice consequences of transportation system changes. Transactions GIS 1999; 3:239-58.

Churg A, Myers JL, Tazelaar H, Wright JL.. Thurlbeck's pathology of the lung. 3rd edition, New York, NY: Thieme. 2005.

de Kok TM, Driece HA, Hogervorst JG, Briedé JJ. Toxicological assessment of ambient and traffi c-related particulate matter: a review of recent studies . Mutat Res . 2006; 613(2-3): 103- 122.

Delfino RJ, Zeiger RS, Seltzer JM, Street DH. Symptoms in pediatric asthmatics and air pollution: differences in effects by symptom severity, anti-inflammatory medication use and particulate averaging time. Environ Health Perspect. 1998

Delfino RJ. Epidemiologic evidence for asthma and exposure to air toxics: linkages between occupational, indoor, and community air pollution research. Environ Health Perspect. 2002

Dockery DW , Pope CA III , Xu X , et al . An association between air pollution and mortality in six U.S. cities . N Engl J Med . 1993 ; 329 (24): 1753 - 1759 .

EPA, 1996. https://www3.epa.gov/scram001/guidance/guide/policymem33d.pdf

Every Breath We Take. Evidence submitted to the Royal College of Physicians https://www.rcplondon.ac.uk/projects/outputs/every-breath-we-take-lifelong-impact-air pollution 2016.

Finch GL, Hobbs CH, Blair LF, Barr EB, Hahn FF, Jaramilllo RJ, et al. Effects of subchronic inhalation exposure of rats to emissions from a diesel engine burning soybean oil-derived biodiesel fuel. Inhal Toxicol 2002; 14 (10): 1017-48.

Gerber A, Hofen-Hohloch A V, Schulze J, Groneberg D A, Tobacco smoke particles and indoor air quality (ToPIQ-II) – a modified study protocol and first results. Journal of Occupational Medicine and Toxicology, 2015; 10:5.

Green, G. M. Alveolobronchiolar transport mechanisms. Arch. Intern. Med. 131: 109. 1973

Gunier RB, Hertz A, Von-Behren J, Reynolds P. Traffic density in California: socioeconomic and ethnic differences among potentially exposed children J Expo Anal Environ Epidemiol 2003; 13:240-6.

Habermann M., Gouveia N. Socioeconomic Position and Low Birth Weight among Mothers Exposed to Traffic-Related Air Pollution. Plos One 9(11); 2014.

Hiraiwa, K. & Van Eeden, S. F. 2013. Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants. Mediators Inflamm, 2013, 619523.

Hoek G. Association between mortality and indicators of traffic-related air pollution in the Netherlands: a cohort study. Lancet, 2002.

How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). Atlanta (GA): Centers for Disease Control and Prevention (US); 2010.

IBGE, Brazilian Institute of Geography and Statistics. http://www.ibge.gov.br/english/estatistica/populacao/censo2010/default.shtm

International Agency for Cancer Research, 2013. http://www.iarc.fr/en/media-centre/pr/2013/pdfs/pr223_E.pdf

Jacobs L, Emmerechts J, Hoylaerts MF, Mathiew C, Hoet PH, Nemery B, Nawrot TS. Traffic air pollution and oxidized LDL. Plos One 2011; 6(1): e1620.

James Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema. E life Sciences, 2015.

Kato A, Kagawa J. Morphological effects in rat lungs exposed to urban roadside air. . Inhal Toxicol. 2003; (8):799-818.

Kulkarni NS, Prudon B, Pandotti SL, Abebe Y, Grigg J. Carbon loading of alveolar macrophages in adults and children exposed to biomass smoke particles. Sci Total Environment 2005; 45 (1-3):23-30.

Kulkarni N., Pierse N., Rushton L,, Grigg J. Carbon in Airway Macrophages and Lung Function in Children. New England Journal of Medicine; 2006. Vol 355:21-30.

Lim S et al. (2012) „A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010): a systematic analysis of the Global Burden of Disease Study 2010." The Lancet 380: 2224-60. 2010

Martonen TB, Bell KA, Phalen RF, Wilson AF, Ho A. Growth rate measurements and deposition modelling of hygroscopic aerosols in human tracheobronchial models. Ann Occup Hyg. 1982

Matter and Tissue Remodeling in Human Lung. Environmental Health Perspectives, November, 2000. Vol. 108 n. 11.

Maynard D, Coull BA, Gryparis A, Schwartz J. Mortality risk associated with short-term exposure to traffic particles and sulfates. Environ Health Perspect. 2007

Mills NL, Törnqvist H, Robinson SD, Gonzalez MC, Söderberg S, Sandström T, Blomberg A, Newby DE, Donaldson K. Air pollution and atherothrombosis. Inhal Toxicol; 1:81-9. 2007.

Niewoehner DE, Kleinerman J, Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. N Engl J Med. 1974; 291(15):755-8.

Nwokoro C, Ewin C, Harrison C, Ibrahim M, Dundas I, Dickison I, Mushtaq N, Grigg J. Cycling to work in London and inhaled dose of black carbon. Eur Respir J 2012; 40 (5): 1091-7.

Pearson RL, Wachtel H, Ebi KL. Distance-weighted traffic density in proximity to a home is a risk factor for leukemia and other childhood cancers. J Air Waste Manag Assoc 2000; 50:175–180.

Pepelko WE, Mattox JK, Yang YY, Moore W Jr. Pulmonary function and pathology in cats exposed 28 days to diesel exhaust. J Environ Pathol Toxicol., 1980. (2-3):449-57.

Perfetti TA, Rodgman A. 2013. The chemical components of tobacco and tobacco smoke. 2nd edition, Boca Raton, FL: CRC Press.2013

Pinkerton, K. E., Green, F. H. Y., Saiki, C., Vallyathan, V., Plopper, C. G., Gopal, V., Hung, D., Bahne, E. B., Lin, S. S., M??Nache, M. G. & Schenker, M. B. 2000. Distribution of particulate matter and tissue remodeling in the human lung. Environmental Health Perspectives.

Pope, C. A., Thun, M. J., Namboodiri, M. M., Dockery, D. W., Evans, J. S., Speizer, F. E. & Heath, C. W. 1995. Particulate air pollution as a predictor of mortality in a prospective study of US adults. American Journal of Respiratory and Critical Care Medicine, 151, 669-674.

Pope CA III, Burnett RT, Turner MC, Cohen A, Krewski D, Jerrett M, Gapstur SM, Thun MJ. Lung cancer and cardiovascular disease mortality associated with ambient air pollution and cigarette smoke: shape of the exposure-response relationships. Environmental Health Perspectives 119:1616–1621. 2011.

Pope CA III, Dockery DW. Health effects of fine particulate air pollution: lines that connect. J Air Waste Manag Assoc 2006;56:709-42.

Pratt, P C and Kilburn, K H. extend of pigmentation in autopsied human lungs as an indicator of particulate environmental air pollution. Chest 1971; 59, Suppl: 39S+

Reynolds P, Behren JV, Gunier RB, Goldberg DE, Hertz A, Smith D. Traffic patterns and childhood cancer incidence rates in California, United States. Cancer Causes Control 2002; 13:665-73.

Robert D. Brook et al. Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement from the American Heart Association. Circulation. 2010;121:2331-2378. 10th May 2010.

Ryman-Rasmussen, Jessica P, Cesta, Mark F, Brody, Arnold R, Shipley-, Jeanette K, Everitt, Jeffrey,, Tewksbury, Earl W , Moss, Owen R ,Wong, Brian a , Darol, E , Andersen, Melvin E, Bonner, James C. Inhaled Carbon Nanotubes Reach the Sub-Pleural Tissue in Mice. NIH Public Access; 747-751, 2010.

Saieg MA, Cury PM, Godleski JJ, Stearns R, Duarte LG, D'Agostino L, Kahn H, Pinto EM, Mauad T, Saldiva PH, Bernardi FD. Differential elemental distribution of retained particles along the respiratory tract. Inhal Toxicol. 2011 Jul; 23(8):459-67.

Salvi S. Tobacco smoking and environmental risk factors for chronic obstructive pulmonary disease. Clinics in Chest Medicine 35:17–27. 2014.

Saxena RK, McClure ME, Hays MD, Green FH, McPhee LJ, Vallyathan V, Gilmour MI. Quantitative assessment of elemental carbon in the lungs of never smokers, cigarette smokers, and coal miners. J Toxicol Environ Health A., 2011. 74(11):706-15.

Scarborough P, Bhatnagar P, Wickramasinghe K et al. The economic burden of ill health due to diet, physical inactivity, smoking, alcohol and obesity in the UK: an update to 2006–07 NHs costs. Journal of Public Health, May 2011.

Schwartz J . Short term fl uctuations in air pollution and hospital admissions of the elderly for respiratory disease . Thorax . 1995 ; 50 (5): 531 - 538 .

Souza MB, Saldiva PH, Pope CA 3rd, Capelozzi VL. Respiratory changes due to long-term exposure to urban levels of air pollution: a histopathologic study in humans. Chest. 1998 May; 113(5):1312-8)

Stafoggia M, Cesaroni G, Peters A, Andersen ZJ, Badaloni C, Beelen R, Caracciolo B, Cyrys J, de Faire U, de Hoogh K, Eriksen KT, Fratiglioni L, Galassi C, Gigante B, Havulinna AS, Hennig F, Hilding A, Hoek G, Hoffmann B, Houthuijs D, Korek M, Lanki T, Leander K, Magnusson PK, Meisinger C, Migliore E, Overvad K, Ostenson CG, Pedersen NL, Pekkanen J, Penell J, Pershagen G, Pundt N, Pyko A, Raaschou-Nielsen O, Ranzi A, Ricceri F, Sacerdote C, Swart WJ, Turunen AW, Vineis P, Weimar C, Weinmayr G, Wolf K, Brunekreef B, Forastiere F. Long-term exposure to ambient air pollution and incidence of cerebrovascular events: results from 11 European cohorts within the ESCAPE project. Environ Health Perspect. 2014

Tour M David B Corry Farrah Kheradmand. Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema. eLife 2015;4:e09623.

U.S. Department of Health and Human Services, Public Health Service, The Health Consequences of Smoking; A Public Health Service Review: 1967

Wang B, Ho SSH, Ho KF, Huang Y, Chan CS, Feng NSY. An environmental Chamber study of the characteristics of air Pollutants released from environmental tobacco smoke. Aerosol and Air Quality Research. 2012.

Weigert C (1898) Über eine Methode zur Färbung elastischer Fasern. Zentralbl. Allg. Pathol. 9, 289-292.ing and Health; 1984. DHHS Publication No. (PHS) 84-50205.

Weinmayr GC, Wolf K, Brunekreef B, Forastiere F. Long-term exposure to ambient air pollution and incidence of cerebrovascular events: results from 11 European cohorts within the ESCAPE project. Environ Health Perspect. 2014

World Health Organisation 2016 http://www.who.int/gho/publications/world_health_statistics/2016/EN_WHS2016_Chapter3.pdf?ua=1

World Health Organisation 2014 http://www.who.int/mediacentre/factsheets/fs310/en/

World Health Organisation 2013. http://www.who.int/whr/2013/report/en/

World Health Organisation: Air Quality Guidelines - Global Update 2005 www.who.int/phe/health_topics/outdoorair/outdoorair_aqg

Wellenius GA, Burger MR, Coull BA, Schwartz J, Suh HH, Koutrakis P, Schlaug G, Gold DR, Mittleman MA. Ambient air pollution and the risk of acute ischemic stroke. Arch Intern Med. 2012

You R, Wen Lu Ming Shan Jacob M Berlin Errol LG Samuel Daniela C Marcano Zhengzong Sun William KA Sikkema Xiaoyi Yuan Lizhen Song Amanda Y Hendrix