The Air Pollution Impact to Maternal Mice and Offspring

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Certificate of original authorship

I, Baoming WANG declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy (PhD), in the School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

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Publications arising from my PhD candidature:

Unfortunately I started my PhD in a group which was not able to supervise me correctly, and after one year I changed supervisors and group to Prof Oliver. The data in my thesis is from only the work which I did as part of Prof Oliver's group.

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Conference Presentations

1. Thoracic Society of Australia and New Zealand meeting (2018): Poster presentation

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Maternal L-Carnitine Supplement Relieves Lung Damage in Offspring from Cigarette Smoke Exposed Mothers

2. Centre for Air pollution, energy and health Research (2019): Poster presentation

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Chronic low dosage of maternal particulate matter exposure can affect dam's and offspring's lung health

3. New Horizon 2019: Poster presentation

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver L-Carnitine mitigates impact of maternal smoking on lung health in mice offspring

4. Thoracic Society of Australia and New Zealand branch meeting (2019): Oral presentation

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Maternal exposure to low dose particulate matter induced transgenerational hyperresponsiveness in mice

5. 19th NSW Asthmatic Meeting (2019): Poster presentation

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Maternal exposure to low dose particulate matter induced transgenerational hyperresponsiveness in mice

6. European Respiratory Society Meeting (2019) :

Poster presentation:

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Impact of chronic maternal particulate matter exposure during pregnancy on offspring's lung health

Poster discussion:

Baoming WANG, Yik Lung Chan, Sonia Saad, Hui Chen, and Brian Oliver. Impact of chronic maternal particulate matter exposure during pregnancy on offspring's lung health

Awards and Prizes

2016: 4 years PhD scholarship from China Scholarship Council

2019: Best presentation from Thoracic Society of Australia and New Zealand branch meeting

2019: Best poster from 19th NSW Asthmatic Meeting

Abstract

Epidemiological studies have shown that maternal exposure to cigarette smoke and air pollution are two predominant *in utero* environmental toxicants which can increase the risk of developing multiple respiratory diseases in the offspring. The proposed mechanisms include reducing mitochondrial function and mitochondrial renewal mechanisms (mitophagy) and activating inflammasome and other inflammatory pathways. However, whether maternal smoking could induce the sex-dependent susceptibility in respiratory disorders and whether chronic low dose particulate matter (PM) exposure which is within the international standard could induce any transgenerational pulmonary disease has not been widely studied.

Firstly, Female Balb/c mice (8 weeks) were exposed to cigarette smoke (SE) for 6 weeks prior to mating, during gestation and lactation. Half of the SE dams (mothers) were given L-Carnitine supplementation (1.5mM in drinking water, SE+LC) during gestation and lactation. Then, another Male Balb/c Mice (6 weeks, Animal Resources Centre, WA, Australia) batch was intranasally exposed to saline or traffic-related PM_{10} (1 μg or 5 μg/day) for 3 weeks. Furthermore, the female BALB/c mice (6 weeks) were exposed to $PM_{2.5}$ ($PM_{2.5}$, 5 μg/day) or saline (SHAM) 6 weeks before pregnancy and during pregnancy and lactation; or for only 6 weeks before pregnancy (Cessation, 5 μg/day). Lung tissues from models were analysed.

Results: Compared to female offspring, maternal SE significantly increased levels of inflammatory markers (phosphorylated(p)-extracellular signal-regulated kinase (ERK1,2), p-p38 Mitogen-activated protein kinase (P38) MAPK, p-Mitogen-activated protein kinase (NF-kB). Three weeks of PM exposure (5 μg/day) significantly increased total macrophages and lymphocytes number in the bronchoalveolar lavage fluid (BALF) accompanied by increased levels of NLRP3 and Interlukin-1 (IL1-β). Chronic exposure to low dose PM significantly increased tissue elastance and damping during lung function tests, followed by increased leukocytes in the BALF, mitochondrial dysfunction, and airway remodelling, including alveolar membrane damage and increased collagen deposition. Maternal exposure to low dose

PM also significantly increased tissue elastance and damping during lung function test followed by increased leukocytes in the BALF and mitochondrial dysfunction without airway remodelling. The mouse model of asthma induced by olvabumin (OVA) showed that maternal exposure to the low dose PM could significantly increase tissue elastance during lung function test in the offspring, suggesting the worse asthmatic symptoms.

In conclusion, male offspring are more susceptible to the adverse effects of maternal smoking. Chronic exposure to the low dose PM could induce chronic obstructive pulmonary disease (COPD)-likes pathology in the dams and worsen asthmatic symptoms in the female offspring.

Chapter 1 General Introduction

Section 1.2 has been published as a review article in,

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- Writing original draft and finalising the printed version

1.1 Ambient particulate matter in air pollution

1.1.1 Different sources of PM

Air pollution plays an important role in the global burden of disease. Epidemiological cohort studies have identified that Sulphur dioxide[1], Nitrogen dioxide[2], Nitrous oxide[3], Carbon monoxide[4], Ozone[5], and particulate matter (PM) are the top 6 factors in air pollution and can induce various diseases. Those components of air pollution have diverse health impacts, such as the development of asthma, chronic obstructive pulmonary disease (COPD) and even lung cancer [6]. When compared with other components of air pollution, PM was detected as the main putative culprit to cause morbidity and mortality [7,8]. More than 3 million global premature deaths each year can be attributed to the PM pollution[9].

The prenatal stage, crucial to the organogenesis of the developing foetus, is highly susceptible to the environmental toxicants exposure [10]. Current study proved that the chemical composition on the PM surface can be transferred to the foetal circulation via blood placental barrier and induce various adverse impacts in the offspring[11]. Particulate matter, especially fine PM with a diameter of less than ten microns (PM_{10}), has significant adverse effects on pregnant women[12]. The underlying mechanisms for the influence of maternal PM exposure on adverse birth outcomes are not clearly understood. The current consensus opinion is that it is related to oxidative stress and inflammation during pregnancy[13,14].

Although PM can be produced through volcanic eruption and forest fire, the main source of PM comes from anthropogenic activities. These pollutants can be broadly divided into two categories: indoor and outdoor contaminants and both categories consist of similar particulate matter[15]. During the global urbanization, emissions from vehicle exhausts have become an important source of outdoor air pollution in both developed and developing countries. Urbanisation has resulted in residents living within 500 metres of busy roads which exposes them to a variety of adverse respiratory health outcomes[16,17].

Indoor air pollution mainly comes from residential energy use such as heating and cooking which is prevalent in India and China, where it has the largest impact on premature mortality globally. Acute respiratory infections in children and patients with COPD are also strongly associated with living in poorly ventilated homes[18].

1.1.2 Classification of PM

Researchers define the inhaled fraction of ambient PM as PM_{10} with a median aerodynamic diameter less than 10um. According to the size, PM_{10} can be further divided into three major fractions based on the size: coarse PM (2.5-10 um) which can easily deposit in the upper airways, being removed by mucociliary clearance; fine PM (0.1-2.5 um), and ultrafine particulate matter(<0.1 um), which can reach deep into the lung (ie. alveoli) [19] and can even reach other organs through blood circulation. Suspended PMs with a median diameter of less than 2.5 um are defined as $PM_{2.5}$, including the fine and ultrafine particles. Sometimes, PM_1 (median diameter less than 1 um) is also be used. In fact, due to the complexity of PM, the content of PM_{10} also encompasses more than 50% of fine and ultrafine particles. Furthermore, most researchers believe that the smaller components ($PM_{2.5}$ or $PM_{0.1}$) in PM_{10} have higher toxicity compared to PM_{10} itself [20]. Furthermore, smaller particles have a larger surface area which can adsorb more metal and other toxic components.

Many publications have explored the health risks of exposure to various sizes of PM. The study havs proved that $PM_{2.5}$ has a higher chance to be retained in the airways and alveoli [21]. This study was further confirmed by the analytical electron microscopy measurements, which showed that $PM_{2.5}$ could retain in the lung parenchyma with more than 90% efficiency. Numerous studies also found that increasing in $PM_{2.5}$ exposure during pregnancy could significantly increase various respiratory diseases in the offspring, such as asthma, COPD [22,23]. Therefore, the size of the PM plays an important role in the cytotoxic effects.

During the PM forming, carbonaceous cores will be surrounded by the diverse chemicals, minerals and organic components. PM produced in different cities and countries is produced from different sources such as combustion sources and climate change. Because of this PM can also be defined by chemical composition in addition to classifying by size. Suspended PM consists of a mixture of organic material (polycyclic aromatic hydrocarbons (PAH) and endotoxins), minerals (quartz and silicates), salts (sulphates and nitrates), and other inorganic components (transition metals)[24].

No single compound in the PM was identified to explain the cytotoxic effects of PM. The biotic contaminants, such as endotoxin, allergens, and pollen fragments, can be adsorbed on the carbon core of PM[25]. A study in Germany suggested that the concentration of endotoxin in coarse particles were 10-fold higher than $PM_{2.5}$[26]. Endotoxin exerts its effects on the intact and isolated lung cells as well, such as activating Toll-like receptors (TLR2/4) signaling pathways which are closely associated with asthma [27]. In animal models, endotoxin was also found to change the physiological functions of both airways and pulmonary circulations resulting in changes in lung function[28]. $PM_{2.5}$ with polycyclic aromatic hydrocarbons (PAH)-like characteristics can form an undesired mutagenic risk[29]. In mice, high PAH concentration could induce higher a chance of mutations[30]. Another study demonstrated that PAH can even cross the blood placental barrier and interfere with the development process of the foetus. Metals, another contaminant on the surface of PM, can penetrate cellular organelles and interfere with their functions [31]. For example, lead could accelerate Ca2+ release from the cell and reduce membrane potential, leading to the reduced mitochondrial function and further cellular dysfunction [31]. Metals, like iron, copper and chromium could induce redox cycling and further damage cell function. Other metals like lead, cadmium could cause oxidative stress by using up the antioxidants. It has been shown that these metals caused an increase in the production of ROS, such as hydroxyl radical (HO^-), leading to oxidative stress in cardiopulmonary

damage[32]. The same process may also happen in the other organs, including the lung, liver and even the growing foetus [33].

1.1.3 Inflammation in the lung

Inhalable PM smaller than 10μm (PM_{10}) can reach the lower airways, while smaller particles can reach the alveoli and are therefore considered to be more damaging to the lung [34]. The inhalation of PM regardless of the size can induce oxidative stress and result in increased inflammation [35]. Nuclear factor-kB (NF-κB) and Mitogen-activated protein kinase (MAPK) are the common inflammatory signalling pathways which could be activated through the endocytosis or phagocytosis of $PM_{2.5}$ [36,37]. NF-κB, is a major transcription factor that is crucially involved in inflammation, apoptosis, and proliferation in lung [38]. A previous study found that $PM_{2.5}$ exposure triggered nuclear translocation, DNA-binding, and transcriptional activation of the NF-κB pathway in human alveolar epithelial cell line A549 [39]. NF-κB can also activate MAPK signalling cascades, including extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK), and p38 Mitogen-activated protein kinase (p38) [40]. The activated NF-κB and MAPK pathways result in the release of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), leading to heightened inflammatory responses.

In order to avoid the excessive damage induced by inflamaiton, autophagy is a selfregulation mechanism important for stress adaptation and cellular homeostasis [41]. Autophagy can modulate inflammation through eliminating unwanted intracellular components, eg. proteins, to reduce the inflammatory stimuli to suppress the inflammation [42]. $PM_{2.5}$ has been shown to induce autophagy in A549 cells through oxidative stress [43].

1.2 Intrauterine exposure to particulate matter and cigarette smoke and asthma

Why do intrauterine exposure to air pollution and cigarette smoke increase the risk of asthma?

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Abstract

The prevalence of childhood asthma is increasing worldwide and increased in-utero exposure to environmental toxicants may play a major role. As current asthma treatments are not curative, understanding the mechanisms underlying the aetiology of asthma will allow better preventative strategies to be developed. This review focuses on the current understanding of how in-utero exposure to environmental factors increases the risk of developing asthma in children. Epidemiological studies show that maternal smoking and particulate matter exposure during pregnancy are prominent risk factors for the development of childhood asthma. We discuss the changes in the developing foetus due to reduced oxygen and nutrient delivery affected by intrauterine environmental change. This leads to foetal underdevelopment and abnormal lung structure. Concurrently an altered immune response and aberrant epithelial and mesenchymal cellular function occur possibly due to epigenetic reprogramming. The sequelae of these early life events are airway remodelling, airway hyperresponsiveness, and inflammation, the hallmark features of asthma. In summary, exposure to inhaled oxidants such as cigarette smoking or particulate matter increases the risk of childhood asthma and involves multiple mechanisms including impaired foetal lung development (structural changes), endocrine disorders, abnormal immune responses, and epigenetic modifications. These make it challenging to reduce the risk of asthma, but knowledge of the mechaisms can still help to develop personalised medicines.

Keywords: asthma; foetus; placental; smoking; particulate matter.

Introduction

Asthma is a disease that generally affects 5-20% of children globally (1, 2). It is a complex condition in which symptoms are mainly caused by bronchoconstriction (3). Airway constriction occurs rapidly in response to a variety of inhaled substances, for example, allergens such as pollen and house dust mite, and environmental sources such as dust and smoke, which usually can be fully or partially reversed by bronchodilators. Pathologically it is defined by airway remodelling, typified by increased smooth muscle and epithelial layer thickness, and increased numbers of inflammatory cells. However, the type of inflammation varies. For example, sputum based phenotyping of inflammation categorises people into eosinophilic, neutrophilic, or paucigranulocytic asthma. The other factors that can add to the complexity of asthma including the age of onset, aetiological cause (if known), co-existence of other respiratory diseases, comorbidities, the degree of reversibility, and the ability for the symptoms being effectively controlled by pharmaceutical interventions.

The susceptibility to asthma is complex, which involves both genetic sucipitibility, environmental insults (both pre and post birth), and is further complicated by asthma syptoms initating and sometimes ceasing at different ages, as well as differences in asthma prevalence between the male and female sexes.

It is known that boys are more susceptible than girls before puberty, but less than girls after puberty. Many therories exsist to explain this phenomina including: dysnapsis due to different sized lungs in boys and girls, increased allergy (more IgE production in boys), different innate and adaptive immune responces in boys and grils, and the influence of sex hormones (4-6). The incidence of asthma is also related to the use of life saving medical inteventions in premiture and newborn children such as oxygen supplementation or mechanical ventilation due to physical permanent damage to the newborn's lungs (7).

However, it has increasingly been recognised that certain factors during the intrauterine period affects childhood asthma susceptibility. In particular, maternal smoking (MSE) and particulate matter (PM) exposure (8, 9), are the best described/researched *in-utero* challenges which affect asthma sucipitibility. This review will discuss the current understanding of multiple mechanisms underlying these two factors, which may help to develop personalised medicines.

Epidemiology of asthma

The prevalence of allergic disorders has been rising since the early 1980s. The average global rate of allergic disorders is 22%, ranging from 15%-35% of the population in different countries (10). According to the WHO, the number of children with asthma is around 14% globally (11). Severe asthma is common in children. A recent study reported that the prevalence of severe asthma was 4.9% in 6-7 years old children, however, the incidence was increased to 6.9% in 13-14 years olds. These phenomena demonstrated that age is an important factor for the onset of asthma (12).

Environmental toxicant exposure during pregnancy is a significant factor that has been shown to increase the incidence of asthma (13). In particular, maternal smoke exposure (MSE) is the largest modifiable risk factor for the development of asthma. Although the harmful effect of smoking is well-known in the general public, smoking mothers find it difficult to quit due to nicotine addiction, even during pregnancy when nicotine metabolism is faster than non-pregnant status(14). A systematic review and meta-analysis in the Lancet showed that the top 3 countries with the highest smoking rate during pregnancy are Ireland (38.4%), Uruguay (29.7%) and Bulgaria (29.4%) (15). Even in Australia where anti-smoking legislation is one of the most aggressive in the world, the smoking rate in pregnant women is 11.7% (16).

Epidemiological studies have demonstrated a dose-dependent increase in asthma risk in offspring due to MSE (Table 1). Currently, several cohort studies have confirmed the association between MSE and asthma risk in the offspring (17-20). For example, a birth cohort study has found that women smoking during pregnancy could increase asthma incidence in the offspring with an adjusted hazard ratio of 1.79 (95% CI 1.20–2.67) (21). The same outcome has been found in another cohort study where MSE during pregnancy caused higher asthma risk in the child in the first year of life with an odds ratio (OR) of 1.83 (22). Similarly, a systematic review of 14 studies revealed a wheezing (OR 1.41 (95% CI 1.19–1.67)) and asthma risk (OR 1.85 (95% CI 1.35–2.53)) in 2 years old and younger children, followed by a higher asthmatic risk in 5–18 years old children (OR 1.23 (95% CI 1.12–1.36)) caused by smoking during pregnancy (18). One study found a strong asthma risk in 14 year old girls whose mothers smoked during pregnancy, however this was not found in boys (23); whereas a different study found that boys at the age of 11 are more susceptible to the maternal and postnatal secondhand smoke (24). These differences might be related to the changes in asthma prevelance in boys and girls around puberty.

Around 91% of the world's population are living in the areas where the levels of air pollutants exceed the WHO limits (25). Epidemiological studies demonstrated a strong association between pulmonary disease and particular matter (PM) exposure(9). Compared to cigarette smoking which can be avoided through quitting, the dangers of airborne pollution are hard to avoid in heavily polluted countries, such as China and India. In China, 74,000 premature deaths were attributed to $PM_{2.5}$ exposure in the year 2013 (26). It was estimated that 22% of these deaths could have been avoided if indoor $PM_{2.5}$ level met National Class I standards (26).

There are many different types of airborne pollution, but simplistically these can be divided into gasses and particulate matter (PM). PM is considered as particularly dangerous as respirable particles can remain airborne over large distances.

As shown in Table 2, prenatal PM exposure is also associated with childhood asthma. A cohort study found that prenatal PM_{10} exposure could cause pulmonary function changes with higher minute ventilation in newborns (27). Another birth cohort study including pre-school and school-age children demonstrated that prenatal PM_{10} exposure increased the risk of developing asthma in both age groups, especially for those pregnant mothers who lived near the highways (28). The correlation between maternal PM exposure and asthma risk in different genders was also investigated. High levels of $PM_{2.5}$ exposure during mid-gestation increased the development of asthma by the age of 6 years in boys, but not in girls (29). The above evidence indicates that maternal PM exposure during pregnancy has similar effects to MSE in terms of increasing the risks of developing asthma in childhood.

The difference of asthma prevalence between boys and girls and the change in prevalence which occurs around pubertiy naturally gives credance to the involvement of sex hormones. Animal models of estrogen receptor knockouts suggests that estrogen promotes the development of the asthma (30); while male mice lacking testosterone showed more severe asthma symptom (31). These studies help to explain why boys are more susceptible to asthma before puberty, and girls more susceptible after puberty. However, the eitology of asthma is complex and is multifactorial.

The role of oxidative stress in the development of asthma in children

Various chemicals can be found in both cigarette smoke and PM. It is unlikely that a single chemical is responsible for all the adverse effects of in-utero exposure to cigarette smoke or

PM on lung health in the offspring. Cigarette smoke and PM are two major environmental sources of inhaled free radicals and strong oxidants. The balance between excessive oxidant activity and the antioxidant capacity can tip in favour of excess oxidants causing oxidative stress. However, it is important to note that the production of oxidants is necessary to maintain healthy cell function, and important in regulating processes such as inflammatory responses. Oxidative stress induces adverse effects in tissues. The developing foetus is highly vulnerable to oxidative stress injury, as the immune system remains immature during the prenatal period (32). Free radicals and chemicals inhaled during MSE and maternal PM exposure can pass the blood-placental barrier to directly increase the level of oxidative stress in the offspring. Therefore, we propose the first common and prominent mechanism underlying these two factors to induce pathological changes in the offspring is oxidative stress.

Our previous studies in mice have repeatedly shown that MSE can reduce the level of endogenous antioxidant Manganese Superoxide Dismutase in the brain, kidney, and lungs of adult offspring accompanied by increased Reactive Oxygen Species (ROS) levels in those organs; interestingly, antioxidant supplementation during pregnancy could completely or partially reverse the adverbse effects on those organs induced by MSE (33-35). The endogenous antioxidant enzyme system is established in the second and third trimester of pregnancy and continues to develop in early childhood (36). Interestingly, lung development also matures in the early postnatal period, suggesting that the antioxidant system may protect early-life lung development from the adverse impacts of environmental oxidant pollutants (37). After all, the function of the respiratory system is vital for survival immediately after birth. Vitamin C is an antioxidant which contributes to cellular antioxidant defence(38, 39). A study in pigs found that vitamin C deficiency during pregnancy could cause brain damage in the offspring (40). Giving smoking women vitamin C during pregnancy was shown to improve lung function (better airflow and less wheezing) in children during the first year of life (41). This again provided evidence that oxidative stress and insufficient capacity of antioxidants play a key role in organ dysfunction in the offspring due to MSE. PM consists of metals and endotoxins (polycyclic aromatic hydrocarbons) which also can generate ROS (42) and produce oxidative damage (43). Therefore, the pathways associated with oxidative stress are regarded as playing an important role in inducing adverse respiratory outcomes after the exposure to environmental pollutants (44, 45).

In utero, any adverse effects that occur during foetal development can have long-lasting

182 negative influences on organ development and later function after birth (46, 47). In fact, local
183 tissue oxidative stress and injury due to the imbalance between free radicals and antioxidant
184 capacity is a key factor in asthma pathogenesis. As such we propose that oxidative stress is the
185 pathological insult that drives changes in the intrauterine environment and disturbs normal
186 foetal development which subsequently increases the risks of developing asthma. It is also
187 worth noting that maternal smoking is a strong risk factor for miscarriage, a process also linked
188 to oxidative stress (48).
189
190 **Intrauterine growth restriction – The Barker Hypothesis**
191 In 1990, the epidemiologist David Barker presented his hypothesis which linked chronic and
192 degenerative diseases, such as heart disease, to the poor intrauterine environment caused
193 intrauterine growth retardation (IUGR), low birth weight, and premature birth. This theory
194 inspired scientists and has been expanded to the other organ systems including the respiratory
195 system (49). Numerous studies have confirmed that environmental toxicant exposure during
196 pregnancy, such as cigarette smoke, can cause IUGR and subsequently abnormal lung
197 development in the offspring (49). Nicotine is the most widely studied component in cigarette
198 smoke due to its addictive effects. Early studies showed that cotinine, the stable metabolite of
199 nicotine, can be found in foetal circulation and body fluids (50). This indicates that chemicals
200 in cigarette smoke can cross the blood-placental barrier and reach the foetus. A more recent
201 study by Geelhoed *et al* showed that MSE can decrease blood flow in the ascending aorta
202 because of higher arterial resistance in the uterus, which can reduce the oxygen and nutrient
203 delivery to the growing foetus resulting in IUGR (51). Inadequate nutrient availability in the
204 developing foetus, especially during the periods of rapid lung growth, has been shown to induce
205 lung developmental defects (52, 53) and respiratory morbidity in the offspring (54, 55). Animal
206 studies have demonstrated a decrease in both alveolarisation and vessel density in the lung of
207 sheep with IUGR (56).
208
209 **How do MSE and maternal PM exposure impact on foetal lung development?**
210 In brief, MSE can induce such effects in two ways: the direct influence on the developing
211 foetus, and indirect effects on the fetoplacental unit. Recently, studies have demonstrated that
212 a small fraction of the circulating nicotine in the mothers can cross the trophoblastic membrane
213 and reach the unborn child, and as such cotinine can accumulate in the foetal circulation and
214 fluids in measurable concentrations (57, 58). Furthermore, a similar concentration of cotinine
215 in both foetal lung tissue and blood was found, suggesting cotinine may bind to the receptors

in the lung to directly affect foetal lung development (59). Maternal air pollution exposure can also cause foetal growth restriction (60). Polycyclic aromatic hydrocarbons on the surface of PM can easily cross the blood-placental barrier and circulate in the foetal blood because of its small size (61). Therefore, lung development in the foetus can be directly affected by the PM inhaled by the mothers.

The fetoplacental unit has a significant influence on foetal development. The damage to fetoplacental unit caused by maternal smoking can be seen during early pregnancy. For example, MSE significantly increases villous membrane thicknesses and trophoblastic layer in the placenta during the first trimester (58). There are also signs of reduced capillary volume in placental vasculature in pregnant smokers (62). The consequence of reduced capillary volume is nutrient delivery decrement. Intrauterine nutrient deficiency has been suggested as the major factor contributing to foetal growth restriction and low birth weight due to MSE (63). Low birth weight can increase the asthma risk in later life, evidenced by a meta-analysis including 1.1 million people (64). In rat models, maternal PM exposure was found to change placental morphology, and decrease placental weight, size and surface area (65). Similar findings have also been confirmed in humans, where PM_{10} exposure can decrease placental weight with higher anti-angiogenic factors in cord blood (66). As a result, increased vascular resistance can be predicted, which will reduce uteroplacental perfusion and lead to various maternal and foetal complications, such as low birth weight and miscarriage (67-69).

The abovementioned evidence indicates that MSE and maternal PM exposure during pregnancy can impair foetal lung development through a direct effect on the foetus and indirect influence on placental morphology and function. However, the molecular mechanisms underlying the increased risk of asthma due to MSE and maternal PM exposure are not well understood. In monkeys, MSE upregulated nicotinic acetylcholine receptors in the foetal lung, associated with lung function decline after birth (70, 71). Several *in vitro* and *in vivo* animal models have also shown that both MSE and PM exposure during pregnancy affects the development of the neonatal immune system, lung structure, and lung function in the offspring, making them more susceptible to the development of asthma(72, 73). These will be discussed in greater detail later.

The development of asthma in children

The role of altered lung structure

Just as discussed above, MSE and maternal PM exposure during pregnancy can result in oxidative stress, and cause nutrition deficiency resulting in IUGR, which eventually alters lung development and structure. Foetal lung development starts from embryo Weeks 3-5 when the laryngotracheal groove forms on the floor of the foregut and matures during the early postnatal year. Therefore, inhaled environmental toxicants by pregnant mothers may change lung morphology and function as early as gestational Weeks 5-17 when epithelial and smooth muscle cell differentiation takes place. Epidemiological evidence well supports this theory, where significant lung function impairment was found in the newborns of mothers who smoked during pregnancy or inhaled high levels of PM (74, 75). Such lung function disorders can last until later childhood (76, 77). It needs to be noted that lung function deficiency in early life has been correlated with increased asthma incidence later on (78).

Lung dysfunction after birth can be attributed to lung structural changes during foetal development. Animal studies have shown that both MSE and maternal PM exposure could decrease lung volume, alveoli number and mean linear intercept in the offspring as well as reduced alveolar–bronchiolar attachment points (72, 73, 79). Nicotine as the 'addictive substance' in tobacco smoke has often been used in animal models to investigate the potential mechanisms underlying the adverse effects of maternal tobacco smoking. For example, increased airway collagen deposition and altered vascular structure were found in a monkey model after prenatal nicotine exposure (80, 81). However, it is uncertain if these results can be translated to humans as nicotine replacement therapy during pregnancy has not been found to be associated with the same adverse outcomes as maternal cigarette smoking (82) or nicotine administration in animal models (80, 81). This suggests that the whole constituent of tobacco smoke is needed to study the mechanism in animals.

The role of endocrine disorders.

Endocrine disruption during pregnancy is a potential cause of adverse pregnancy outcomes. Endocrine glands form an important part of the fetoplacental unit that can secrete a significant amount of hormones including the oestrogen to support pregnancy. Oestrogen plays a key role in regulating neuroendocrine homeostasis in the developing foetus and promotes Th2 immune cell development in the foetus (83, 84). A human study demonstrated that abnormal oestrogen level in pregnant mothers affects foetal development (85). A reduction in oestrogen and oestrone (a weak oestrogen) levels in the cord blood has been found if the mother smoked during pregnancy (86)(87). This is because smoking can produce an anti-oestrogenic effect

and induce androgenisation in pregnant mothers to disturb hormonal homeostasis (88). Such changes may influence the risk of asthma in offspring (89).

The evidence to prove the relationship between maternal PM exposure and its impact on endocrine homeostasis are scarce. It has been shown that the endocrine-disrupting chemicals (EDCs) on the surface of PM can disrupt sex hormone synthesis (90). Polycyclic aromatic hydrocarbons in both tobacco smoke and PM, can also affect steroidogenesis through inhibiting steroidogenic enzymes (91). However, there is no direct evidence suggesting the correlation between hormone change induced by maternal PM exposure and foetal lung development, neither is known about the risk of asthma in the offspring (92).

However, the information collected from cord blood at birth can't accurately reflect the changes in foetal lung development during particular sensitive windows of embryo development induced by MSE and Maternal PM exposure. Amniocentesis is an alternative method to measure hormone levels at different time points and explore endocrine disruption, but access is limited. Animal modelling may shed a light on the correlation between placental hormone changes and foetal lung development, as well as postnatal lung function and susceptibility to asthma. Future research can focus on this aspect to better understand the niche factors contributing to lung development and the risk of asthma.

The role of epigenetic programming

Programming is a term used to describe an altered phenotype due to changes in the *in utero* environment. Epigenetic programming describes stable inheritable phenotypic changes without the alteration in the DNA sequence. Such a process controls mRNA expression and protein production through changing the transcriptome, including DNA methylation and histone modifications. Mounting evidence has closely linked asthma to epigenetic programming due to intrauterine environmental changes. For example, asthma is also an inheritable disease (93). The parent-of-origin effect which is usually due to epigenetic mechanism, also shows a prominent influence on the development of asthma, eg. asthmatic mothers are more likely to have offspring with asthma than the asthmatic fathers (94). As mitochondrial DNA is 100% inherited from the mothers, epigenetic modification of this genome may largely contribute to this phenomenon. In addition, the foetal period is a vulnerable stage and thus very sensitive to environmental toxicant exposure, when maternal protection is vital. During embryogenesis,

cells divide rapidly and therefore the genome is in a relatively unstable status. During this period, oxidative stress induced by environmental toxicant exposure may easily interrupt genomic duplication process (95), leading to abnormal epigenetic modifications or even mutation, rendering the foetus susceptible to future chronic diseases after birth, such as asthma.

In a cohort study on MSE, CpGs methylation has been found on genes responding to the pollutants in tobacco smoke in the newborns of smokers who smoked during pregnancy (96). In addition, CpG methylation was also found in the genes involved in foetal development in cord blood by MSE, suggesting a mechanism by which MSE results in intrauterine underdevelopment (96). Previous studies have shown that maternal PM exposure could alter DNA methylation in the offspring. Prenatal PM_{10} exposure induced superoxide dismutase 2 (SOD2) protomer methylation in cord blood cells (97), which is related to phthalate and diisocyanate-induced asthma (98, 99). As the epigenetic changes are inheritable, they will change gene expression to affect normal embryo development and persist throughout life, resulting in the susceptibility to chronic diseases in later life (100). It may also result in the transfer of certain respiratory diseases to subsequent generations, such as asthma, establishing a family history. For a detailed review on epigenetic changes due to *in utero* oxidative challenges, please see Zakarya *et al.* 2019 (101).

The role of the immune response

The mother's immune system plays a central role in the protection of foetal development. The foetus and newborns need maternal antibodies (Ig) to protect them from infectious diseases (102). Previous studies have shown that parental smoking and PM exposure increased Ig E levels in the cord blood (43, 103). MSE and maternal PM exposure can also alter immune responses through activating inflammatory macrophages and memory B cells in the offspring (104, 105). These changes in immune responses suggest that MSE and maternal PM exposure can alter the innate and adaptive immune response in the offspring. In addition, MSE and maternal PM exposure have also been shown to delay the maturation of immune system [106],[107], which may also make such offspring more susceptible to allergic disorders.

Toll-like receptors (TLRs) play an important role in the neonatal immune response (108). MSE can inhibit neonatal immune system maturation through impairing TLR mediated responses (such as TLR2 and TLR9) (109). We also have similar observations in the brains of mice who are offspring which had MSE. At postnatal day 1, mRNA expression of TLR4 was decreased

in the offspring from MSE compared to those from Sham-exposed mothers, suggesting suppressed immune response or delayed maturation of immune response (110). However, TLR4 mRNA expression was increased in 13 weeks old offspring which had MSE along with increased inflammatory cytokines expression (110), suggesting that MSE has a sustainable influence on the immune system leading to heightened inflammatory cytokines production. Maternal PM exposure could induce similar adverse effects. High levels of TLR2 and TLR4 expression were found in the human offspring and animals from mothers exposed to increased levels of PM during pregnancy(106).

Asthma is typified by T cell dysregulation, including Th1, Th2 and Th17 cells (111). In most asthmatic patients, accumulating evidence shows the suppression of Th1 cytokines (for example IFNγ) with higher Th2 cytokine expression (IL-4, IL-5, and IL-13) (112). Furthermore, clinical data showed that allergic responses are more prevalent among the children who have developed attenuated Th1 responses during infancy (113). Similar changes were found in animal studies. In pregnant C57BL/6 mice, intranasal exposure to diesel exhaust particles has been shown to increase the Th2 cell percentage in the bronchoalveolar lavage fluid with higher levels of pro-inflammatory cytokines (IL-4 and IL-5) in the offspring with asthma (114). MSE was also shown to increase Th2 cytokines (IL-4 and IL-5) and other pro-inflammatory cytokines (such as IL6) with suppressed Th1 cytokines (IFN-γ) due to reduced NK cell activities (115, 116).

However, the immune response is complicated, and difficult to investigate from a broader spectrum. A study has found that $PM_{2.5}$ exposure differentially impacts the immune system at different stages of gestation. High level of CD3+ and CD4+ lymphocytes and low percentage of CD19+ lymphocytes and NK cells can be found in the cord blood during the early gestation; however, the opposite changes with low level of CD3+ and CD4+ lymphocytes and high percentage of CD19+ lymphocytes and NK cells were found if PM exposure occurs during late gestation (117). These studies suggest that immune response has been programmed by *in-utero* exposure to air pollution, however, future studies are needed to fully understand the extent of the changes in this system.

Conclusion and perspectives

In conclusion, cigarette smoking and PM exposure during pregnancy is detrimental to foetal development and increase the risk of childhood asthma. As summarised in Fig 1, Fig 2 and Fig

3, oxidants inhaled by the mother result in increased oxidative stress in the intrauterine environment. This results in persistent changes to both the structure of the lung and the epigenome, altering immune and endocrine systems. Collectively these changes increase the risk of childhood asthma. Although smoking cessation is preferred, the success rate remains low during pregnancy. Given the similarity between MSE and maternal PM exposure, antioxidant supplementation during pregnancy may be a plausible prophylactic strategy, which is yet to be confirmed by large clinical trials.

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Figure 1. MSE and maternal PM exposure can increase the rate of childhood asthma. MSE and maternal PM exposure can induce various adverse impacts on the foetus during different intrauterine developmental stages, such as DNA methylation, oxidative stress, inflammatory responses, and placental dysfunction. The resulting intrauterine growth retardation, low birth weight, and premature birth can increase the risk of childhood asthma with a lower alveolar number and reduced lung function, as well as increased lung inflammation.

Figure 2. MSE and maternal PM exposure increase oxidative stress in the womb which increases the risk of developing asthma due to the epigenetic modification of fetal DNA. Environmental toxicants can induce histone modifications and DNA methylation, which results in Th2 cytokine overproduction, eosinophils accumulation, goblet cell hyperplasia, and mucin hypersecretion.

Figure 3. MSE and maternal PM exposure can dysregulate the immune system in the foetus. The numbers of Th2 and Th17 cells are increased with a lower number of Th1 cells. This is caused by several epigenetic mechanisms, for example, miRNA 223 is increased in Treg cells. B cell and macrophages differentiation are also affected, and a lower number of NK cells are found.

801 **Table 1. Maternal smoking during pregnancy and the risk of asthma in children**

Smoking exposure	Age	Relative risk Odds ratio (95% CI)		References
		Male	Female	
Smoker at some stage	14 years	1.15 (1.01-1.72)	1.25 (0.85-1.22)	(118)
>20 cigarettes (early and late)	14 years	0.57 (0.20-1.60)	1.09 (0.47-2.51)	(118)
Total of 1–9 cigarettes/day	4-16 years	1:19 (0.98, 1.43)		(119)
< 10 Cigarettes per day	7 years	1.20 (1.04, 1.38)		(120)
Total of ≥10 cigarettes/day	<5 years	1.68 (1.10 to 2.58)		(121)
> 10 Cigarettes per day	7 years	1.31 (1.09, 1.58)		(120)
Total of ≥10 cigarettes/day	4-16 years	1:66 (1.29, 2.15)		(119)
Smoking during pregnancy	First 3 years	1.88 (1.14 – 3.12)		(122)
Smoking during pregnancy	4-6 years	1.65 (1.18–2.31)		(123)
Smoking during pregnancy	2-7 years	1.7(1.2-2.2)		(124)
Smoking during pregnancy	5-9 years	0.97 (0.51 to 1.84)		(125)
Smoking during pregnancy	14 years	1.49 (0.91–2.45)		(126)
Smoking during pregnancy	7-16 years	0.99 (0.78 to 1.25)		(127)

802

803 **Table 2. Maternal PM exposure and the development of asthma in offspring**

Pollutant	Age	Concentration increase	Relative Risk	References
$PM_{2.5}$	6 years	1.7 μg/m^3 (per IQR)	1.15(1.03-1.26)	(128)
$PM_{2.5}$	3-4 years	1 μg/m^3 (exposure interval)	0.95 (0.91–1.00)	(129)
$PM_{2.5}$	0-5years	1.45 μg/m^3 (per IQR)	0.99 (0.97–1.01)	(130)
$PM_{2.5}$	6-10 years	1.46 μg/m^3 (per IQR)	1.01 (0.97–1.06)	(130)
$PM_{2.5}$	0-6years	3.7 μg/m^3 (per IQR)	1.01 (0.99 – 1.04)	(131)
PM_{10}	3-6 years	12 μg/m^3 (per IQR)	0.89 (0.68, 1.16)	(132)
PM_{10}	3-4 years	1 μg/m^3 (exposure interval)	1.09 (1.05–1.13)	(129)
PM_{10}	0-5years	1.3 μg/m^3 (per IQR)	1.12 (1.05–1.19)	(130)
PM_{10}	6-10 years	1.36 μg/m^3 (per IQR)	1.09 (0.96–1.24)	(130)

804 IQR: interquartile range.

Figure 1.TIF

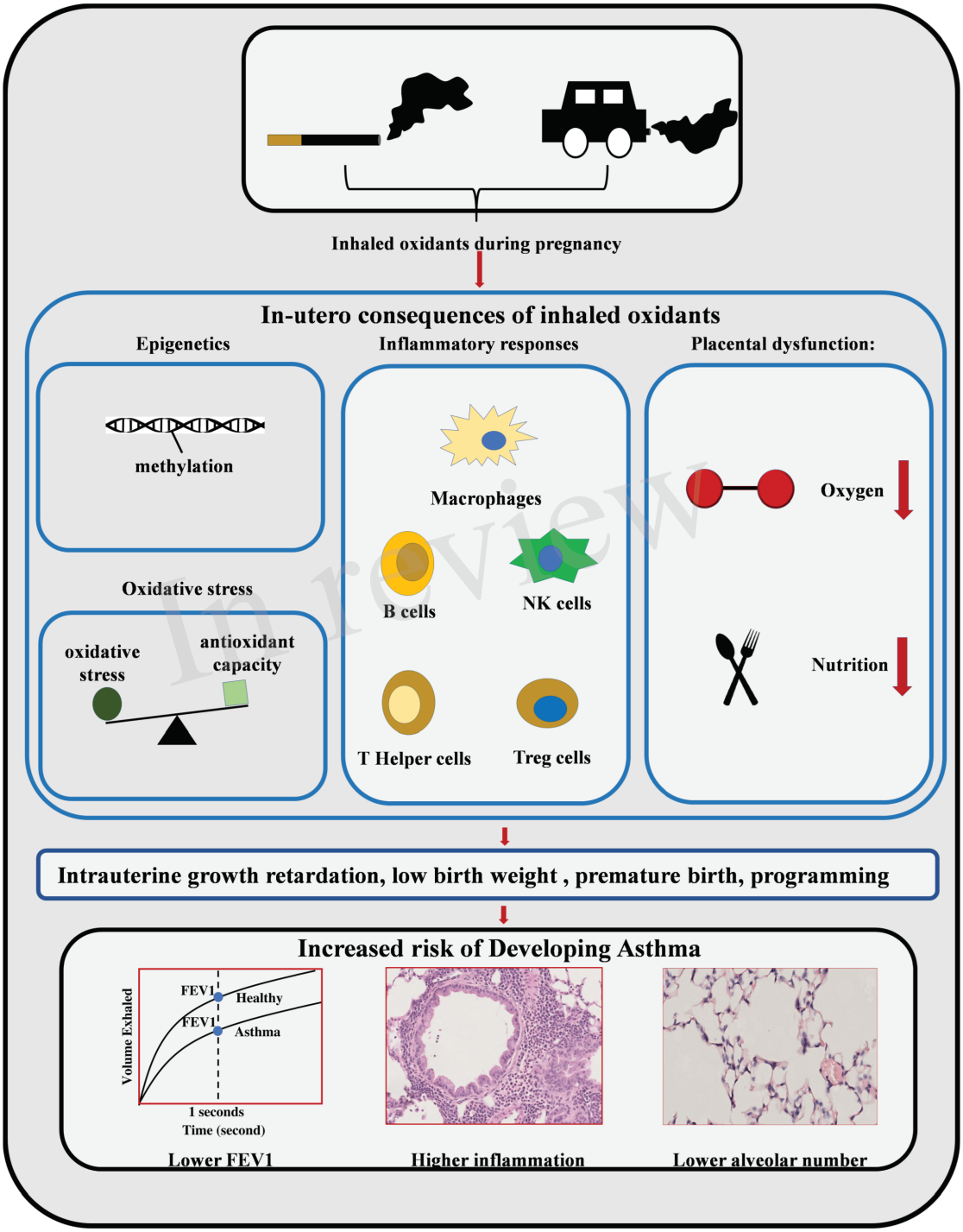


Figure 2.TIF

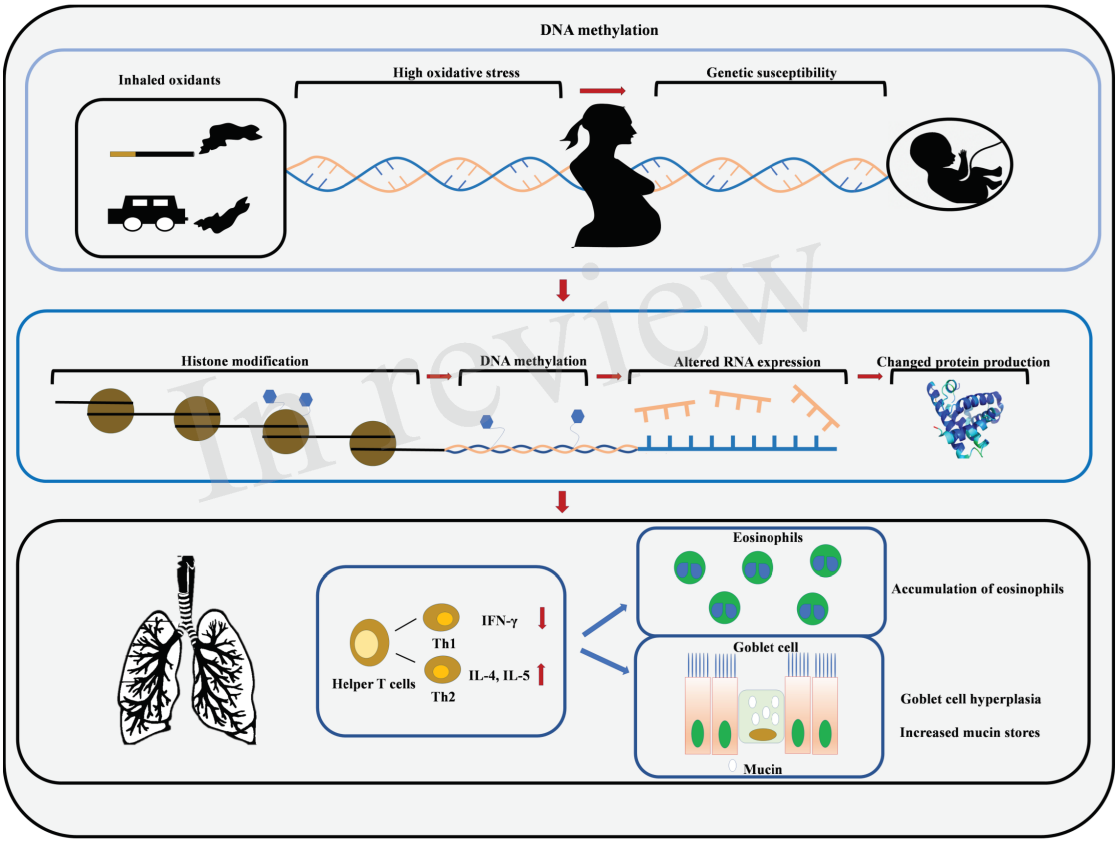
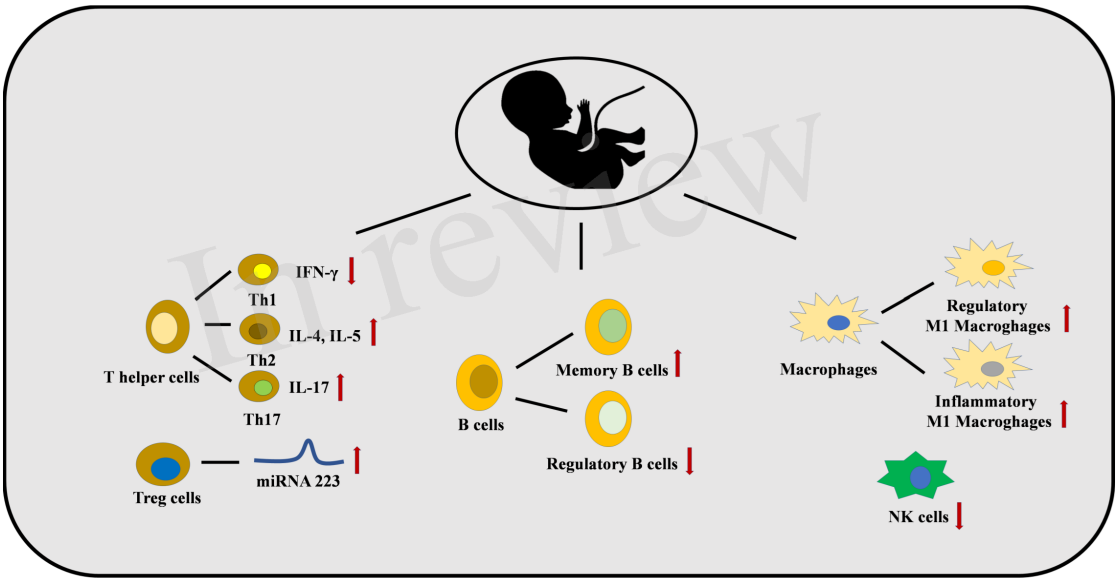


Figure 3.TIF



1.3 Overall hypothesis and aims of this thesis

We hypothesised that, in utero exposure to environmental pollutants such as cigarette smokeand PM would cause respiratory diseases in offspring.

To address this hypothesises, in mouse models, we aimed to investigate,

1. The inflammatory response in the offspring. This was evaluated by measuring the number of inflammatory cells BAL, and in tissue sections, as well as individual factors by both PCR and Western blotting
2. Mitochondrial dysfunction and oxidative stress in the offspring. This was evaluated by measuring the mitophagy markers by Western blotting and ROS level in cryo sections by immunofluorescence.
3. Respiratory hyper-responsiveness and lung remodelling in the offspring. This was evaluated by measuring lung function by FlexiVent measurements, and pathology in tissue sections using histological staining and immunohistochemical staining of individual factors .

The abovementioned aims were addressed in the studies in Chapters 2, 3, and 4.

Chapter 2 Offspring sex affects the susceptibility to maternal smoking-induced lung inflammation and the effect of maternal antioxidant supplementation in mice

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Contribution:

- All tissue analysis
- Draft preparation
- Finalising the manuscript

Offspring sex affects the susceptibility to maternal smoking-induced lung inflammation and the effect of maternal antioxidant supplementation in mice

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Abstract

Background: Cigarette smoke exposure (SE) during pregnancy is the largest modifiable risk factor for the development of lung disorders in offspring. We have previously shown that maternal L-Carnitine treatment can reduce the adverse impacts of maternal SE on renal and brain disorders in offspring. Here, we investigated the effect of maternal L-Carnitine supplementation on lung inflammatory pathways, autophagy, and mitophagy markers in the offspring in response to maternal SE.

Female Balb/c mice (8 weeks) were exposed to cigarette smoke for 6 weeks prior to mating, during gestation and lactation. Half of the SE mothers were given L-Carnitine supplementation (1.5mM in drinking water, SE+LC) during gestation and lactation. Lungs from the offspring were studied at birth and adulthood (13 weeks) in both genders.

Results: At birth, in male offspring, there were increased levels of inflammatory markers (phosphorylated(p)-ERK1,2, p-P38 MAPK, p-NF-kB), and inflammasome marker (NLRP3), as well as mitophagy fission marker Drp-1 and autophagosome marker (LC3A/B-II) in the lung. Maternal L-Carnitine supplementation significantly reduced NLRP3 level. In contrast, maternal SE only increased IL1-β in female offspring, which was reversed by maternal L-Carnitine supplementation. At 13 weeks, there was an increase in LC3A/B-II and p-NF-kB in the male SE offspring with reduced p-JNK1,2, which were partially normalised by maternal L-Carnitine treatment. Female offspring were not affected by maternal SE at this age.

Conclusion: Maternal SE had adverse impacts on the male offspring's lung, which were partially alleviated by maternal L-Carnitine supplementation. Females seem to be protected from the adverse effects of maternal SE.

Keywords: antioxidant, sex differences, inflammasome, mitophagy.

Background

Smoking during pregnancy is a major cause of maternal and newborn morbidity and mortality (1), with pulmonary diseases being a major adverse outcome (2, 3). In-utero smoke exposure (SE) reduces lung function in human newborns (4, 5). Animal models have shown a decreased number of saccules, septal crests, and decreased elastin fibres in foetuses (6) and suckling pups (7), as well as increased airway thickness, collagen deposition, inflammation, and airway hyper-responsiveness due to intrauterine SE (8-10).

In humans, certain diseases including chronic obstructive pulmonary disease (COPD), occur disproportionately in males and females (11). The common pathophysiological process includes increased inflammation, oxidative stress, impaired mitochondrial renew mechanism (mitophagy), and cellular self-cleaning mechanism (autophagy)(12). In keeping with this, our previous murine studies found that the changes in inflammation, oxidative stress, mitophagy, and autophagy have a marked sex difference in the offspring's brain and kidney following in-utero SE, wherein female offspring are more protected from such adverse effects(13, 14).

A previous study only demonstrated that maternal SE causes lung inflammatory response in the male offspring (15); whereas a recent study suggested that prenatal SE can differentially affect methylation in mice lungs in different sexes, which found hypo and hypermethylation (CpG-site-specific methylation) in male in female offspring respectively because of the various exons in the Igf1 gene response to the hormone (estrogen) activation (16). Therefore, we hypothesised that sex differences also exist regarding the effects of in-utero SE on other pulmonary changes.

The regulation of inflammation involves several signalling pathways, such as NF-κB and

MAPK pathways (17). Three well-characterised subfamilies of MAPK include the extracellular signal-regulated kinase (ERK)1/2, Jun N-terminal kinase (JNK) stress-activated protein kinase, and p38 (18). NF-κB is often regarded as the master controller of inflammation (19). Inflammatory response requires a considerable amount of energy derived from the mitochondria (20), whereas mitochondrial function is often compromised during this process. There is a close relationship between the activation of the nucleotide-binding domain and leucine-rich repeat-containing family pyrin domain containing 3 (NLRP3) inflammasome (increasing IL-1β activity) and mitochondrial dysfunction (21). Thus, the inflammasome is regarded as the bridge between inflammatory response and subsequent mitochondrial damage (21), including oxidative stress (21) and mitochondrial DNA impairment (22). This has been observed in conditions like COPD but has not been investigated in the setting of maternal SE(23).

The autophagic elimination of injured mitochondria is termed mitophagy, which is regulated by fusion and fission (24). The balance between fusion and fission is essential to mitochondrial integrity. Fission is to separate damaged mitochondrial fragments from the healthy part, while fusion is to generate a new mitochondrion from two healthy mitochondrial fragments (25, 26). We have observed dysregulated mitophagy in the brain and kidney caused by maternal SE which was associated with organ pathology(13, 27); however, whether this also occurs in the lung is unknown.

In-utero SE results in considerable foetal oxidative stress and inhibits the endogenous antioxidant activity (28). Therefore, improving the antioxidant ability may alleviate the adverse effects of maternal SE. L-Carnitine has been shown to attenuate age-related disorders by reducing oxidative stress and increasing antioxidant capacity in rats (29, 30). A clinical study

also showed that L-Carnitine supplementation can suppress serum levels of inflammatory cytokines in humans (31). We have shown that maternal L-Carnitine supplementation during pregnancy and lactation can alleviate brain (13) and renal dysfunction (32) in offspring from the SE mothers. As such, this approach may also ameliorate the adverse impact of maternal SE on lung health in the offspring.

Given the known differences in the susceptibility of developing lung diseases between males and females(33), we hypothesised that *in-utero* smoke exposure would result in chronic hyperactivation of inflammatory markers and dysregulated autophagy and mitophagy in male offspring, but not in female offspring. Maternal L-carnitine may ameliorate the adverse impact of maternal SE on the offspring's lung.

Results:

Effect of maternal SE on body weight

At P1, both male and female offspring from the SE dams appeared smaller than the SHAM offspring (Table 1). Maternal L-Carnitine supplement during gestation and lactation increased the birth weight of both male and female offspring ($P<0.05$ vs SE). There were no differences in body weight among the 3 groups at 13weeks for both males and females (Table 1).

Effect of maternal SE on lung p-ERK, p-p38, p-JNK, and p-NF-kB in the offspring.

At P1, maternal SE significantly increased the levels of p-ERK1,2 ($P<0.01$ vs SHAM, Figure 1A), p-P38 ($P<0.01$ vs SHAM, Figure 1E) and p-NF-kB ($P<0.01$ vs, SHAM, Figure 1G) in the male offspring. Only p-NF-kB appeared to be partially reversed by maternal L-Carnitine treatment without statistical significance (Figure 1G). In the female offspring, maternal SE did

not significantly affect phosphorylated ERK1,2, JNK1,2, p38, or NF-kB levels, whereas maternal L-Carnitine supplementation significantly reduced p-ERK1,2 ($P<0.05$ vs SHAM, $P<0.01$ vs SE, Figure 1B) and p-P38 ($P<0.05$ vs SHAM, Figure 1F) levels.

At 13 weeks, p-JNK1,2 level was lower and p-NF-kB was higher in the male offspring ($P<0.05$ vs SHAM offspring, Figure 2C, G). which was not reversed by maternal L-Carnitine supplementation. In the adult females, neither maternal SE nor maternal L-Carnitine supplementation had any effect on the abovementioned proteins (Figure 2).

Effect of maternal SE on lung NLRP3 and IL1-β levels in the offspring.

In P1 offspring, a trend towards increased NLRP3 and IL-1β was observed in male and female offspring, however only NLRP3 in the male ($P<0.01$ vs SHAM, Figure 3A) and IL-1β in female ($P<0.05$ vs SHAM, Figure 3D) were significant. Maternal L-Carnitine treatment normalised both markers ($P<0.05$ vs SE, Figure 3A).

At 13 weeks, maternal cigarette smoke exposure significantly increased NLRP3 expression in female offspring ($P<0.01$ vs SHAM, Figure 3F); maternal L-Carnitine supplementation did not have any effect ($P<0.01$ vs SE, Figure 3F). Maternal smoke exposure significantly increased IL-1β level ($P<0.01$ vs SHAM, Figure 3G) in the male offspring, which was further increased after maternal L-Carnitine treatment ($P<0.01$ vs SE, Figure 3G.

Effect of maternal SE on lung mitophagy markers in the offspring

At P1, total cell autophagy marker LC3A/B-II and mitochondrial fission marker Drp-1 protein levels were significantly increased in the male SE offspring ($P<0.05$, Figure 4A, C). Maternal L-carnitine supplementation further increased LC3A/B-II level, but normalised Drp-1 levels in the SE+LC offspring ($P<0.001$ vs SHAM, Figure 4A, C). No changes in autophagy and

mitophagy markers were found in P1 female offspring among the 3 groups (Figure 4B, D, E).

At 13 weeks, LC3A/B-II protein was significantly increased by maternal SE in the male offspring ($P<0.01$, Figure 5A) which was not affected by maternal L-Carnitine supplementation. In the females offspring, no difference in autophagy and mitophagy markers was observed among the 3 groups (Figure 5B, D, E).

Discussion

Maternal smoking during pregnancy is well-documented to cause long-term adverse effects on the offspring's health outcomes in multiple organs, including respiratory, neurological, and renal systems (34). However, the sex difference in such susceptibility in respiratory disorders has not been broadly studied, perhaps due to the preference of using one gender to model asthma or COPD.

In this study, male offspring from the SE dams had smaller body weight from birth to adulthood, consistent with previous studies and human birth weight suggesting the reproducibility and human relevance of our model (35, 36). Maternal SE activated inflammatory NF-kB and MAPK pathways, which were more prominent in the male offspring at P1. It is well known that cigarette smoking can induce inflammation via the MAPK signalling cascade (37), reflected by increased phosphorylation of ERK and P38 (38, 39). MAPK pathway activation can also lead to increased phosphorylation of certain transcription factors, such as NF-kB (40). In the current study, these effects in P1 male SE offspring are likely due to the chemicals including free radicals in cigarette smoke accessing the foetal circulation via the placenta. NLRP3 inflammasome activation in the male offspring at P1 is in accordance with other inflammatory pathways especially NF-kB. However, only NF-kB hyperactivation was

maintained at adulthood. This may be due to a lack of a second insult after birth. As NF-kB regulates acute responses to external stimuli, its innate hyperactivation may enhance the response to postnatal environmental factors, such as an increased risk of asthma or COPD (41). This requires further investigation with additional modelling in the offspring.

It is not surprising to observe that female offspring are mostly protected from the adverse effect of maternal SE compared with the male littermates. Such a lack of response in the females is consistent with our previous observations in the brain and kidney(14, 42, 43). One possible reason is the different innate and adaptive immune responses in the boys and girls, and the influence of sex hormones (44, 45).

A recent study found that inflammasomes can be regarded as the bridge between inflammation and mitochondrial function(46). There is increasing recognition that mitochondrial dysfunction plays a key role in the development of various diseases including COPD and asthma(24, 47, 48). Maternal smoking can induce high oxidative stress levels in the developing foetus (49) persistent until adulthood which can directly damage mitochondria(14, 50). Injured mitochondria can also induce more oxidative stress and inflammation. As such, mitophagy and autophagy are key to recycle intact mitochondrial fragments and eliminate damaged ones to maintain cellular homeostasis (51).

Increased fission maker Drp-1 and autophagosome marker LC3A/B-II in the male SE offspring at birth, suggest increased damaged mitochondria due to maternal SE. The fusion marker Opa-1 was not increased accordingly suggesting less healthy fragment can be recycled. At adulthood, only LC3A/B-II remained elevated, suggesting a higher demand to eliminate other injured cellular elements by maternal SE. This may drive the development of lung disorders in the SE

offspring (52). Interestingly, mitophagy markers in the lung were not changed in the female offspring at any age, again suggesting gender-specific protection from maternal SE. These results are consistent with our previous research in other organs (13).

In vivo and *in vitro* studies have demonstrated that L-Carnitine can prevent oxidative stress-induced injury (53-55). In this study, maternal L-Carnitine supplementation increased the birth weight in both male and female SE offspring. This suggests that L-Carnitine can ameliorate *in-utero* underdevelopment caused by maternal SE. Additionally, maternal L-Carnitine supplementation exhibited some anti-inflammatory effects in newborns from the SE dams, by partially supressing NF-kB activation and NLRP3 inflammasome formation in the males as well as MAPK pathway and IL-1β in the females. This may be due to its ability to inhibit oxidative stress induced by maternal SE in utero. However, the protection of maternal L-Carnitine supplementation on the lung did not persist until adulthood, especially in the male offspring.

The protection effects of L-Carnitine were observed in the other organs (13, 27, 32, 56), however, we did not found significant protective effect in the lung. This is surprising, but may be explained by the limitation of how we assessed the lung in this study. firstly, we did not collect BAL fluid, and as such typical markers of inflammation such as cytokine levels in bronchoalveolar lavage fluid to perform inflammatory cell counts which is a more direct way to access lung inflammation. The hyperactivation of signalling cascades may represent an increased ability to respond to external stimuli such as allergens or cigarette smoke, but in and of itself may not cause lung disorders. We did not measure reactive oxygen species (ROS) levels, and as such whilst it is likely that L-Carnitine supplementation acts via scavenging ROS, we can not definitively say this was the case.

Conclusions

In conclusion, there are gender differences in the susceptibility to lung disorders in response to maternal smoking, with male offspring more vulnerable to increased inflammatory changes. Maternal L-Carnitine supplementation during pregnancy may partially alleviate the adverse effects of maternal SE on lung health outcomes only in the newborn offspring.

Materials and Methods:

Animals

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A). All protocols were performed according to the Australian National Health and Medical Research Council Guide for the Care and Use of Laboratory Animals. Female Balb/c mice (8 weeks, Animal Resources Centre, Perth, WA, Australia) were housed at 20 ± 2°C and maintained on a 12 h light, 12 h dark cycle (lights on at 06:00 h) with ad libitum access to standard rodent chow and water. Female Balb/c mice were divided into 3 groups. The SHAM group (n=12) was exposed to air in a 15L perspex chamber for 6 weeks prior to mating, during gestation and lactation, SE group (n=12) was exposed to cigarette smoke generated from 2 cigarettes (Winfield Red, 1.2 mg nicotine; VIC, Australia) per session (5-minute interval between), twice daily during the same period of time as we have previously described (43). A sub-group of the SE dams (n=12) was provided with L-Carnitine in drinking water (1.5 mM, SE+LC) during gestation and lactation as we have previously described (13). L-Carnitine dose was determined according to a previous publication(57). Mice pregnancy was detected through continuous weight gain during matting. P1 mice were sacrificed by decapitation, while animals older than 20 days were sacrificed by anesthetic overdose (Pentothal®, 0.1 mg/g, i.p., Abbott Australasia Pty. Ltd., Macquarie Park,

NSW, Australia) between 9:00–12:00 h. The lungs from the offspring were collected at birth (postnatal day (P)1) and adulthood (13 weeks) and stored at -80℃ for later analysis.

Western Blotting

The protein levels of the markers of interest were measured in the lung, including inflammatory markers, phosphate(p)-ERK1,2 (1:2,000; Cell Signalling Technology), p-JNK1,2 (1:2,000; Cell Signalling Technology), p-p38 MAPK (1: 2,000; Cell Signalling Technology), p-NF-kB (1:2,000; Cell Signalling Technology), and autophagy markers light chain 3 microtubule-associated protein A/B (LC3A/B)-II (1:2,000; Cell Signalling Technology), mitophagy fission marker dynamin-related protein (Drp)-1 (1:2,000; Cell Signalling Technology) and mitophagy fusion marker optic atrophy (OPA)-1 (1:2,000; Cell Signalling Technology), inflammasome marker NLRP3 (1:2,000; Abcam), IL-1β (1:2,000; Cell Signalling Technology). B-actin (1:10000; Cell Signalling Technology).

The lung was homogenised using cell lysis buffers for whole protein and mitochondrial protein extraction through differential centrifugation as previously described (32). Protein concentrations were measured using DC Protein assay (Bio-Rad, Hercules, CA); 15ug of proteins were separated on Ctiterion^{TM}TGX Stain Free Precast Gel (BIO-RAD, USA) and then transferred to PVDF membranes (BIO-RAD, USA), which was then blocked with TBST. The membranes were incubated with the primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Protein expression was detected by SuperSignal West Pico Chemiluminescent substrate (Thermo, MA, USA) by exposure of the membrane in ChemiDoc (BIO-RAD, USA). The density of the protein band was determined using Image J (National Institute of Health, Bethesda, Maryland, USA).

Statistical Methods

The results are expressed as mean ± SEM. Normality was tested prior to the statistical analysis. If the data were not normally distributed, they were log transformed to research normality. The differences between groups were analysed by one-way ANOVA followed by Tukey's post hoc tests. $P<0.05$ was considered statistically significant.

Abbreviations:

SE: smoke exposure;

COPD: chronic obstructive pulmonary disease;

ERK: extracellular signal-regulated kinase;

JNK: Jun N-terminal kinase;

NLRP3: nucleotide-binding domain and leucine-rich repeat-containing family pyrin domain containing 3;

p38: p38 Mitogen-activated protein kinase;

NF-kB: Nuclear factor-Kb;

SE+LC, maternal smoke exposure with L-Carnitine supplement;

Declarations:

Ethics declarations:

The animal experiments were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-313A).

Consent for publication

N/A

Availability of data and materials

The datasets herein used and analyzed are available from the corresponding author on reasonable request.

Interest conflict: There is no conflict of interest.

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Author Contributions

HC (University of Technology Sydney), SS (University of Sydney), and BGO (University of Technology Sydney) designed the study. BW (University of Technology Sydney), SZ (Shandong University, China) and YLC (University of Technology Sydney) performed all the experiments. BW, HC, SS, BGO, and YLC contributed to the writing of the manuscript. BW and YLC prepared all the figures. All the authors reviewed the final manuscript

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Table 1: Body weight of the offspring at different ages.

	Day 1			13 weeks		
Male offspring	SHAM n=8	SE n=9	SE+LC n=8	SHAM n=8	SE n=7	SE+LC n=8
Body weight (g)	1.53±0.29	1.30±0.12	1.62±0.2*	25.6±0.9	24.7±0.9	25.7±1.24
Female offspring	SHAM n=8	SE n=6	SE+LC n=8	SHAM n=8	SE n=8	SE+LC n=8
Body weight (g)	1.48±0.38	1.21±0.06	1.68±0.19*	22.0±1.2	20.7±1.0	21.0±0.7

Results are expressed as mean ± SEM. Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. *$P < 0.05$, compared with the SE offspring at the same age. SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Table 2 Litter demographics

	SHAM	SE	SE+LC
Litter size (pup / litter)	6.2 ± 0.8	5.3 ± 1.0	6.0 ± 1.0
Male pup / litter	3.5 ± 0.5	2.9 ± 0.7	3.0 ± 0.6
Female pup / litter	2.6 ± 0.6	2.4 ± 0.6	3.0 ± 0.5

Results are expressed as Mean ± SEM. n = 9–12. The data were analysed by One-way ANOVA followed by Turkey's post hoc tests. SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Figure legends

Figure 1. Lung p-ERK1,2, p-JNK1,2, p-p38 and p-NF-kB in the offspring at P1.

Protein expression of p-ERK1,2 (A, B), p-JNK1,2 (C, D), p-p38 (E, F) and p-NF-kB(G, H) in the lung of the male and female offspring at P1. Results are expressed as means ± SE, (male *n* = 8, female, *n* = 6-8). Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. **P* < 0.05, ***P* < 0.01, *** P < 0.001, *****P* < 0.0001. ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; p38, p38 Mitogen-activated protein kinase; NF-kB: Nuclear factor-kB. SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Figure 2. Lung p-ERK1,2, p-JNK1,2, p-p38 and p-NF-kB in the offspring at 13 weeks.

Protein expression of p-ERK1,2 (A, B), p-JNK1,2 (C, D), p-p38 (E, F) and p-NF-kB(G, H) in the lung of the male and female offspring at 13 weeks. Results are expressed as means ± SE, (male *n* =7-8, female *n* = 8). Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. **P* < 0.05, ***P* < 0.01. ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; p38, p38 Mitogen-activated protein kinase; NF-kB: Nuclear factor-kB. SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Figure 3. Lung inflammasome markers NLRP3 and IL-1 β in the offspring at P1 and 13 weeks.

Protein expression of NLRP3 (A, B) and IL-1β (C, D) in the lung of male and female offspring at P1. Protein expression of NLRP3 (E, G) and IL-1β (F, H) in the lung of male and female offspring at 13 weeks. Results are expressed as means ± SE (male *n* = 8, female *n* = 6-8). Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. NLRP3, nucleotide-binding domain and leucine-rich repeat-containing (NLR) family pyrin domain containing 3; SE; maternal smoke exposure; SE+LC, maternal smoke

exposure with L-Carnitine supplement.

Figure 4. Lung LC3A/B-II, Drp-1 and Opa-1 in the offspring at P1.

Protein expression of LC3A/B-II (A, B), Drp-1 (C, D) and Opa-1 (E, F) in the lung of male and female offspring at P1. Results are expressed as means ± SE, (male n = 8, female n =6-8). Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001. LC3A/B, light chain 3 microtubule-associated protein; Drp-1, dynamin-related protein; Opa-1, optic atrophy-1; SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Figure 5. Lung LC3A/B-II, Drp-1 and Opa-1 in the offspring at 13 weeks.

Protein expression of LC3A/B-II (A, B), Drp-1 (C, D) and Opa-1 (E, F) in the lung of male and female offspring at 13 weeks. Results are expressed as means ±SE, (male n = 7-8, female n =8). Data were analysed by one-way ANOVA followed by Tukey's post hoc tests. ** P < 0.01. Drp-1. LC3A/B, light chain 3 microtubule-associated protein; dynamin-related protein; Opa-1, optic atrophy-1; SE, maternal smoke exposure; SE+LC, maternal smoke exposure with L-Carnitine supplement.

Fig. 1

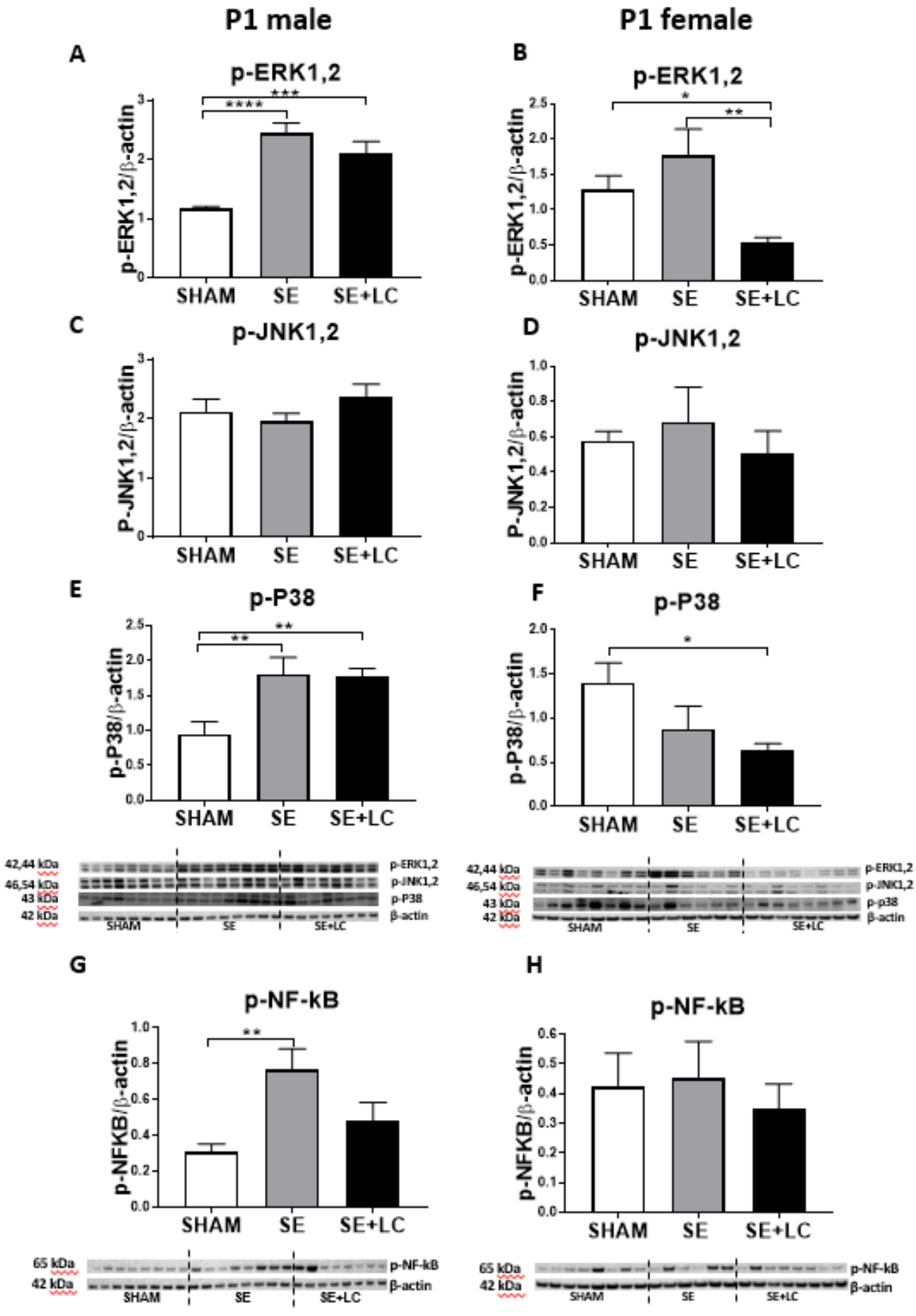

P1 male
P1 female
A
p-ERK1,2
p-ERK1,2/β-actin
SHAM
SE
SE+LC
B
C
p-JNK1,2
P-JNK1,2/β-actin
D
E
p-P38
p-P38/β-actin
F
42,44 kDa
46,54 kDa
43 kDa
42 kDa
p-ERK1,2
p-JNK1,2
p-P38
β-actin
G
p-NF-kB
p-NFKB/β-actin
H
65 kDa
42 kDa
p-NF-kB
β-actin

Fig. 2

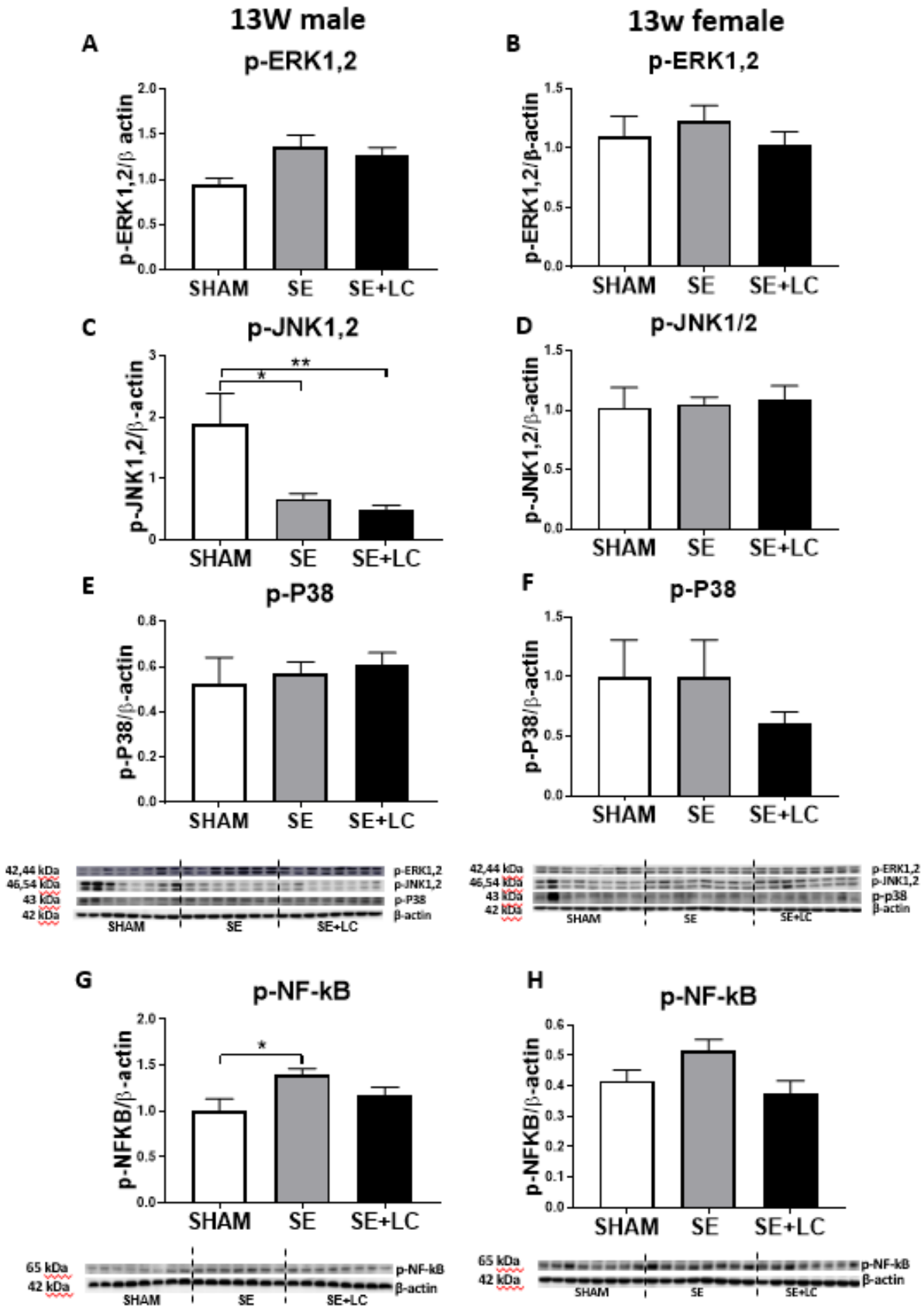

13W male
A
p-ERK1,2
p-ERK1,2/β actin
SHAM
SE
SE+LC
13w female
B
p-ERK1,2
p-ERK1,2/β-actin
C
p-JNK1,2
p-JNK1,2/β-actin
**
*
D
p-JNK1/2
p-JNK1,2/β-actin
E
p-P38
p-P38/β-actin
F
p-P38
p-P38/β-actin
42,44 kDa
46,54 kDa
43 kDa
42 kDa
p-ERK1,2
p-JNK1,2
p-P38
β-actin
p-p38
G
p-NF-kB
p-NFKB/β-actin
H
p-NF-kB
p-NFKB/β-actin
65 kDa
42 kDa

Fig 3.

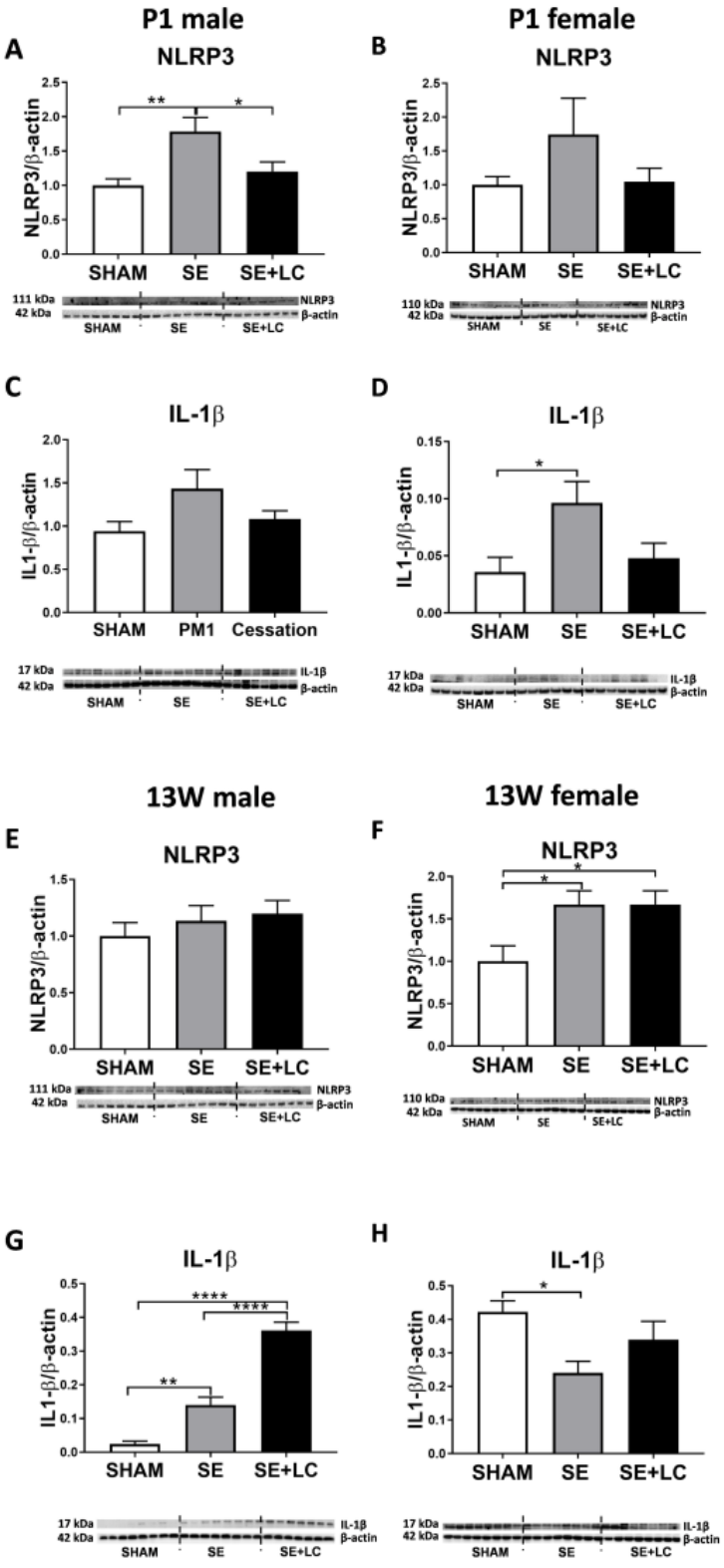

P1 male
A
NLRP3
NLRP3/β-actin
SHAM
SE
SE+LC
111 kDa
42 kDa
NLRP3
β-actin
P1 female
B
NLRP3
NLRP3/β-actin
SHAM
SE
SE+LC
110 kDa
42 kDa
NLRP3
β-actin
C
IL-1β
IL1-β/β-actin
SHAM
PM1
Cessation
17 kDa
42 kDa
IL-1β
β-actin
D
IL-1β
IL1-β/β-actin
SHAM
SE
SE+LC
17 kDa
42 kDa
IL-1β
β-actin
13W male
E
NLRP3
NLRP3/β-actin
SHAM
SE
SE+LC
111 kDa
42 kDa
NLRP3
β-actin
13W female
F
NLRP3
NLRP3/β-actin
SHAM
SE
SE+LC
110 kDa
42 kDa
NLRP3
β-actin
G
IL-1β
IL1-β/β-actin
SHAM
SE
SE+LC
17 kDa
42 kDa
IL-1β
β-actin
H
IL-1β
IL1-β/β-actin
SHAM
SE
SE+LC
17 kDa
42 kDa
IL-1β
β-actin

Fig. 4

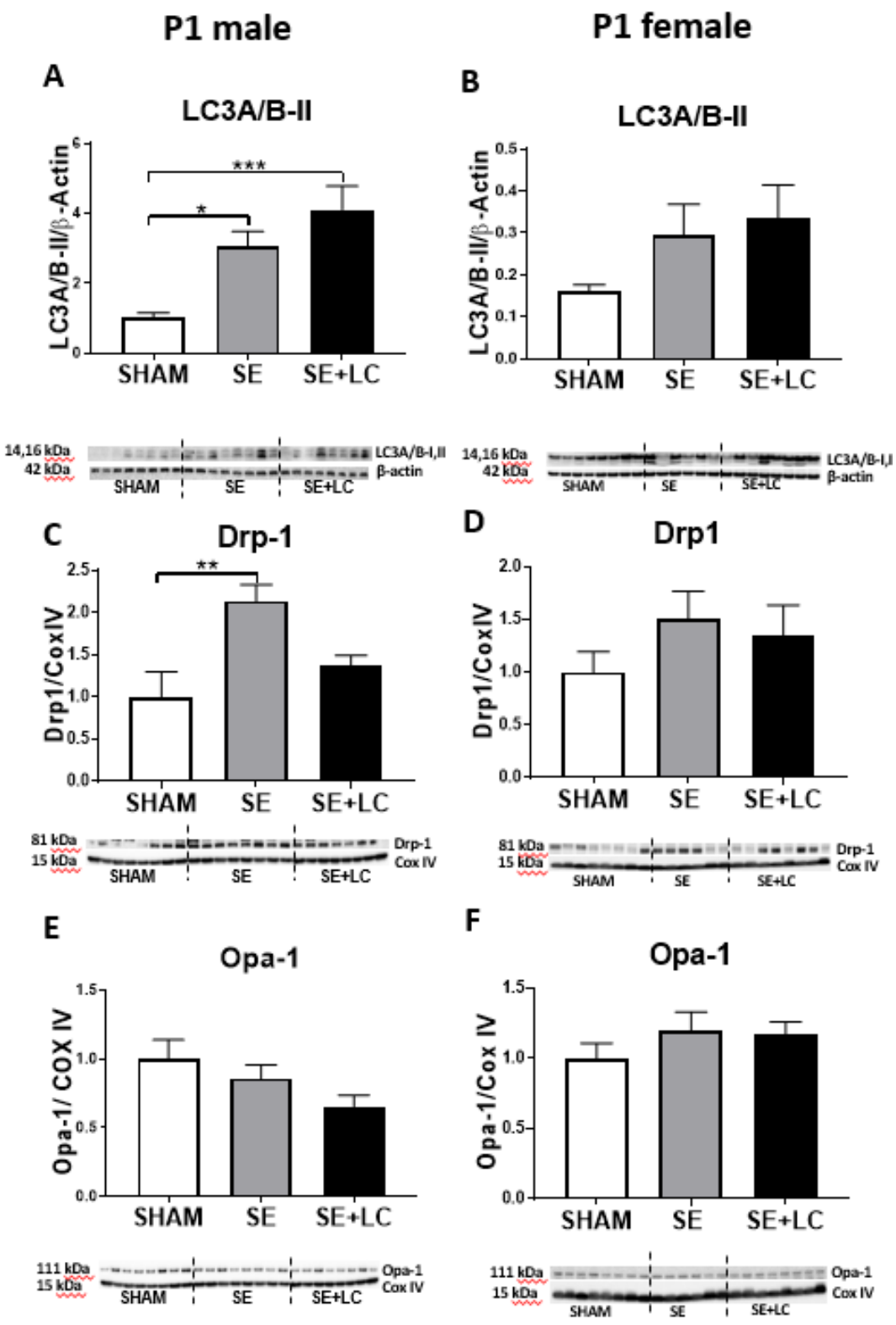
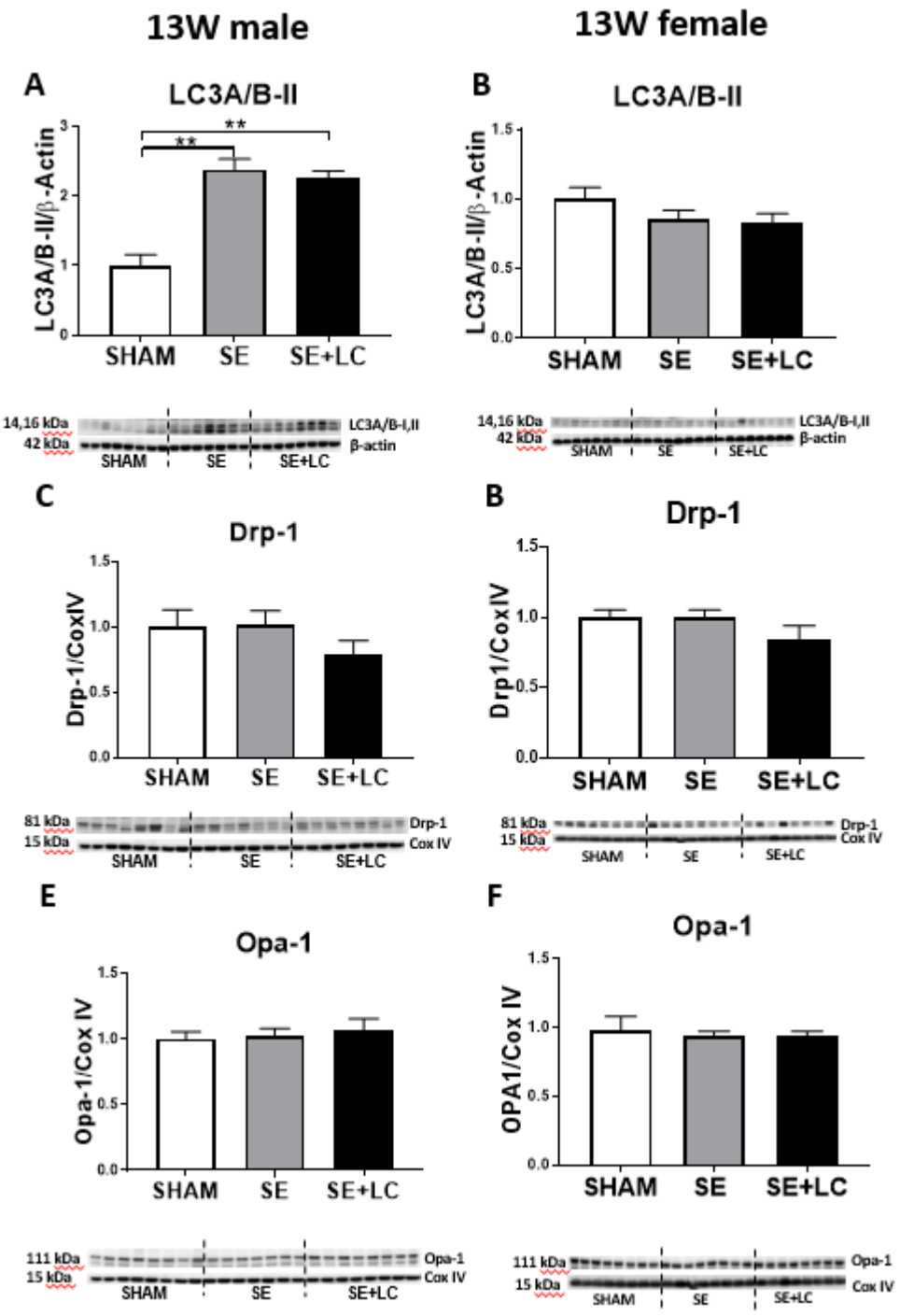

P1 male
P1 female
A
LC3A/B-II
LC3A/B-II/β-Actin
***
*
SHAM
SE
SE+LC
14,16 kDa
42 kDa
LC3A/B-I,II
β-actin
B
LC3A/B-II
LC3A/B-II/β-Actin
C
Drp-1
Drp1/CoxIV
**
81 kDa
15 kDa
Drp-1
Cox IV
D
Drp1
E
Opa-1
Opa-1/ COX IV
111 kDa
Opa-1
F
Opa-1
Opa-1/Cox IV

Fig. 5


13W male
13W female
A
LC3A/B-II
LC3A/B-II/β-Actin
SHAM
SE
SE+LC
14,16 kDa
42 kDa
LC3A/B-I,II
β-actin
B
C
Drp-1
Drp-1/CoxIV
81 kDa
15 kDa
Cox IV
E
Opa-1
Opa-1/Cox IV
111 kDa
F
OPA1/Cox IV

Chapter 3 Pulmonary inflammation induced by low-dose particulate matter exposure in mice

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*Joint first author

Contribution:

- All tissue analysis in Figures 1 and 2
- wrote the first draft of the manuscript

Pulmonary inflammation induced by low dose particulate matter exposure in mice

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Running title: Low dose PM causes inflammation and affects mitochondria

Abstract

Air pollution is a ubiquitous problem and comprises gaseous and particulate matter (PM). Epidemiological studies have clearly shown that exposure to PM is associated with impaired lung function and the development of lung diseases such as chronic obstructive pulmonary disease and asthma. To understand the mechanisms involved, animal models are often used. However, the majority of such models represent high levels of exposure and are not representative of the exposure levels in less polluted countries, such as Australia. Therefore, in this study we aimed to determine whether low dose PM_{10} exposure has any detrimental effect on the lungs. Mice were intranasally exposed to saline or traffic-related PM_{10} (1μg or 5μg per day) for three weeks. Bronchoalveolar lavage (BAL) and lung tissue were analysed. PM_{10} at 1μg did not significantly affect inflammatory and mitochondrial markers. At 5μg, PM_{10} exposure increased lymphocytes and macrophages in BAL fluid. Increased NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and IL-1β production occurred following PM_{10} exposure. PM_{10} (5μg) exposure reduced mitochondrial antioxidant manganese superoxide (antioxidant defence system) and mitochondrial fusion marker (OPA-1) whilst increased fission marker (Drp-1). Autophagy marker Light chain 3 microtubule-associated protein (LC3)-II and phosphorylated-AMPK were reduced, and apoptosis marker (Caspase-3) was increased. No significant change of remodelling markers was observed. In conclusion, a sub-chronic low level exposure to PM can have an adverse effect on lung health, which should be taken into consideration for the planning of roads and residential buildings.

Introduction

The World Health Organisation (WHO) air quality model demonstrates that ambient air pollution annually causes 4.2 million deaths, and 91% of the world's population lives in places where air quality exceeds the limits of WHO guidelines. Air pollution causes 1.8 million deaths from lung diseases (1). Forty three percent of chronic obstructive pulmonary diseases (COPD) and 29% of lung cancer deaths are attributable to air pollution (2). PM is the sum of all particles suspended in the air which includes both organic and inorganic particles such as dust, pollens, and vehicle emissions. Respirable PM is thought to be the most detrimental to human health. PM sized equal or below 10 microns (PM_{10}) is capable of entering the lungs, whilst PM sized equal or below 2.5 microns ($PM_{2.5}$) can reach the distal lung segments including alveoli (17) .

In adults, every 5 $\mu g/m^3$ increment of PM exposure is associated with a 39% to 56% increased risk of developing COPD (13). In developed countries such as the UK, traffic related air pollution (TRAP) accounts for 13% of total PM (4). In Sydney Australia, the levels of TRAP are amongst the lowest in the world, accounting for 14% of total PM (5), which often assumed to be safe. However, a study on 65,000 children in Canada found that children exposed to TRAP, even in urban areas with low levels of pollution, had a 25% increased risk of developing asthma by the age of 5 years.

PM is a strong oxidant, with its oxidant capacity regulated by antioxidants such as manganese superoxide dismutase (16). However, in humans, even short-term exposure of PM_{10} increased circulating levels of Interleukins (IL)-1β, IL-6 and TNF-α (28). PM_{10} contains approximately 10^{16} free radicals/g which can increase oxidative stress in human macrophages and lung epithelial cells (8, 29). ROS can induce inflammatory responses via the activation of the

nucleotide-binding domain and leucine-rich repeat protein (NLRP)3 inflammasome, which in-turn cleaves pro-interleukin (IL)-1β into IL-1β. Interestingly, Hirota et al have shown that PM activates the NLRP3 inflammasome resulting in increased IL-1β in bronchial epithelial cells (14).

Mitochondria can be damaged by both oxidative stress and the activation of NLRP3 inflammasome, resulting in reduced capacity to produce ATP. Mitophagy is a quality control process where fission removes damaged mitochondria fragments and fusion merges healthy mitochondrial fragments to regenerate new mitochondria (7), which has been shown to ameliorate inflammatory disorders (23). The impact in low level PM exposure on mitophagy markers has not been reported.

TRAP contains both gaseous and PM components. While the gaseous components are equally toxic as PM, gases dissipate quicker in air than the PMs which can remain airborne for long periods of time. However, most PM / TRAP exposure models used very high PM exposure regimens (e.g. 50 to 200 μg (11, 21)), which are not relevant to the PM/TRAP levels in countries with low levels of air pollution. We hypothesized that exposure to low levels of PM would be detrimental for lung health. Our objective was to establish an environmentally relevant model of TRAP-related PM exposure and to characterise pulmonary changes including inflammasome activation (NLRP 3 and IL-1β), IL-6 production, mitochondrial fission and fusion markers (Optic atrophy (Opa)-1 and dynamin-related protein (Drp)-1), autophagy markers and fibrotic markers (fibronectin, collagen III and transforming growth factor beta 1 (TGFβ1)).

Materials and Methods

PM collection

Twenty-four-hour integrated PM_{10} were collected through a 47-mm Teflon (Pall Life Sciences, Ann Arbor, MI) and pre-fired (800 °C, 3 hr) 47-mm quartz-fibre filters (Whatman Inc., Clifton, NJ) from a busy roadside in Hong Kong (114,000 vehicles per day) with URG PM samplers (URG-2000-30EH) in the summer (24th June to 11th July, 2017) with a flow rate of 8 L/min at each channel. Filter preparation (e.g. equilibrated for 24 hr at 25 °C and relative humidity of 40% before and after sampling) and gravimetric analysis were conducted in a high-efficiency particulate absorption clean room (ISO 14644 Class 7) at The Hong Kong Polytechnic University. All filters were stored at -20 °C and in dark prior to the analysis. PM was extracted in 90% ethanol with 5 minutes of sonication, followed by freeze drying overnight.

PM analysis

Energy-dispersive x-ray fluorescence spectrometry (PANalytical Epsilon 5) was used to determine concentrations of Al, Si, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ba and Pb. Each sample was analysed for 30 min. Thin-film standards were used for calibration (MicroMatter, Arlington, USA) (34). All reported chemical concentrations were corrected for field blanks, and duplicated samples were analyzed for quality assurance.

Ion chromatography (IC) for water-soluble inorganic ions analysis. One quarter of the filter was extracted with 10 mL of distilled deionized water and the extract underwent IC (Dionex DX-600) analysis (IonPac CS12A and AS14A columns) Six species were analysed as previously described (36). Analysis of organic carbon and elemental carbon were by thermal optical reflectance (TOR) technique on a thermal/optical carbon analyser (DRI Model 2001, Atmoslytic Inc., Calabasas, CA as described in Pathak et al (22).

In vivo PM exposure.

Animal experiments were approved by the Animal Care and Ethics committee at the University of Technology Sydney (ACEC#ETH16-0886). Male Balb/c mice (6 weeks, Animal Resources Centre, Perth, Australia) were housed at 20 ± 2 ^{o}C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h) with *ad libitum* access to standard laboratory chow and water. After the acclimatisation period, mice were assigned to 3 groups (n =10) which were exposed to either particulate matter with 1μg (PM_{10}(1μg)) or 5μg (PM_{10}(5μg)) or saline as control (SHAM). In urban Sydney, the average PM_{10} levels are 17 μg/m^3, equating to a daily human exposure of 181μg (3). Based on the breathing volumes, mice should be exposed to around 5μg/day to reflect air pollution levels in Sydney. Mice were exposed intranasally by instillation of 40μl of saline or saline resuspended PM_{10} daily for three weeks.

At the endpoint, the animals were sacrificed via cardiac puncture after deep anaesthesia (3% isoflurane). Lungs were perfused with phosphate buffered saline to obtain bronchoalveolar lavage (BAL) fluid. Lungs were then harvested, snap frozen and stored at -80^{o}C for protein analysis. Anthropometry measurements were done following dissection and measurement on a microbalance.

BAL analysis.

The BAL cells evaluated by Diff-Quik staining (Polyscience Inc, Taipei, Taiwan). Differential cell counts were performed for macrophages, lymphocytes, eosinophils and neutrophils.

Western blotting.

Lung tissue homogenates (20μg) were analysed using standard techniques, as described previously (9). Antibodies were purchased from Cell Signaling Technology, USA: IL-1β and IL-6 (1:1000); Caspase-3, p-Akt, Akt, p- AMP-activated protein kinase (AMPK), AMPK, light chain 3 microtuble-associated protein (LC)3A/B-I/II (1:2000); from Novus Biotechnology, USA: Drp-1, Opa-1 (1:2000) and Collagen-III (1:1000); from Millipore, USA: MnSOD (1:2000,); from Sigma-Aldrich, USA Fibronectin (1:2000); and R&D systems, USA: TGF-β1 (1:500).

Mitochondrial DNA copy number.

mtDNA was measured using qPCR on DNA as we have previously published (25, 26).

Statistical methods.

The data conformed to the normal distribution and differences between groups were analysed using one-way ANOVA followed by a Bonferroni post-hoc tests. $P<0.05$ was considered significant.

Results

PM characterisation

The main components of the PM were organic carbons. Sulphate, elemental carbon, chloride and nitrate were the other components in abundance in the PM sample. Traces of other substances such as titanium, manganese, lead, chromium and nickel were also detected, see Table 1.

Anthropometry markers

Weight gain was used as a generic indicator of health status. As shown in Table 2, body weight was not affected by PM exposure (Table 2). However, PM_{10} (5ug)-exposed animals had significantly more retroperitoneal fat mass compared to the SHAM group ($p<0.05$). There were no significant changes in liver or muscle weights.

Bronchoalveolar (BAL) cell count

PM_{10} (5μg) exposure increased leukocyte counts in BAL fluid ($P<0.01$, PM_{10} (5μg) vs SHAM, Figure 1A), as well as lymphocytes and macrophages (both $P<0.01$ vs SHAM, Figure 1A, B). There were no neutrophils or eosinophils observed.

Lung Inflammation

NLRP 3 and IL-1β were increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$, Figure 1D/E), but not IL-6 (Figure 1F).

Markers of matrix remodelling

Protein levels of fibronectin, TGF-β1 and collagen-III were not changed in any of the PM groups compared to the SHAM group (Figure 1G-I).

Mitochondrial antioxidant, mitophagy markers and mitochondrial DNA copy number

PM_{10} (5μg) exposure significantly increased mitochondrial fission protein Drp-1 ($P<0.05$, PM_{10} (5μg) vs SHAM, Figure 2A) and reduced mitochondrial fusion protein OPA-1 and the antioxidant MnSOD levels (both $P<0.05$, PM_{10} (5μg) vs SHAM, Figure 2B/C). Mitochondrial DNA copy number was not changed between SHAM and PM_{10} (5μg) (Figure 2D).

Autophagy and apoptosis

Autophagy marker LC3A/B-II, LC3A/B-II to I ratio were reduced in PM_{10} (5μg) compared to SHAM ($P<0.05$, Figure 2E/F). Apoptotic marker Caspase-3 was increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$, Figure 2G). The upstream marker of autophagy, p-AMPK and p-AMPK to AMPK ratio were reduced by the exposure to PM_{10} (5μg) compared to the SHAM exposure ($P<0.05$ vs SHAM, Figure 2K/M). Akt and AMPK protein levels were increased in the PM_{10} (5μg) group compared to the SHAM group ($P<0.05$ vs SHAM, Figure 2I/L), but there were no changes in p-Akt protein levels and p-Akt to Akt ratio by PM_{10} exposure (Figure 2I/J).

Discussion

We found that the exposure to low levels of traffic related PM_{10} induced marked pulmonary activation of NLRP3 inflammasome, and inflammation, as well as reduced mitochondrial antioxidants, and impaired mitophagy capacity.

PM_{10} exposure for three weeks did not affect the overall wellbeing of the mice reflected by body weight, suggesting low toxicity. However, fat mass was increased following the exposure to 5μg of PM_{10}, consistent with other human and mouse studies (27, 31).

We found increased lymphocytes and macrophages, which has also been observed with high dose PM exposure (8). However, PM_{10} (5μg) did not induce eosinophilic or neutrophilic inflammation. Increased IL-1β was accompanied by NLRP3 inflammasome activation as expected. Zheng et al (37) also found that 3 weeks exposure to 50μg of $PM_{2.5}$ daily increased IL-1β and TGF-β1 levels in BAL. Inflammasome activation has been observed in asthma, COPD and during pulmonary inflammation (10, 18, 35), suggesting that continuous exposure to even low level of PM may increase the susceptibility to these conditions.

Mitochondrial dysfunction is associated with various pulmonary diseases. COPD patients have mitochondrial fragmentation through an increase in Drp-1. In-vitro prolonged cigarette smoke exposure increased mitochondrial fission (6, 15). Damaged mitochondria increase oxidative stress which can consume the antioxidative MnSOD. Our study shows that 5µg of PM reduced MnSOD, suggesting reduced antioxidant capacity. Mitochondrial DNA copy number was unaffected, suggesting mitochondrial biogenesis was not changed by PM in this model. The reduction in LC3A/B-II protein in the PM_{10} (5µg) group indicates that there was reduced capacity of autophagy which can increase apoptosis. This was confirmed with the increased protein levels of caspase-3 in our study.

Activated AMPK was reduced by PM_{10} exposure. AMPK is a stress sensor which is crucial for maintaining intracellular homeostasis during oxidative stress and importantly, AMPK deficient mice have increased progression of COPD (19). AMPK typically supresses Akt, but we found no change in Akt levels, suggesting dysregulation of AMPK/Akt signalling. In our study we found PM reduced AMPK activation with reduced autophagy, however *in-vitro* studies have found PM increases AMPK and autophagy. We postulate that such differences are related to the 10-20 times higher dose of PM used *in-vitro* which induce cell death, in-addition to activating AMPK and autophagy (20, 30, 32). The *in-vitro* response is consistent with the notion that autophagy generally acts to keep cells alive, and is upregulated in response to stress (for a review see (12)). Differences may also occur due to PM processing for *in-vitro* studies in which steam sterilisation to remove LPS may also remove other PM components. Interestingly LPS inhibits AMPK activation (33).

Inflammasome activation by asbestos or crystalline silica is strongly associated with the development of lung fibrosis (24). However, in this study, exposure to a low level of PM did not induce fibrosis. The negative findings are most likely attributable to the low PM dose and the short duration of this study.

This study has several limitations. PM_{10} composition varies by generation source, and as such future studies need to compare different types of PM. We did not assess endotoxin levels in PM which are likely to influence the proinflammatory capacity of the PM. The lung tissues were not fixed to assess any histological changes or mitochondrial morphology, which need to be addressed in future studies.

In conclusion, this study shows that the exposure to low levels of roadside PM has detrimental effects on lung health. As such people living alongside major traffic corridors need to be aware of the potential adverse effects on their respiratory health. Our results also have implications for government agencies responsible for urban planning.

Acknowledgements

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Interest conflict: There are no conflicts of interest.

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400

401 **Figure Legends**

402 Figure 1. Leukocytes counts bronchoalveolar lavage (A-C). Lung protein levels of NLRP3
403 (D), IL-1β (E), IL-6 (F), fibronectin (G), TGF-β1 (H) and collagen-III (I) in Sham, particulate
404 matter $(PM)_{10}$ (1μg) and PM_{10} (5μg) groups. Results are expressed as mean ± SEM, n = 8-10
405 (one-way ANOVA followed by Bonferroni post hoc test). *p<0.05, **p<0.01, compared with
406 SHAM; #P<0.05, ##p<0.01, compared with PM_{10} (1μg).

407

408 Figure 2. Lung mitochondrial protein levels of Drp-1(A), Opa-1(B), MnSOD (C),
409 Mitochondrial DNA copy number (D) , Lung protein levels of LC3A/B-II (E), LC3A/B-II to
410 I ratio (F), Caspase-3 (G), p-Akt (H), Akt (I), p-Akt/Akt ratio (J), p-AMPK (K), AMPK (L)
411 and p-AMPK to AMPK ratio (M) in Sham, PM_{10} (1μg) and PM_{10} (5μg) groups. Results are
412 expressed as mean ± SEM, n=5-8. (one-way ANOVA with Bonferroni tests). *P<0.05
413 compared to SHAM. **P<0.01 compared to SHAM, #P<0.05, compared to PM_{10} (1μg). Akt,
414 protein kinase 3; AMPK, 5' adenosine monophosphate-activated protein kinase; Drp-1,
415 dynamin related protein 1; LC3A/B, Light chain 3 microtubule-associated protein A/B;
416 MnSOD, manganese superoxide dismutase; Opa-1, optic atrophy 1; PM, particulate matter.

417

418 **Chemical components of PM**

419 **Table 1. Chemical characteristic of PM_{10}**

	$\mu g/m^3$		$\mu g/m^3$
PM_{10} mass	22.61±1.26	**Ammonium**	0.16±0.03
Organic Carbon (OC)	4.19±0.20	**Barium**	0.08±0.003
Sulfate	4.00±0.34	**Zinc**	0.08±0.01
Elemental Carbon (EC)	3.26±0.17	**Copper**	0.04±0.03
Chloride	2.52±0.41	**Titanium**	0.02±0.004
Nitrate	1.92±0.13	**Manganese**	0.02±0.002
Iron	0.85±0.04	**Lead**	0.02±0.002
Calcium	0.43±0.03	**Vanadium**	0.01±0.002
Silicon	0.35±0.02	**Chromium**	0.01±0.001
Aluminium	0.17±0.02	**Nickel**	0.01±0.001

420 Results are expressed as mean ± SEM. Data showing different components inside the traffic
421 related air pollutants (n=10).

422

423

424 **Table 2. The effects of PM_{10} exposure on anthropometry markers**

	SHAM	**PM_{10} (1µg)**	**PM_{10} (5ug)**
Body Weight	22.39±0.31	22.26±0.36	22.13±0.37
Liver (g)	1.26±0.045	1.21±0.037	1.15±0.037
Liver %	5.62±0.0015	5.47±0.0011	5.21±0.0015
Muscle (g)	0.073±0.0024	0.075±0.0023	0.072±0.0032
Muscle %	0.33±0.00013	0.34±0.00011	0.33±0.00019
Retroperitoneal fat weight (g)	0.077±0.0037	0.109±0.014	0.12±0.012*
Retroperitoneal fat %	0.34±0.00016	0.50±0.00064	0.55±0.00052*
Glucose (mM)	9.13±1.14	9.6±1.07	9.27±1.1

425 Results are expressed as mean ± SEM, n = 10. Data were analysed by one-way ANOVA
426 followed by Bonferroni post hoc test. *$p<0.05$, compared with SHAM. PM_{10}: particulate
427 matter.

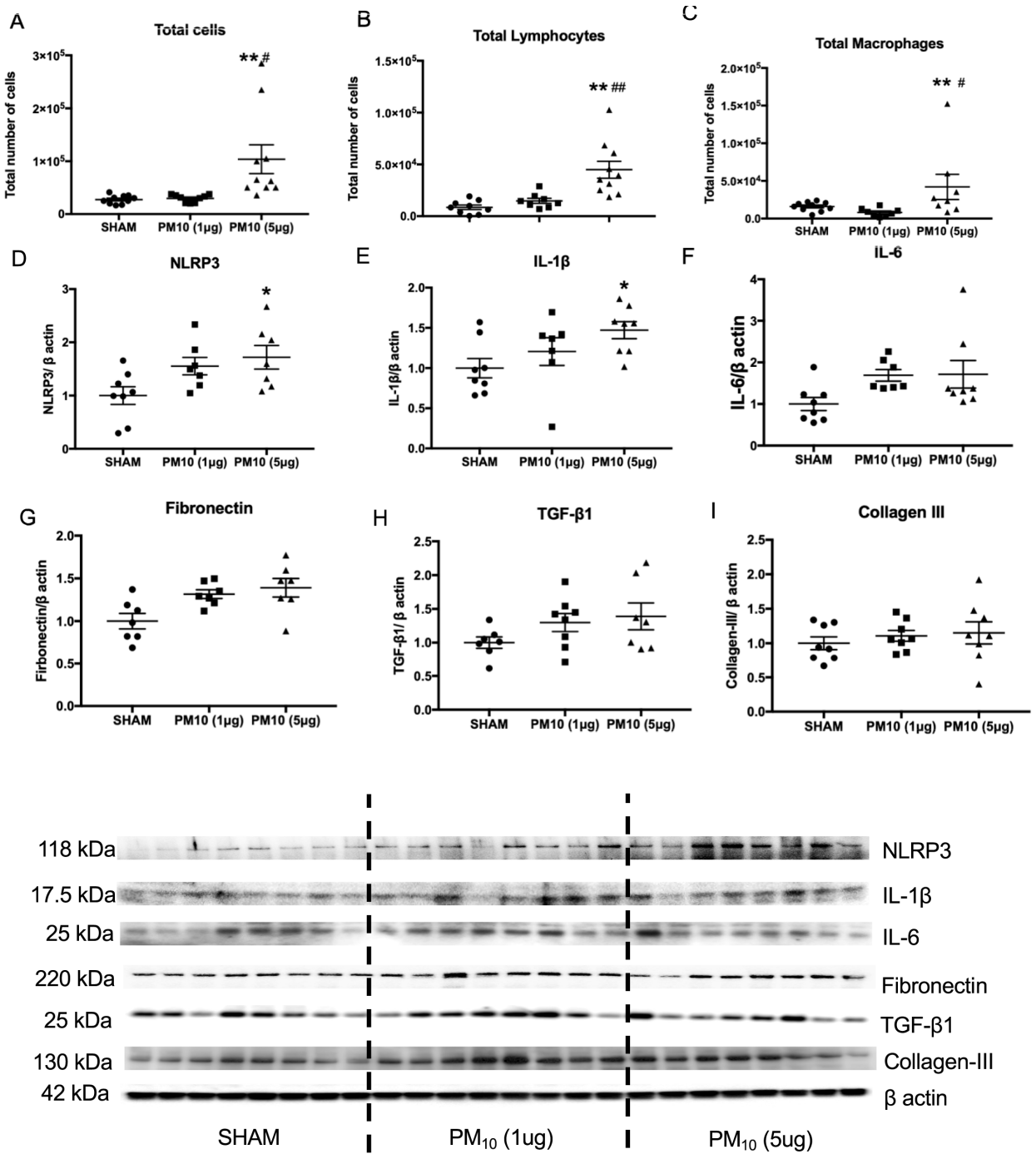
A
Total cells
Total number of cells
SHAM
PM10 (1μg)
PM10 (5μg)
B
Total Lymphocytes
C
Total Macrophages
D
NLRP3
NLRP3/ β actin
E
IL-1β
IL-1β/β actin
F
IL-6
IL-6/β actin
G
Fibronectin
Firbonectin/β actin
H
TGF-β1
TGF-β1/ β actin
I
Collagen III
Collagen-III/ β actin
118 kDa
NLRP3
17.5 kDa
IL-1β
25 kDa
IL-6
220 kDa
Fibronectin
25 kDa
TGF-β1
130 kDa
Collagen-III
42 kDa
β actin
SHAM
PM10 (1ug)
PM10 (5ug)

Figure 1.

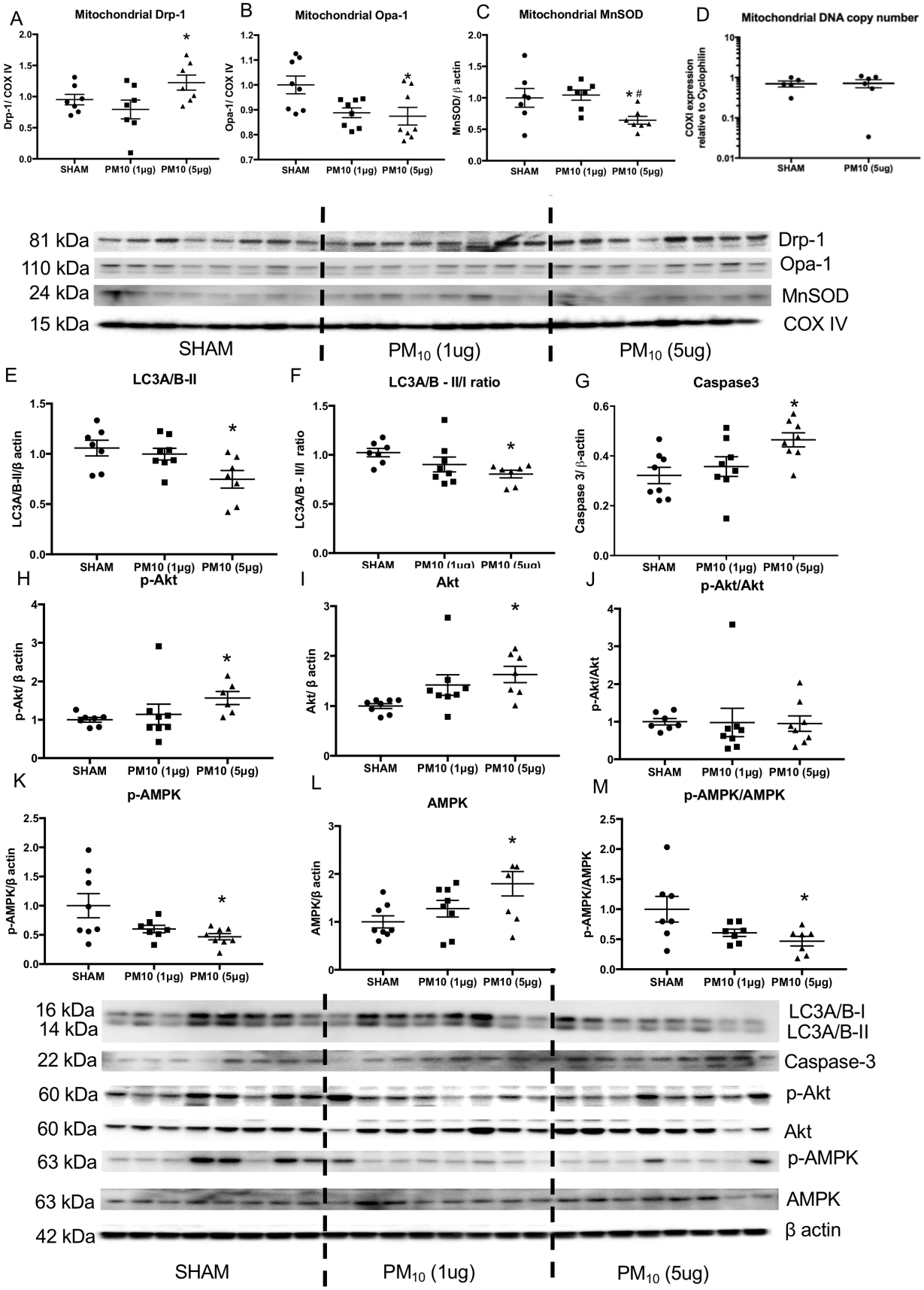
A
Mitochondrial Drp-1
Drp-1/ COX IV
SHAM
PM10 (1μg)
PM10 (5μg)
B
Mitochondrial Opa-1
Opa-1/ COX IV
C
Mitochondrial MnSOD
MnSOD/ β actin
D
Mitochondrial DNA copy number
COXI expression relative to Cyclophilin
PM10 (5ug)
81 kDa
110 kDa
24 kDa
15 kDa
Drp-1
Opa-1
MnSOD
COX IV
SHAM
PM10 (1ug)
PM10 (5ug)
E
LC3A/B-II
LC3A/B-II/β actin
F
LC3A/B - II/I ratio
LC3A/B - II/I ratio
G
Caspase3
Caspase 3/ β-actin
H
p-Akt
p-Akt/ β actin
I
Akt
Akt/ β actin
J
p-Akt/Akt
p-Akt/Akt
K
p-AMPK
p-AMPK/β actin
L
AMPK
AMPK/β actin
M
p-AMPK/AMPK
p-AMPK/AMPK
16 kDa
14 kDa
22 kDa
60 kDa
60 kDa
63 kDa
63 kDa
42 kDa
LC3A/B-I
LC3A/B-II
Caspase-3
p-Akt
Akt
p-AMPK
AMPK
β actin
SHAM
PM10 (1ug)
PM10 (5ug)

Figure 2.

Chapter 4 Maternal particulate matter exposure impairs transgenerational lung health and is associated with mitochondrial damage

This chapter is currently under review at the *Journal of Hazardous Materials*

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Contribution:

- Animal experiments and all tissue analysis
- preparing the manuscript
- Finalising the manuscript

Maternal particulate matter exposure impairs transgenerational lung health and is associated with mitochondrial damage

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HC and BGO designed the study. BW, BS, JL, GL, and YLC performed all the experiments. BW, YLC, GL, KFH, AA, BS, GH, BJ, CH, PST, JL DC, SS, PSF, HC, and BGO contributed to the writing of the manuscript. BW and YLC prepared the figures and tables. All the authors reviewed the final manuscript.

Abstract:

Relatively little is known about the transgenerational effects of chronic maternal exposure to low-level traffic-related air pollution (TRAP) on the offspring lung health, nor are the effects of removing such exposure prior to pregnancy. Female BALB/c mice were exposed to $PM_{2.5}$ ($PM_{2.5,}$ 5 μg/day) for 6 weeks before mating and during gestation and lactation; in a subgroup, PM was removed after mating to model mothers moving away from TRAP during pregnancy to protect their unborn child (Cessation). Lung pathology was characterised in both dams and offspring. A subcohort of offspring were also exposed to ovalbumin to model allergic airways disease. $PM_{2.5}$ and Cessation dams exhibited airways hyper-responsiveness (AHR) with mucus hypersecretion, increased mitochondrial reactive oxygen species (ROS) and mitocondrical dysfunction in the lung. Offspring from $PM_{2.5}$ and Cessation dams displayed AHR with increased lung inflammation and mitochondrial ROS production. After the ovalbumin challenge, airway resistance was worse in offspring from $PM_{2.5}$ dams compared with those from control dams. Using an in-vitro model the mitochondria-targeted antioxidant MitoQ reversed mitochondrial dysfunction by PM stimulation, suggesting the lung pathology is oxidative stress-driven. In conclusion, chronic exposure to low dose $PM_{2.5}$ exerted transgenerational impairment on lung health.

Keywords: air pollution, lung function, reactive oxygen species, mitochondrial dysfunction, asthma

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Chapter 5 General Discussion and future perspective

Epidemiological studies have shown that maternal smoking is one of the prominent *in utero* environmental risk factors for the development of respiratory diseases in childhood. Smoking during pregnancy is a risk factor for asthma, COPD and lung cancer in the offspring [83,84]. However, the sex difference in such susceptibility to respiratory disorders has not been well studied, perhaps due to the preference in animal models to use only one gender to model asthma or COPD.

Our study in Chapter 2 shows that male offspring are more vulnerable to the adverse effects of maternal cigarette smoke exposure during pregnancy, which was demonstrated by the smaller body weight, and higher levels of inflammatory markers in the lung, such as increased expression level of p-ERK1,2, total p-P38, and p-NF-kB. Maternal cigarette smoke exposure also increased the levels of mitochondrial fission marker Drp-1. The fusion marker Opa-1 was not increased accordingly suggesting less healthy mitochondrial fragment can be recycled. Those results demonstrated that maternal smoking during pregnancy could induce mitochondrial dysfunction in the offspring.

Maternal cigarette smoke exposure is also an *in utero* environmental toxicant which can induce excessive oxidative stress. *In vivo* and *in vitro* studies have demonstrated that the antioxidant L-Carnitine can prevent oxidative stress-induced injuries to the kidneys and cardiometabolic systems in mouse models [85-87]. In our study (Chapter 2), maternal L-Carnitine supplementation increased the birth weight of both male and female SE offspring and partially ameliorated the adverse impacts of maternal cigarette smoke on lung health outcome. This suggests that oxidative stress may be the primary mechanism of *in-utero* underdevelopment by maternal smoking and L-Carnitine is capable of ameliorating such oxidative stress.

Summary of the markers expression levels in chapter 2

		P1 Male		P1 Female		13w Male		13w Female	
		Smoking	L-carnitine	Smoking	L-carnitine	Smoking	L-carnitine	Smoking	L-carnitine
Inflammation	P-ERK1,2	↑	↑	–	↓	–	–	–	–
	p-JNK1,2	–	–	–	–	↓	↓	–	–
	p-P38	↑	↑	–	↓	–	–	–	–
	P-NF-kB	↑	–	–	–	↑	–	–	–
Inflammasome	NLRP3	↑	↓	–	–	–	–	↑	↑
	IL1-β	–	–	↑	–	↑	↑	↓	–
Autophagy	LC3A/B-II	↑	↑	–	–	↑	↑	–	–
Mitophagy	Drp-1	↑	–	–	–	–	–	–	–
	Opa-1	–	–	–	–	–	–	–	–

Another well-known environmental toxicant to foteal lung development is air pollution [88]. It is common sense that high levels of air pollution could induce adverse impacts on foetal lung development and health. Previous mouse models also demonstrated the possible mechanisms, such as changed immune cell development [59,89]. Those mouse models using high levels of PMs may reflect the high levels of annual ambient air pollution in Asia and Africa [60]. However, the population weighted mean annual PM concentrations in Europe, North America, and Oceania are lower (5-15 μg/m^3) than Asia and Africa regions [60]. Few studies explored whether exposure to PM lower than the WHO air quality guideline (50 μg/m^3 24-hour mean) can also induce adverse impacts on lung development.

The study in Chapter 3 shows that even low dose PM_{10} exposure (5 μg/day) for 3 weeks can still cause a high level of inflammation in mice. In this study, we found several effects similar to the model using high doses of PM exposure. For example, PM induced marked pulmonary activation of the NLRP3 inflammasome. Inflammasome activation has been observed in asthma and COPD, as well as during pulmonary inflammation [90-92] suggesting that continuous exposure to even a low level of PM may increase the susceptibility to these conditions.

Mitochondria play an important role in lung function. Mitophagy plays a key role in maintaining mitochondrial integrity and normal mitochondrial function through the balance of fusion and fission. Mitochondrial dysfunction is related to several pulmonary diseases, such as asthma, COPD and IPF[93]. In this study, we found PM_{10} exposure impaired mitophagy markers only after 3 weeks of exposure, which may promote lung structure damage and functional impairment in the long term, as we have shown in PM exposed dams in Chapter 4. This suggests that even living in the less polluted areas where the PM concentration is within the WHO air quality guideline still can induce pulmonary diseases.

Summary of the markers expression levels in chapter 3

	PM10 (1ug/day)	PM10 (5ug/day)
Immune cells number		
Total cells number	–	↑
Total lymphocytes	–	↑
Total Macrophages	–	↑
Inflammasome activation		
NLRP3	–	↑
IL1-β	–	↑
Mitophagy		
Drp-1 (Fission)	–	↑
Opa-1 (Fussion)	–	↑
Autophagy		
LC3A/B-II	–	↓

People living near busy roads and industrial areas are exposed to more to air pollution and thus have a higher risk of developing respiratory diseases. A previous study confirmed that air pollution exposure during pregnancy decreases placental growth factor [55], increases cord blood immune biomarkers (e.g. Ig E, IL-33) [56], and causes mitochondrial oxidative DNA damage [57]. Previous studies in mouse models have also found *in-utero* exposure to 100 μg PM from

residential roof spaces impaired somatic growth, reduced lung volume and lung function in offspring [58]. Pregnant mice exposed to combustion generated free radical containing particles (200nm, 50 μg) have systemic oxidative stress and the offspring developed asthma [59]. Those mouse models demonstrated the adverse impacts induced by high levels of air pollution. Our study in Chapter 4 found that chronic exposure to low dose PM (5 μg/day) induced airway hyper-responsiveness, increased inflammation level in the lung, and higher number of leukocytes in the bronchial alveolar lavage fluid. We also observed lung tissue remodelling with increased collagen deposition, excessive mucous production and damaged alveolar membranes.

A previous study showed that chronic exposure to environmental toxicants could induce COPD in humans [94]. The characteristic of the COPD lung is airflow limitation because of the airway obstruction and parenchymal destruction. In the COPD lung, there is increased tissue density (small airway fibrosis) in the places where the alveolar membrane is not damaged [95]. We observed increased tissue elastance and tissue damping, excessive inflammation, and airway remodelling which resemble lung pathology in patients with COPD. Those results demonstrated that chronic low dose PM exposure could induce COPD-liked pathology in the mouse.

Maternal exposure to the low dose PM also induced AHR and higher inflammation in the offspring, which can't be reversed by removing PM exposure during pregnancy. These results indicate that exposure to low dose PM in the dams also can induce respiratory diseases in the offspring, even only exposed before pregnancy. Multiple epidemiological studies confirmed that maternal PM exposure could increase the risk of asthma in the offspring. A classical Ovalbumin (OVA)-sensitized and challenged asthmatic model confirmed that maternal low dose PM exposure can possess the same risk in the offspring and worsen their asthmatic symptoms.

We suspected that the mitochondrial dysfunction was closely associated with these transgenerational adverse impacts induced by maternal low dose PM exposure, as the mitochondria in offspring are exclusively inherited from the mothers [96]. As shown in Chapter 3, 3-week low dose PM exposure, 5 μg/day impaired mitophagy, with increased level of total ROS in the lung tissue further confirming increased oxidative stress. Similar results were found in the female offspring, including high mitochondrial density, mitochondrial ROS and total ROS level. The mitophagy markers were also impaired by maternal PM exposure. In order to further confirm the role of mitochondria in PM exposure induced pathology, we examined the mitochondrial functional change in Beas-2B cells. Results in Chapter 4 show that MitoQ, a mitochondrion targeted antioxidant, significantly ameliorated mitochondrial dysfunction induced by the PM exposure. These results strongly suggest that mitochondrial dysfunction is closely associated with the adverse impacts induced by PM exposure.

Summary of the markers expression levels in chapter 4

	$PM_{2.5}$	Cessation
Lung Function		
Tissue Elastance	↑	↑
Tissue Damping	↑	–
Cells differenatiation		
Macrophages	↑	–
Eosinophils	↑	–
Neutrophils	↑	–
Lymphocytes	↑	–
Inflammation Level		
	↑	↑
Airway Remodelling		
Epithelial thickness	↑	↑
Alveolar Damage (MLI)	↑	–
Fibrosis level	↑	–
Mucus secretion	↑	↑
Muscle thickness	↑	↑

In conclusion, this thesis confirmed that male offspring are more susceptible to *in utero* environmental toxin exposure. Maternal low dose PM exposure can induce transgenerational adverse impacts on pulmonary health in offspring.

Limitations:

Our studies do have limitations. We measured the targeted proteins expression levels in the chapter 2 with western blot without more dimentional approaches, such as PCR and immonohistochemistry. More analysis approaches will be used in the future work.

Take home message

1. Male offspring are more vulnerable to maternal smoking induced lung impairment than their female littermates.
2. Maternal L-Carnitine supplement during pregnancy could partially alleviate the adverse impacts on the offspring's lung induced by maternal smoking.
3. Short-term exposure to low dose PM can increase the pulmonary inflammatory response
4. Chronic exposure to low dose PM could induce COPD-like pathology in the lung
5. *In utero* exposure to low dose PM could exacerbate asthmatic symptoms in the adulthood

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