The association between early life exposure to ambient particulate matter and long-term immune and respiratory health in children

by

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Declaration of Originality

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Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. Ethics Approval Nos H0014875 and H0015236.

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List of Abbreviations

$PM_{2.5}$	Particulate matter with an aerodynamic diameter < 2.5 micrometers
FVC	Forced vital capacity
FEV_1	Forced expiratory volume in 1 s
$FEV_{0.5}$	Forced expiratory volume in 0.5 s
PEF	Peak expiratory flow
PAH	Polycyclic aromatic hydrocarbon
IL	Interleukin
FOT	Forced oscillation technique
Latrobe ELF Study	Latrobe Early Life Follow-up Study
CSIRO	Commonwealth Scientific and Industrial Organisation
PM_{10}	Particulate matter with an aerodynamic diameter less than 10 μm
LUR	Land use regression
OR	Odds ratio
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
CASP	Critical appraisal skills programme
IDW	Inverse distance weighted
GIS	Geographic information systems
PEMS	Personal environmental monitoring samplers
ETS	Environmental tobacco smoke
SES	Socio-economic status
Rrs_5	Rrs at a frequency of 5 Hz
Xrs_5	Xrs at a frequency of 5 Hz

AX	Area under the reactance curve
IQR	Interquartile range
SD	Standard deviation
IRSD	Index of Relative Socio-economic Disadvantage
SHS	Second hand smoke
MICE	Multiple imputation by chained equations
MAR	Missing at random
PMM	Predictive mean matching
GP	General practitioner
MBS	Medicare Benefits Schedule
PBS	Pharmaceutical Benefits Scheme
NO_2	Nitrogen dioxide
IRR	Incidence rate ratio
PAH	Polycyclic aromatic hydrocarbon
SEM	Scanning electron microscopy
LAL	Limulus amebocyte lysate
EU	Endotoxin units
mg	Milligram
GC-MS	Gas chromatography mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
ATCC	American Type Culture Collection
BEGM	Bronchial epithelial cell growth medium
HBSS	Hank’s balanced salt solution

PCA	Principal component analysis
ANOVA	Analysis of variance
HSD	Honestly significant difference

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Abstract

Background: Landscape fire smoke exposure could cause adverse health outcomes, especially in children. The Hazelwood coal mine fire started on 9 February 2014 in Victoria, Australia and lasted for approximately 6 weeks. This event caused increased concentrations of fine particulate matter ($PM_{2.5}$) in the nearby area.

The effect of smoke exposure during the *in utero* period and infancy (i.e. the first two years of life) may have implications for the development and growth of a child's immune and respiratory systems. However, there is very limited evidence regarding the associations between early life exposure to short-to-medium duration fire smoke events, which can result in severe air pollution, and the potential health outcomes in later life.

Aims: This Thesis aimed to investigate possible associations between early life exposure to air pollution from the Hazelwood coal mine fire and later respiratory health and immune function. A range of complementary biomedical and epidemiological research approaches were used to address four specific research questions: 1) to evaluate current epidemiological evidence on the associations between intrauterine and infant exposure to particulate matter and subsequent development of asthma and wheezing (Chapter 2); 2) to evaluate children's lung function following infant exposure to the Hazelwood coal mine fire emissions (Chapter 3); 3) to assess the effect on health service utilisation in children after intrauterine and infant coal mine fire smoke exposure (Chapter 4), and; 4) to investigate how fire smoke-related particulate matter, and the chemical components, affect respiratory health by conducting toxicological studies in human lung cells (Chapter 5).

Methods: 1) A systematic review and meta-analysis was conducted to answer Aim 1. Epidemiological data from relevant literature investigating the associations between ambient

$PM_{2.5}$ exposures during two time points (prenatal or the first two years of life), and wheezing or asthma throughout life was extracted from five databases. All included studies were assessed according to the Critical Appraisal Skills Programme checklists. Meta-analyses were performed if ≥2 studies estimated the effects of continuous $PM_{2.5}$. 2) To answer the second and third Aims, I collected data from the Latrobe Early Life Follow-up (ELF) Study, comprising 571 children born between 01/03/2012 and 31/12/2015 from the Latrobe Valley in Victoria, Australia. Individual exposures to 24-hour average and peak concentrations of $PM_{2.5}$ during the fire were estimated using individual activity/location data, dispersion and chemical transport modelling. Lung function was measured using the forced oscillation technique (FOT), generating standardised Z scores for resistance (Rrs), reactance (Xrs) and the area under the reactance curve (AX). Data on general practitioner attendances, and dispensations of prescribed asthma inhalers, steroid skin creams and antibiotics were collected from the Australian Medicare Benefits Schedule (MBS) and Pharmaceutical Benefits Scheme (PBS). Multiple regression analyses were used to assess the associations. 3) For Aim 4, roof space particulate matter samples from 36 different homes and their particle characteristics (i.e. size, endotoxin and chemical composition) were analysed using standardised techniques. The cytokine production of BEAS-2B cells after exposure to either media alone, 5.7 or 57 μg/mL of particulate matter suspension for 4 h or 24 h, was assessed using ELISA. Principle component analysis (PCA) and linear regression analyses were employed to evaluate the associations between cytokine production and the particle composition.

Results: For Aim 1 (Chapter 2), while evidence was limited and inconsistent, epidemiological literature was suggestive of an association between early life $PM_{2.5}$ exposure and wheezing/asthma. Meta-analyses conducted for the associations between: (1) intrauterine exposure and asthma (n=4); (2) infant exposure and asthma (n=5); and (3) infant exposure and wheezing (n=3), found no significant associations. While meta-analysis of intrauterine

exposure and wheezing (n=5) was not possible due to inconsistent exposure and outcome assessments, four studies found strong positive associations with wheeze by age 2. High heterogeneity was present among studies of intrauterine exposures and asthma, while studies of other associations showed low heterogeneity.

Data in Aim 2 (Chapter 3), using FOT assessment, showed a 10 μg/m^3 increase in infant average $PM_{2.5}$ exposure was significantly associated with worsening AX (β-coefficient, 0.26; 95%CI 0.02, 0.50), while the association between a 100 μg/m^3 increase in peak $PM_{2.5}$ and AX was not significant (0.17; 95%CI -0.00, 0.33). In the analysis of MBS/PBS data (Aim 3 – Chapter 4), 10- and 100- μg/m^3 increases in average and peak $PM_{2.5}$ exposure during infancy were associated with a greater incidence of antibiotics being dispensed during the year following the fire: the adjusted incidence rate ratios were 1.24 (95% CI 1.02, 1.50, $p<0.05$) and 1.14 (1.00, 1.31, $p<0.05$) respectively. No other significant associations were observed.

For Aim 4 (Chapter 5), exposure to roof space particulate matter caused significant dose (IL-6, $p<0.05$ for all comparisons; IL-8, $p<0.05$ for comparisons after 24 h exposure) and time (IL-6, $p<0.05$ for all comparisons; IL-8, $p<0.05$ for all comparisons) dependent increases in cytokine production that was evident 4 and 24 h post-exposure with the exception of IL-8 production 4 h post exposure to 5.7 μg/mL particulate matter which was not elevated above control levels ($p>0.05$). Higher concentrations of Fe, Al, Mn in particulate matter were significantly associated with increased cytokine production.

Conclusions: Current evidence on the associations between early life $PM_{2.5}$ exposure and adverse respiratory outcomes during childhood is limited. My analyses provided novel findings of significant associations between infant exposure to $PM_{2.5}$ from coal mine fire emissions and later adverse immune and respiratory health outcomes, including worse lung reactance, and increased use of antibiotics. The underlying mechanisms might be the pro-inflammatory

capacity of $PM_{2.5}$ on human lung cells. Further follow-up studies are needed to confirm these findings, to investigate whether these effects persist as children develop and to further explore potential mechanisms.

Chapter 1

General Introduction

Chapter 1. General Introduction

1.1 Coal mine fire smoke exposure and human health

Coal mine fires are widespread and currently active around the world, generating air pollutants including particulate matter, gases and condensation by-products[1]. In February 2014, an open-cut coal mine fire (Hazelwood coal mine fire) was ignited by embers from wildfires and lasted for 45 days in the Latrobe Valley, Victoria, Australia. Several regional towns near the mine were affected by smoke during the fire period with air quality impacts ranging from minor to severe. The nearest town of Morwell experienced severe air pollution exposure with a peak 24-hour average $PM_{2.5}$ concentration of 731 μg/m^3, which is remarkably higher than the Australian air quality standard of 25 μg/m^3[2-3]. However, the potential health effects of coal mine fire smoke exposure have been poorly investigated[4].

Air pollutants generated from coal mine fire emissions are thought to be similar to those from landscape fires including burning forest, grass and peat[4], which make a significant contribution to air pollution[5] and is an increasing global concern because of the rising frequency and severity of fires resulting from climate change[6]. Exposure to air pollutants from landscape fire smoke has been demonstrated to adversely affect human health, especially the respiratory and immune systems. For example, epidemiological studies have consistently found that short-term fire smoke exposure is significantly associated with decreased lung function among non-asthmatic children, and increased hospitalisations, physician and emergency department visits for respiratory problems and asthma among general population[7-8]. There is also strong evidence suggesting an association between fire smoke exposure and increased respiratory infections[8-9]. However, evidence on the health effects from early life fire smoke exposure is very limited[7]. A study of rhesus macaque monkeys suggested that infant exposure to fire

smoke was associated with immune dysregulation and reduced lung volume in adolescence[10] indicating that further work is warranted.

Particulate matter with an aerodynamic diameter < 2.5 micrometers ($PM_{2.5}$) is one of the primary emissions from landscape fires[11-12]. $PM_{2.5}$ from other sources such as traffic and industrial emissions is well known to be harmful to respiratory and immune health, both for short-term and long-term exposures. For example, daily exposure to $PM_{2.5}$ has been found to be positively associated with increased hospital admissions and/or emergency department visits for pneumonia and asthma in children and adolescents[13-15], while long-term exposure has also been associated with asthma development during childhood[16]. There is a small, but growing, body of evidence indicating an association between short-term fire smoke-related $PM_{2.5}$ exposure and adverse health outcomes. A study of the 2007 San Diego landscape fires observed a significant association between daily fire smoke-related $PM_{2.5}$ exposure and increased emergency department presentations for respiratory issues such as asthma, respiratory infections and other symptoms[17]. In line with this, similar associations were also found in studies of landscape fires from other areas of America and Canada between short-term exposure to $PM_{2.5}$ from fire emissions and respiratory diseases including asthma/wheezing and bronchitis[18-21]. However, the effects of fire smoke $PM_{2.5}$ exposure in later life have not been well documented. Additionally, despite the similarity in toxic components from coal mine fire and landscape fire emissions, individual fire emissions vary significantly depending on the substrate burned, the nature of combustion and meteorological conditions[4]. Coal mine fires are often of a longer duration than landscape fires, and are characterised by predominantly smouldering combustion. Therefore, it is important to understand the association between coal mine fire smoke exposure and human health to guide public health responses.

1.2 Developmental susceptibility to the effects of air pollution

The development and growth of human respiratory and immune systems starts *in utero* and lasts throughout the whole childhood. For the respiratory system, the prenatal period is critical for cellular differentiation and branching morphogenesis[22]. The embryonic stage starts from the first week of pregnancy and lasted for nearly 7 weeks, followed by the pseudoglandular stage (5-17 weeks of pregnancy), the canalicular stage (16-26 weeks of pregnancy) and the saccular stage (24-38 weeks of pregnancy) successively[23]. The alveoli develop and grow from 36 weeks of pregnancy to 1-2 years after birth, which is known as the alveolar proliferation stage[22-23]. Development of the human immune system begins with the formation and migration of hematopoietic stem cells, followed by the expansion of progenitor cells and the colonisation of the bone marrow and thymus. All these processes occur during the *in utero* period[22, 24]. After birth, the immune system matures to immunocompetence during the first year of life[22].

Infants and young children have higher oxygen consumption rates compared with adults[25]. On a body weight basis, the rate of oxygen consumption of a resting infant is nearly twice the rate of a resting adult. Therefore, the volume of air pollutants reaching the lung of an infant, per body weight, are likely to be much higher than that of an adult under the same conditions[25].

Therefore, the *in utero* and early post-natal periods (i.e. first two years of life) may be periods of heightened susceptibility to adverse health outcomes resulting from air pollution exposure due to the developing respiratory and immune systems, and the faster breathing rates of infants.

1.3 Respiratory and immune effects of early life $PM_{2.5}$ exposure

Current literature on the respiratory and immune health outcomes resulting from early life ambient $PM_{2.5}$ exposure have focussed on wheezing/asthma, lung function, respiratory mortality, respiratory symptoms (e.g. cough), allergy and infections. A few studies have suggested that early life immune responses, that shape conditions such as lower respiratory

infections, are associated with reduced lung function and increased risk of asthma development during childhood[26-28]. Early life allergic sensitisation to mold could also increase the risk of childhood asthma[28]. There are limited, but increasing, studies investigating the associations between $PM_{2.5}$ exposure during *in utero* or the first two years of life and respiratory and immune health.

1.3.1 Respiratory effects of intrauterine $PM_{2.5}$ exposure

A systematic review and meta-analysis published in 2017 did not find evidence of an association between intrauterine $PM_{2.5}$ exposure and the development of childhood wheezing or asthma[29]. However, more recent studies, that were not included in this systematic review, have suggested that $PM_{2.5}$ exposure during pregnancy is positively associated with the incidences of both wheezing[30] and asthma[31-33] by age 6.

There are also a few studies investigating the effects of intrauterine $PM_{2.5}$ exposure on children's lung function. For example, a study in Krakow found significant deficits in forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1) and forced expiratory volume in 0.5 s ($FEV_{0.5}$) of 176 children at age 5 after intrauterine $PM_{2.5}$ exposure[34]. Another study suggested an association between intrauterine $PM_{2.5}$ exposure and reduced FVC in children at age 7, while a significant association with reduced FEV_1 was only found in boys[35].

1.3.2 Respiratory effects of infant $PM_{2.5}$ exposure

Current evidence regarding the associations between infant $PM_{2.5}$ exposure and wheezing/asthma is mixed. For example, there are studies indicating non-significant associations between $PM_{2.5}$ exposure during the first year of life and the development of childhood wheezing or asthma[36-38], while an American study of 24,608 children suggested that exposure to $PM_{2.5}$ during the first year of life was significantly associated with increased risk of asthma from age 2 to 6[32].

In terms of the association between infant $PM_{2.5}$ exposure and lung function, a study of 2,307 children observed significant negative associations between exposure to $PM_{2.5}$ during the first year of life and reduced peak expiratory flow (PEF) and forced expiratory flow at 25% and 50% of FVC ($FEF_{25\%}$ and $FEF_{50\%}$, respectively) in 9-10 year old children, but no significant associations were observed for FEV_1 and FVC[39]. Similarly, a study investigating FEV_1 and FVC also reported weak but non-significant associations with infant $PM_{2.5}$ exposure in 614 children with a median age of 7.7 years[40].

1.3.3 Immune effects of intrauterine $PM_{2.5}$ exposure

Studies investigating the associations between intrauterine $PM_{2.5}$ exposure and adverse immune health have mainly focused on infections and allergy. For example, a study in Krakow showed that intrauterine $PM_{2.5}$ exposure was associated with increased recurrent bronchitis and pneumonia (≥5 physician-diagnosed episodes) during early childhood (odds ratio (OR): 2.44, 95%CI: 1.12-5.36)[41]. Similar results were seen in a study of 953 Singapore children, suggesting that intrauterine $PM_{2.5}$ exposure was associated with increased risk of bronchiolitis/bronchitis in the first two years of life[30]. The Krakow research group did not find independent associations between intrauterine $PM_{2.5}$ exposure and childhood eczema, however significant joint effects of $PM_{2.5}$ exposure were observed with maternal paracetamol usage during pregnancy and postnatal tobacco smoke exposure[42-43].

1.3.4 Immune effects of infant $PM_{2.5}$ exposure

There is limited and inconsistent evidence of the association between exposure to $PM_{2.5}$ during the first two years of life and childhood infections. For infant bronchiolitis, one study indicated that chronic $PM_{2.5}$ exposure could increase the risk of bronchiolitis during infancy[44], while other studies suggest weak and non-significant associations[45-47]. For other infections, no significant associations have been observed in studies which have assessed the relationship

between infant $PM_{2.5}$ exposure and ear/nose/throat infections, flu/serious cold or other respiratory infections during the first two years of life[48-50].Evidence regarding the association between infant $PM_{2.5}$ exposure and atopic dermatitis in children is also limited. One Chinese study suggested that exposure to $PM_{2.5}$ during the first year of life was associated with increased risk of eczema in 3383 children aged 3-6 years[51], while another study found no association between lifetime $PM_{2.5}$ exposure and doctor-diagnosed eczema or itchy rash by age 2[48].

Overall, there is still limited and inconsistent evidence regarding the association between early life exposure to $PM_{2.5}$ and later immune and respiratory health. In addition, most studies have focused on traffic-related $PM_{2.5}$, while no study has evaluated the effect of early life exposure to $PM_{2.5}$ from fire smoke. One study indicated that infant exposure to fire smoke was associated with reduced immune and lung function in rhesus monkeys during adolescence[52].

The physiochemical and toxicological characteristics of particulate matter vary by source. For example, in a study comparing wildfire and traffic emissions in California, fire smoke-related PM contained higher concentrations of potassium, levoglucosan and water-soluble organic carbon compared with traffic-related PM[53]. The dithiothreitol activity, which was mainly influenced by polar organic compounds, increased for fire smoke-related PM compared with traffic-related PM, while the reactive organic species activity (influenced by transition metals) was unaffected by fire smoke-related PM[53]. Therefore, the health effects of exposure to fire smoke-related $PM_{2.5}$ might be different from traffic-related $PM_{2.5}$. More studies are needed to further explore this field.

1.4 How does particulate matter exposure affect the respiratory and immune health?

While epidemiological evidence on the associations between intrauterine and infant exposure to particulate matter and adverse respiratory and immune health outcomes during childhood is

limited, experimental studies have demonstrated that the associations are biologically plausible. $PM_{2.5}$ can bypass the upper airway defences and reach the lower respiratory tract[54]. The potential mechanisms of $PM_{2.5}$ induced respiratory and immune health are still unclear, however, many *in vitro* studies investigating the effects of $PM_{2.5}$ using human macrophages and lung cells consistently show that $PM_{2.5}$ induces a proinflammatory response through gene damage and oxidative stress[55-58]. $PM_{2.5}$ could induce the release of interleuken-6 (IL-6) and IL-8 in human lung cells[55], which were found to be associated with reduced lung function and asthma development. For example, IL-6 was found to play a pathogenic role in allergic asthma in mice[59]. In addition, a Japanese study suggested that the association between lung function in schoolchildren and daily $PM_{2.5}$ exposure differed by PM's ability to induce IL-8 production[60].

Importantly, the effect of particulate matter on human lung and immune cells is heavily influenced by particle size and composition[61-63]. For example, total iron content in coarse particulate matter is associated with the magnitude of lung inflammatory cell infiltrations and plasma creatine kinase levels in mice[64], while the polycyclic aromatic hydrocarbons (PAHs) components of $PM_{2.5}$ are negatively associated with IL-6 production in macrophage cell line (RAW 264.7)[65]. Taken together, previous work suggests that $PM_{2.5}$ can induce an inflammatory response in lung cells and that the magnitude of the response is influence by particle properties. However, most of this work has been conducted using traffic-derived $PM_{2.5}$ with very little work on $PM_{2.5}$ derived from other sources such as landscape fires[66].

1.5 Aims

The Hazelwood coal mine fire caused significant community concern, however, as discussed above the evidence for the health effects of exposure to this severe, short-to-medium duration

of air pollution is extremely limited, especially the effects of early life exposure. Therefore, the aim of this Thesis was to explore respiratory and immune health effects resulting from early life exposure to $PM_{2.5}$ from coal mine fire emissions by:

1. Summarising current epidemiological evidence on the associations between intrauterine and infant exposure to $PM_{2.5}$ and subsequent development of asthma and wheezing by conducting a systematic review and meta-analysis (Chapter 2);
2. Measuring children's lung function 3 years after exposure to the Hazelwood coal mine fire emissions during the first two years of life using forced oscillation technique (FOT) (Chapter 3);
3. Evaluating general practitioner visits and medication dispensations in children during the year following intrauterine or infant coal mine fire smoke exposure (Chapter 4);
4. Investigating how fire smoke-related particulate matter and its components affect human respiratory health by conducting toxicological studies using the human bronchial epithelial cell line (BEAS-2B) (Chapter 5).

1.6 Methodology

Three broad Methods were used to address these Aims:

1. A systematic review and meta-analysis (Aim 1 - Chapter 2);
2. Original analyses using data from the Latrobe Early Life Follow-up (ELF) Study (Aims 2 and 3; Chapters 3 and 4), which forms part of the wider research program of the Hazelwood Health Study and is run by a multidisciplinary group of researchers and administrative staff from the University of Tasmania, Monash University, the University of Melbourne, the University of Sydney and Commonwealth Scientific and Industrial Organisation (CSIRO). The Latrobe ELF Study aims to understand the

possible influence of exposure to coal mine fire smoke on the health and development of young children and children born to women who were pregnant at the time. The ELF study has two major streams: an identified cohort study of children from the Latrobe Valley who were recruited during 2015-2016, and a series of anonymous data extraction and data linkage studies. Chapter 3 (Aim 2) of this Thesis investigated the lung function of the identified participants from the Latrobe ELF Study in 2017, while Chapter 4 (Aim 3) evaluated deidentified medical service and medication usage during the year after the Hazelwood coal mine fire; and

3. A cell line study investigating cytotoxicity and pro-inflammatory effects of particulate matter and its components on human bronchial epithelial cells (BEAS-2B cell line) (Chapter 5).

Specific details of the methodology for each study are described in the following Chapters. A description of the forced oscillation technique can be found in the Appendix 3.

Chapter 2

Prenatal and Infant Exposure to Fine Particulate Matter on Wheezing and Asthma: A Systematic Review and Meta-analysis

Chapter 2. Prenatal and Infant Exposure to Fine Particulate Matter on Wheezing and Asthma: A Systematic Review and Meta-analysis

2.1 Preface

As mentioned in Chapter 1 (General Introduction), there is limited evidence regarding the respiratory effects of early life exposure to $PM_{2.5}$. I chose $PM_{2.5}$ specifically because it could travel to the deep lung and is thought to be the most harmful to respiratory health. The systematic review outlined in this Chapter was conducted during 2016-2017, aiming to summarise epidemiological studies regarding the effects of prenatal and infant $PM_{2.5}$ exposure on wheezing and asthma, which were published before December 4, 2017. Studies on all sources of $PM_{2.5}$, rather than fire smoke alone, were included due to the lack of studies focusing on the health effects of fire smoke-related $PM_{2.5}$ exposure during *in utero* or the first two years of life. A modified version of this Chapter has been published in *Environmental Epidemiology*.

2.2 Introduction

Exposure to $PM_{2.5}$ is a well-recognised global public health issue. It has been estimated that mortality from $PM_{2.5}$ exposure increased from approximately 3.5 million in 1990 to 4.2 million in 2015.[67] Globally, the association between $PM_{2.5}$ exposure, wheezing and asthma has been widely studied.[68-70] Short-term (e.g. daily) increases in $PM_{2.5}$ have a well-established association with worsening asthma symptoms and increases in hospital attendance rates,[71] while long-term exposure has been shown to increase the risk of developing asthma.[16] However, few studies have evaluated the impacts of exposure during early life.

The period from *in utero* to the first 2 years of life is a critical window for lung development and growth.[72-73] Increasingly, studies have suggested that exposure to air pollution during this period could increase the risk of developing wheezing and/or asthma in later life. For example, a systematic review has found a significant association between prenatal exposure to particulate matter with an aerodynamic diameter less than 10 μm (PM_{10}) and childhood asthma[29] with *in vivo* laboratory models suggesting that this relationship is causal.[74-75]

However, the identified associations in the literature between $PM_{2.5}$ exposure during this critical period and the long-term risk of wheezing and asthma are inconsistent. For example, an American study suggested that childhood asthma was significantly associated with prenatal $PM_{2.5}$ exposure as estimated by a land use regression (LUR) model (odds ratio (OR): 1.17; 95%CI (confidence interval): 1.04-1.30),[76] while a Canadian study using a similar methodological approach did not observe associations.[77] These inconsistencies might be explained by differences in $PM_{2.5}$ sources, exposure and outcome measurements, and analytic approaches in different studies, making further analysis necessary to better assess this relationship.

Previous systematic reviews have focused on the effects of either prenatal exposure alone [29] or many years of exposure to traffic-related air pollution.[16, 69] The aim of this systematic review was to identity and summarise the available epidemiological evidence for the association between prenatal or infant (less than 2 years of age) exposure to $PM_{2.5}$ and the subsequent development of wheezing and asthma.

2.3 Methods

We followed the Cochrane guidelines[78] and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist[79] [See PRISMA 2009 Checklist, Appendix 2, which provides details of the checklist].

2.3.1 Search strategy

We initially searched PubMed, Scopus, Web of Science core collection, ProQuest and Cochrane library on 11/05/2016 for scientific articles. We used a combination of free text words found in the title, abstract and key words (Table 2-1).

We included all respiratory outcomes in the search terms in order to reduce the loss of potentially relevant papers. There was no restriction on publication date. Articles that were not written in English were excluded. We updated the database search and searched the reference lists of all included studies by 4/12/2017.

2.3.2 Study screening

We screened titles and abstracts of all included papers for potential relevance. After that, full texts of all relevant studies were reviewed based on the following inclusion and exclusion criteria. We included all epidemiological studies which:

1. Were peer-reviewed journal articles, conference proceedings, theses and official reports using a cohort, case-control or cross-sectional design;
2. Evaluated the effects of exposure to $PM_{2.5}$ prenatally or during the first 2 years of life;
3. Assessed the impact of prenatal and infant $PM_{2.5}$ exposure on wheezing and asthma incidence or prevalence ≥1 year after the exposure period investigated.

Studies were excluded if they:

1. Were experimental studies, reviews, meeting abstracts, book sections, blogs, newspaper articles, editorials or non-research letters;
2. Only assessed maternal $PM_{2.5}$ exposure before conception or childhood exposure after 2 years of age;
3. Only assessed indoor air pollution, tobacco smoke, or other air pollution exposure metrics;
4. Only assessed other respiratory illnesses or symptoms.
5. Assessed acute effects of $PM_{2.5}$ exposure.

2.3.3 Data extraction

Data was extracted manually from all eligible studies for information on study design, location, population characteristics, exposure, outcomes, confounding factors and effect estimates with 95%CIs. We contacted the corresponding authors of studies with important data missing.

2.3.4 Critical appraisal

We examined the quality of all included studies using the Critical Appraisal Skills Programme (CASP) checklists[80-81] [See CASP checklist for cohort study and CASP checklist for case-control study, Appendix 2, which provides details of these checklists].

2.3.5 Analysis

We employed random-effects meta-analyses to calculate the weighted effect estimates and 95%CIs for every 10 μg/m^3 increase in $PM_{2.5}$ concentrations. Meta-analysis was conducted if ≥2 studies reporting ORs, RRs or HRs using continuous $PM_{2.5}$ concentrations as an independent variable. Studies reporting ORs, RRs or HRs were combined in a single meta-analysis as this is acceptable for common outcomes with a small effect size[82] and is a well-established approach.[16, 83] All meta-analyses were performed on Review Manager 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) using the generic inverse variance method. Heterogeneity was assessed using the I^2 statistic and *p* value from the Chi-squared test. Publication bias was visually evaluated using funnel plots. We conducted sensitivity analyses by employing fixed-effects models, excluding case-control studies, and excluding studies estimating exposure using techniques other than the most common approach of LUR. Since one study[84] used both LUR and inverse distance weighted (IDW) approaches to estimate $PM_{2.5}$ exposure, we included LUR in the primary meta-analysis and used IDW in the sensitivity analysis.

2.4 Results

2.4.1 Study screening

Our search strategy initially identified 8031 articles (Figure 2-1). After removing duplicates (n=3326) and conducting the first screening of titles and abstracts (n=4705), we reviewed 111 full texts articles which yielded 13 relevant studies. We added five more articles by further searching for new publications and reference lists of all the included articles. Eighteen studies were included in our final review consisting of 17 peer-reviewed journal articles and 1 Thesis [See Table S2-1, Appendix 2, which provides details].

2.4.2 Study setting

All 18 studies were published between October 2002 and January 2018. The majority of the studies were conducted in North American and European countries, including 5 in America,[76, 85-88] 3 in Canada,[77, 84, 89] 3 in Poland,[90-92] 2 in Germany,[49-50] 1 in The Netherlands[48] and 1 in the Czech Republic.[93] One study was conducted in Mexico.[94] The remaining two studies were pooled analyses of multi-centre cohorts conducted in Canada, Germany and The Netherlands.[95-96] Sample sizes ranged from 184 to 41,569 and follow-up periods ranged from 2 to 10 years. Most of the studies (n=16) focused on the general population (2 to 21 years of age), except one study of high-risk children (i.e. ≥1 first-degree asthmatic relative or ≥2 first-degree relatives with other IgE-mediated allergic disease)[89] and another on ethnic minorities.[87]

2.4.3 Study design

Most of the studies were pregnancy or birth cohort studies (n=15) including two pooled analyses of multiple birth cohorts from different locations.[95-96] The remaining three[77, 84, 87] were matched case-control studies in which two were nested within birth cohorts.[77, 84]

2.4.4 $PM_{2.5}$ sources and measurements

There were 11 studies evaluating outdoor $PM_{2.5}$ from traffic-related sources,[48-50, 76-77, 84, 86, 88-89, 95-96] woodsmoke,[84] industrial points[84] or other sources,[76, 86] while three investigated $PM_{2.5}$ from both outdoor and indoor sources.[90-92] The remaining four studies did not specify the source of ambient $PM_{2.5}$.[85, 87, 93-94]

Various methods were used for estimating prenatal and infant $PM_{2.5}$ exposure. The LUR model was mostly based on Geographic information systems (GIS) [48-50, 77, 84, 89, 95-96] or satellite data.[76, 85-86, 94] Studies estimating prenatal $PM_{2.5}$ exposure[76-77, 84-86, 94] have taken into account participants' residential histories, while studies estimating postnatal exposure[48-50, 89, 95-96] only used birth address. Other studies employed an IDW approach[84, 87] or a dispersion model[88] based on individual's residential histories, personal environmental monitoring samplers (PEMS)[90-92] and data from the central monitoring sites.[93]

2.4.5 Outcome definition

The majority of the included studies (n=13) relied on questionnaires or interviews to define doctor-diagnosed wheezing and asthma (Table 2-1). There were four studies defining asthma from medical records as different combinations of physician diagnoses, hospital admissions and asthma-related medication use.[77, 84, 88, 93] One study diagnosed asthma by a blinded paediatric allergist based on the presence of asthmatic symptoms.[89] We included parental reports of doctor diagnosed asthmatic/spastic/obstructive bronchitis as an indication of asthma in two German studies[49-50] due to the relatively low asthma frequency and the strict diagnostic criteria for pre-school asthma.[95]

2.4.6 Quality assessment

According to the CASP checklists, all the studies were highly [77, 84-86, 88, 93] or moderately qualified[48-50, 76, 87, 89-92, 94-96] [See Table S2-2, Table S2-3, Appendix 2, which provides details].

The major concerns for the validity of the studies were potential for information bias (n=13), selection bias (n=10), short follow-up duration (n=9) and not accounting for important confounding factors (n=8) [See Table S2-2, Table S2-3 and Notes for CASP quality assessment of all included studies, Appendix 2, which provides details].

2.4.7 Prenatal $PM_{2.5}$ exposure and asthma

Of the six studies assessing prenatal $PM_{2.5}$ exposure and asthma development, four were included in the meta-analysis,[76-77, 84, 93] while the other two either contained overlapping data[86] or investigated the RDs,[88] respectively. The overall risk of developing childhood asthma for a 10 $\mu g \cdot m^{-3}$ increase in prenatal $PM_{2.5}$ exposure was 1.12 (95%CI: 1.00-1.26), with no significance (p>0.05) (Figure 2-2). We found high heterogeneity among those studies (I^2=73%; p<0.05). Sensitivity analyses all found similar but non-significant associations between prenatal $PM_{2.5}$ exposure and asthma development (Table 2-2; see Figure S2-1, Appendix 2, which provides details).

The meta-analyses did not include a recent study using RDs to estimate the effect of prenatal $PM_{2.5}$ exposure on asthma development of nearly 20,000 American children.[88] In this study, the authors found significant positive associations between log-transformed prenatal $PM_{2.5}$ exposure (per 2.7-fold increase) and cumulative asthma incidences from age 2 to age 6 with RDs ranging from 0.015 to 0.035 after adjustment for confounders. Sensitivity analysis of modelling exposure by quintiles also revealed significant associations between prenatal $PM_{2.5}$ exposure and asthma incidence and persistence by age 5. However, modelling $PM_{2.5}$ linearly resulted in positive associations but with no statistical significance [See Table S2-4, Appendix 2, which provides details].

2.4.8 Infant $PM_{2.5}$ exposure and asthma

There were nine studies evaluating the associations between infant $PM_{2.5}$ exposure and asthma. These included one for birth year exposure,[89] four for exposure during the first of life[84, 87-88, 96] and four for exposure during first 2 years of life.[48-50, 95] After excluding four studies either with repeated data[48-49, 89] or estimating the effect by RDs,[88] five remained in the meta-analyses.[50, 84, 87, 95-96] Our meta-analyses showed a trend towards a positive association that was not statistically significant (overall OR: 1.14; 95%CI: 0.96-1.35) with low heterogeneity (I^2=0%; p>0.05) (Figure 2-3). The results were robust to multiple sensitivity analyses (Table 2-2; see Figure S2-2, Figure S2-3, Appendix 2, which provides details).

One study also analysed the outcomes as current asthma or ever asthma plus current wheeze in their regression models,[96] which was not included in the meta-analyses. According to the results of those analyses, infant $PM_{2.5}$ exposure was found to be significantly associated with an increased risk of current asthma of 35% (95%CI: 7%-70%) at age 6 to 8, while ever asthma plus current wheeze did not show statistically significant associations [See Table S2-4, Appendix 2, which provides details].

In the study assessing RDs,[88] significant associations were observed for $PM_{2.5}$ exposure during the first year of life and incident or persistent asthma when modelling exposure as a log-transformed continuous variable and by quintiles. Similar with the results of prenatal $PM_{2.5}$ exposure, modelling the $PM_{2.5}$ as a continuous variable without log-transformation revealed non-significant associations. However, goodness-of-fit analyses suggested that the log-transformed modelling was better than the linear continuous modelling. Other sensitivity analyses all suggested significant associations [See Table S2-4, Appendix 2, which provides details].

2.4.9 Prenatal $PM_{2.5}$ exposure and wheezing

Meta-analysis was not applicable for the five studies of prenatal $PM_{2.5}$ exposure and wheezing since most of the studies categorised $PM_{2.5}$ exposure by median and had different outcome definitions.[85, 90-92]

There was only one study that modelled $PM_{2.5}$ as a continuous variable using regression analyses.[94] The authors evaluated the effect of $PM_{2.5}$ exposure during different trimesters of pregnancy on ever or current wheeze (wheeze in the past year) in 552 4-year-old children. No significant association was observed in any trimester $PM_{2.5}$ exposure and wheezing outcomes.

Another study suggested that higher prenatal $PM_{2.5}$ exposure (>11.22 $\mu g \cdot m^{-3}$) was significantly associated with a 102% increase (95%CI: 20%-240%) in the risk of repeated wheezing in children from birth to 2 years old compared with the lower exposure group (≤11.22 $\mu g \cdot m^{-3}$), with consistent results from multiple sensitivity analyses.[85]

The other three studies were from the same project – the Krakow study[90-92] which used PEMS to measure $PM_{2.5}$ exposure during the 2nd trimester of pregnancy. All studies suggested significant associations between prenatal $PM_{2.5}$ exposure and wheezing duration in the first 2 years of life; however, while the association for ages 3 to 4 was also positive, it was not statistically significant [See Table S2-4, Appendix 2, which provides details].

2.4.10 Infant $PM_{2.5}$ exposure and wheezing

Meta-analyses included three of the four studies investigating the association between infant $PM_{2.5}$ and wheezing,[48, 50, 96] while the other one containing repeated data was excluded.[49] Infant $PM_{2.5}$ exposure was not associated with wheezing development in either random- or fixed-effects models (overall OR: 1.49; 95%CI: 0.99-2.26) (Figure 2-4; see Figure S2-4, Appendix 2, which provides details). Low heterogeneity was found in the three studies as indicated by an I^2=0% and a *p* value>0.05. $PM_{2.5}$ was also not significantly associated with current wheeze at age 6 to 8[96] [See Table S2-4, Appendix 2, which provides details].

2.4.11 Publication bias

Small studies with negative findings have not been published on the associations between prenatal or infant $PM_{2.5}$ exposure and asthma. The distribution was symmetrical in the funnel plot of infant exposure and wheezing, despite the small number of studies included in the meta-analysis [See Figure S2-5, S2-6 and Figure S2-7, Appendix 2, which provides details].

2.4.12 Outcomes by specific characteristics

There were nine studies including stratified analyses by gender,[49, 76-77, 84, 86-88] heredity,[87-88] maternal stress during pregnancy,[76, 94] race,[88] atopic status[87] and other characteristics including birthweight, gestational length, maternal age, parity, neighbourhood SES[77] and genotype[96] [See Table S2-4, Appendix 2, which provides details].

The differences of effects by gender were inconsistent among the seven studies. To illustrate, two studies suggested larger magnitudes of effects in males compared with females,[76, 86] while the other five suggested stronger effects in females.[49, 77, 84, 87-88] Of those studies, Hsu and colleagues[86] reported significant associations in males exposed to $PM_{2.5}$ during the 12-26th gestational weeks with asthma development, while Pennington and colleagues[88] reported significant associations between infant $PM_{2.5}$ exposure and asthma development in females. Other studies did not show significant results among different genders.

Higher risk was shown for children with a family history of asthma than those without in one study,[87] while the other one[88] only found significantly increased risks of asthma in children of mothers without asthma, but not in children of mothers with asthma.

Stratified analyses by maternal stress during pregnancy revealed a consistently significant and increased risk in children whose mothers were highly stressed during pregnancy compared with those slightly stressed.[76, 94]

Only one study[88] tested for potential effect modification by race or ethnicity and found no statistical differences between groups described as ‘white’ or ‘black’.

Studies that evaluated atopic status[87] and other characteristics including birthweight, maternal age, parity, gestational length and SES[77] did not find any significant associations with asthma. However, evidence of effect modification was seen with birthweight. Children with a birthweight <2500 g were at a higher risk of developing asthma associated with prenatal $PM_{2.5}$ exposure. Children with the GSTP1, rs1138272 or rs1695 minor alleles were more susceptible to developing asthma associated with infant $PM_{2.5}$ exposure.[96]

2.5 Discussion

Our meta-analyses demonstrated positive associations between prenatal $PM_{2.5}$ exposure and asthma and infant $PM_{2.5}$ exposure, and both wheezing and asthma; however, there were a limited number of relevant studies, and the results were inconsistent. There was high heterogeneity among the studies for prenatal $PM_{2.5}$ exposure and asthma. This might be due to the variability in children's ages, exposure measurement methods, sources of particulate matter, outcome definitions, and adjustment of confounding factors. Studies investigating prenatal $PM_{2.5}$ exposure and subsequent wheezing were not amenable to meta-analysis but consistently reported significant associations, especially in infants (≤2 years).

This is the largest review assessing the effects of prenatal and infant $PM_{2.5}$ exposure on subsequent wheezing or asthma. We added three more studies[76, 93-94] to a previous systematic review and meta-analysis of the effects of prenatal exposure to all types of air pollutants including $PM_{2.5}$ on the development of wheezing and asthma.[29] Our results of meta-analyses of the association between prenatal $PM_{2.5}$ exposure and asthma were similar to this previous review, observing no significant associations and high heterogeneity. In contrast, the other new study not included in meta-analysis reported significantly increased risk of asthma by age 2 to 6 after prenatal exposure to $PM_{2.5}$.[88] However, the evidence was mixed, with more significant associations seen in children followed to school age[76, 88, 93] than preschool age.[84, 88] This

phenomenon might be explained by the difficulties in the diagnosis of asthma among young children,[97] leading to the underestimation of physician-diagnosed asthma in this population. The significant associations between prenatal $PM_{2.5}$ exposure and wheezing in infants[90-92] rather than in older children[92, 94] could indirectly support this explanation. However, some researchers argue that it is difficult to predict asthma based solely on early life wheezing as less than half of children with episodes of preschool wheezing will have continuing childhood asthma.[98]

For infant $PM_{2.5}$ exposure and the subsequent development of wheezing or asthma, our meta-analyses did not demonstrate an association. However, these studies were of higher risk of bias due to potential for selection bias,[48, 50, 87, 95-96] recall bias,[48, 50, 87, 95-96] not adjusted for important confounding factors,[84, 96] and a case-control design.[84, 87] In contrast, a recent large, high quality cohort study of nearly 20,000 children revealed positive associations between $PM_{2.5}$ exposure during the first year of life and asthma incidence by age 6, despite not adjusting for important confounders.[88] This result was robust to different asthma definitions but sensitive to $PM_{2.5}$ modelling decisions and covariate controls. Overall, the small number of studies identified in this systematic review limited our confidence in conclusively suggesting the presence or absence of associations. Studies with a larger sample size, a standardised exposure estimate method, more accurate outcome assessment approaches and greater statistical power are needed to further explore the effects of prenatal and infant $PM_{2.5}$ exposure on asthma or wheeze development.

Our review also highlights the limited evidence of susceptible populations to prenatal and infant $PM_{2.5}$ exposure. Children whose mothers were exposed to negative life events during pregnancy were more likely to develop wheezing or asthma after prenatal and infant $PM_{2.5}$ exposure than those not exposed. The different effects of $PM_{2.5}$ exposure by gender and heredity were inconsistent between studies. There was insufficient evidence to suggest that race, low birth

weight and specific genotypes could increase the risk of wheezing or asthma development after $PM_{2.5}$ exposure, while the effects of atopic status, gestational length, maternal age, parity and SES require further investigation.

The main strength of our systematic review was the comprehensive search strategy and reproducible evaluation of current evidence. Our findings provide a timely contribution to the rapidly developing field, which could highlight limitations and guide future studies. However, some limitations should also be acknowledged. Firstly, evidence of prenatal and infant $PM_{2.5}$ exposure and wheezing or asthma is still limited. In addition, publication bias might be present in studies evaluating early life $PM_{2.5}$ exposure and asthma. Therefore, any conclusions should be made with caution and confirmed by further investigations. Secondly, high variability was found between studies in study design, exposure estimating methods, outcome assessment approaches, participants' ages at assessment and adjustment of confounders, especially in those evaluating prenatal $PM_{2.5}$ exposure and asthma. Future syntheses of evidence in this area will benefit from more studies using standardised designs and methods. In addition, diagnosis of asthma in young children is difficult and outcome misclassification is inevitable in this population. Finally, the major source of $PM_{2.5}$ in this systematic review was traffic, with scarce evidence regarding the respiratory effects of early life $PM_{2.5}$ exposure from other sources such as wildfire smoke, which is an increasing global concern due to climate change.[99-100] More research on $PM_{2.5}$ from other sources is needed to guide public health responses.

2.6 Conclusions

Prenatal and infant $PM_{2.5}$ exposure was not clearly associated with subsequent development of wheezing or asthma in our review of the literature. The strongest evidence was for an association between prenatal $PM_{2.5}$ exposure and wheezing in infants, while *in-utero* exposure and asthma had a borderline positive overall effect estimate. However, evidence was

insufficient and mixed, indicated by a small number of studies included in the meta-analyses and inconsistent results. Further research is necessary to explore the associations using harmonised exposure methods and appropriate statistical analyses controlling for important covariates. Furthermore, studies of susceptible populations and other sources of $PM_{2.5}$ are needed to help policy makers improving public health.

Table 2-1. Items for database search

Population	Connecting word	Exposure	Connecting word	Outcome
perinatal	AND	"air pollution"	AND	respirat*
post-natal		"air pollutant*"		lung
prenatal		particle*		pulmon*
pre-natal		"particulate matter*"		bronchi*
maternal				"air way"
pregnan*				airway
gestation				asthma
conception				cough
fetus*				wheeze
foetus*				wheezing
fetal				
newborn*				
"new born*"				
infant*				

Notes: Asterisk represents any suffix thereafter; double quotation marks represent that the two words should not be broken apart.

Table 2-2. Prenatal and infant $PM_{2.5}$ exposure (per 10 μg/m^3 increase) on wheezing/asthma from the sensitivity meta-analyses

	Sensitivity analysis 1: fixed-effects OR (95%CI); I^2 (p^* value)	**Sensitivity analysis 2:** excluding case-control studies random-effects OR (95%CI); I^2 (p^* value)	**Sensitivity analysis 3:** excluding studies with other exposure estimates approaches except LUR random-effects OR (95%CI); I^2 (p^* value)
Prenatal $PM_{2.5}$ & asthma	**N.A.**	1.17 (0.99 to 1.37); 78% (0.00)	1.16 (0.91 to 1.48); 77% (0.00)
Infant $PM_{2.5}$ & asthma	1.14 (0.96 to 1.35); 0% (0.48)	1.27 (0.82 to 1.98); 22% (0.28)	1.16 (0.92 to 1.44); 9% (0.36)
Infant $PM_{2.5}$ & wheezing	1.49 (0.99 to 2.26); 0% (0.77)	N.A.	N.A.

Abbreviations: OR, odds ratio; 95%CI, 95% confidence interval; LUR, land use regression; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm; N.A., not applicable. Significant associations are shown in bold. *, *p*-value refers to the test of heterogeneity.

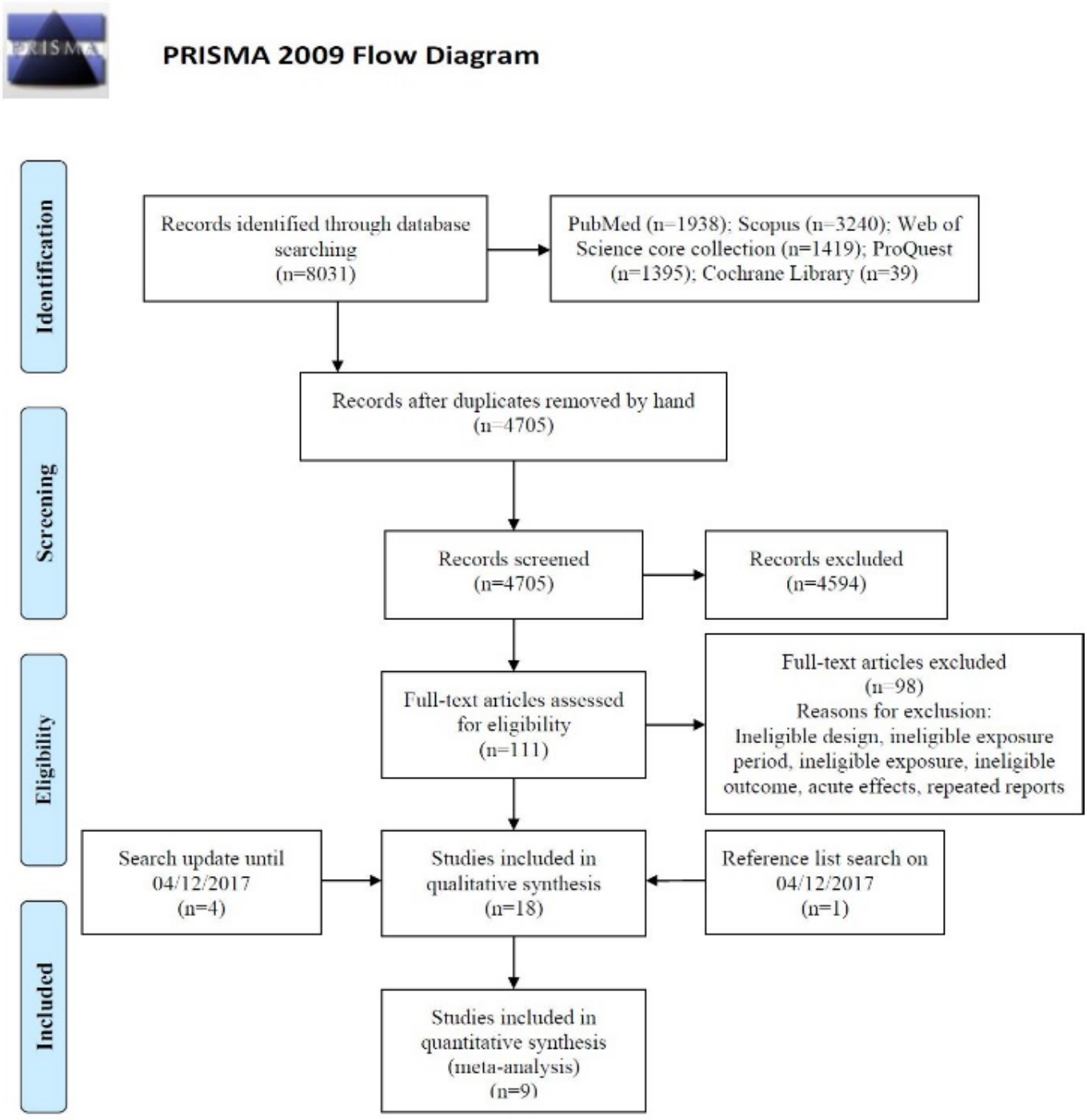

Figure 2-1. PRISMA flow diagram describing the database search and study screening process

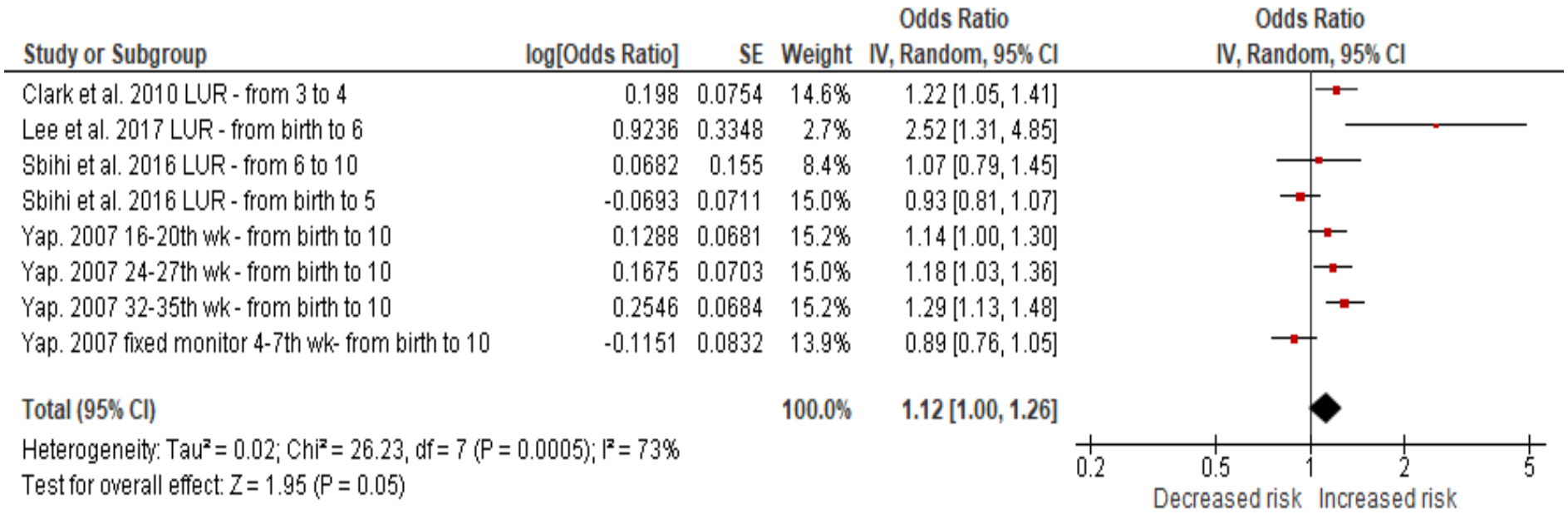

Figure 2-2. Random-effects meta-analysis of the association between prenatal $PM_{2.5}$ exposure (per 10 µg/m^3) and asthma

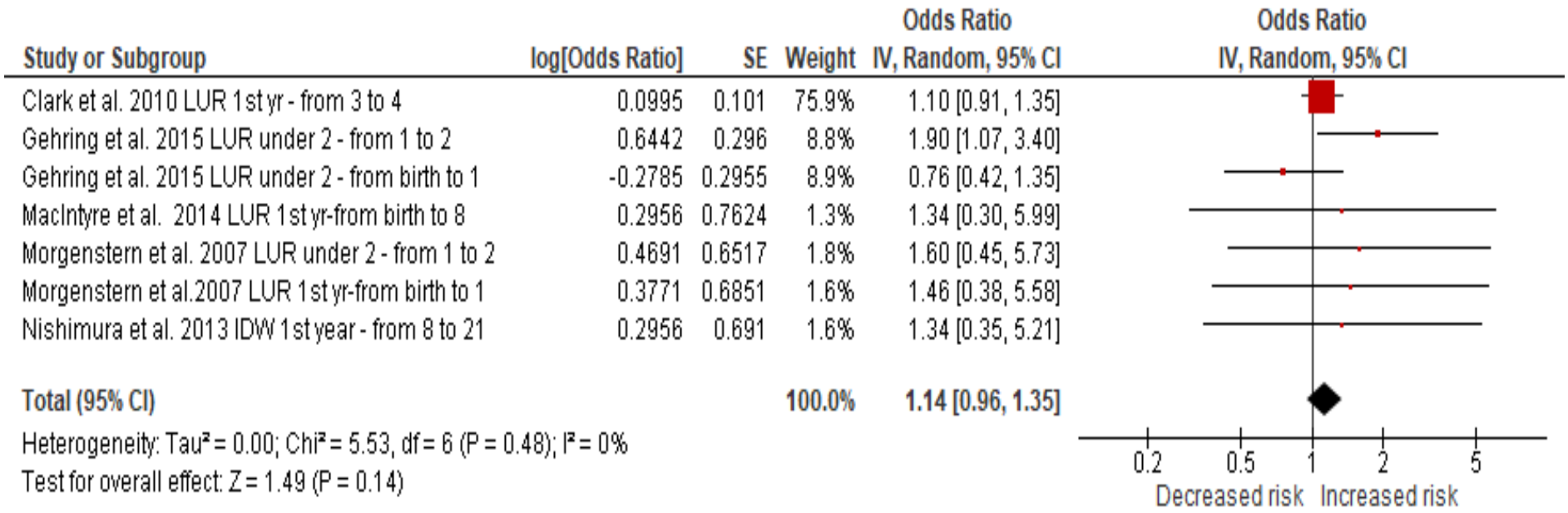

Figure 2-3. Random-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 µg/m^3) and asthma

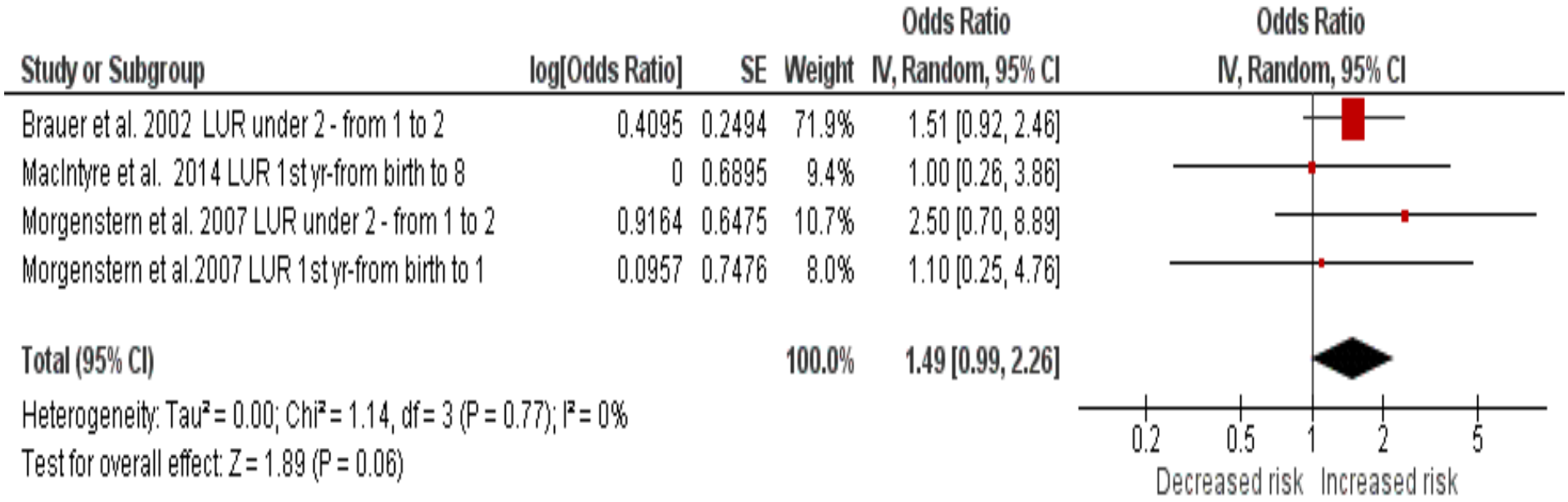

Figure 2-4. Random-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and wheezing

2.7 Appendix 2

Appendix 3 includes the PRISMA 2009 checklist, information on CASP quality assessment, and figures/tables listed below.

Figure S2-1. Sensitivity analysis of the association between prenatal $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and asthma

Figure S2-2. Fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and asthma

Figure S2-3. Sensitivity analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and asthma

Figure S2-4. Fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and wheezing

Figure S2-5. Funnel plot – fixed-effects meta-analysis of the association between prenatal $PM_{2.5}$ exposure and asthma

Figure S2-6. Funnel plot – fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure and asthma

Figure S2-7. Funnel plot-fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure and wheezing

Table S2-1. Summary of studies included in the systematic review

Table S2-2. Risk of bias assessment for cohort studies according to the CASP checklist

Table S2-3. Risk of bias assessment for case-control studies according to the CASP checklist

Table S2-4. Original risk estimates of the 18 studies investigating prenatal and infant $PM_{2.5}$ exposure and wheezing/asthma development

Section/topic	#	Checklist item
TITLE		
Title	1	Identify the report as a systematic review, meta-analysis, or both.
ABSTRACT		
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.
INTRODUCTION		
Rationale	3	Describe the rationale for the review in the context of what is already known.
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).
METHODS		
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.

Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.
Section/topic	**#**	**Checklist item**
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).

Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.
RESULTS		
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).
15-DISCUSSION		
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g.,

		healthcare providers, users, and policy makers).
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.
FUNDING		
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.

CASP checklist for cohort study

Section/Topic	#	Checklist item
Section A. Are the results of the study valid?		
Screening Questions		
	1	Did the study address a clearly focused issue?
Selection bias	2	Was the cohort recruited in an acceptable way?
Detailed Questions		
Measurement of classification bias	3	Was the exposure accurately measured to minimise bias?
	4	Was the outcome accurately measured to minimise bias?
Confounding factors	5a	Have the authors identified all important confounding factors?
	5b	Have they taken account of the confounding factors in the design and/or analysis?
Completion and length of follow-up	6a	Was the follow up of the subjects complete enough?
	6b	Was the follow up of subjects long enough?
Section B. What are the results?		
	7	What are the results of this study?
	8	How precise are the results?
	9	Do you believe the results?
Section C. Will the results help locally?		
	10	Can the results be applied to the local population?
	11	Do the results of this study fit with other available evidence?
	12	What are the implications of this study for practice?

CASP checklist for case-control study

Section/Topic	#	Checklist item
Section A. Are the results of the study valid?		
Screening Questions		
	1	Did the study address a clearly focused issue?
	2	Did the authors use an appropriate method to answer their question?
Detailed Questions		
Selection bias	3	Were the cases recruited in an acceptable way?
	4	Were the controls selected in an acceptable way?
Measurement, recall or classification bias	5	Was the exposure accurately measured to minimise bias?
Confounding factors	6a	What confounding factors have the authors accounted for?
	6b	Have the authors taken account of the potential confounding factors in the design and/or in their analysis?
Section B. What are the results?		
	7	What are the results of this study?
	8	How precise are the results? How precise is the estimate of risk?
	9	Do you believe the results?
Section C. Will the results help locally?		
	10	Can the results be applied to the local population?
	11	Do the results of this study fit with other available evidence?

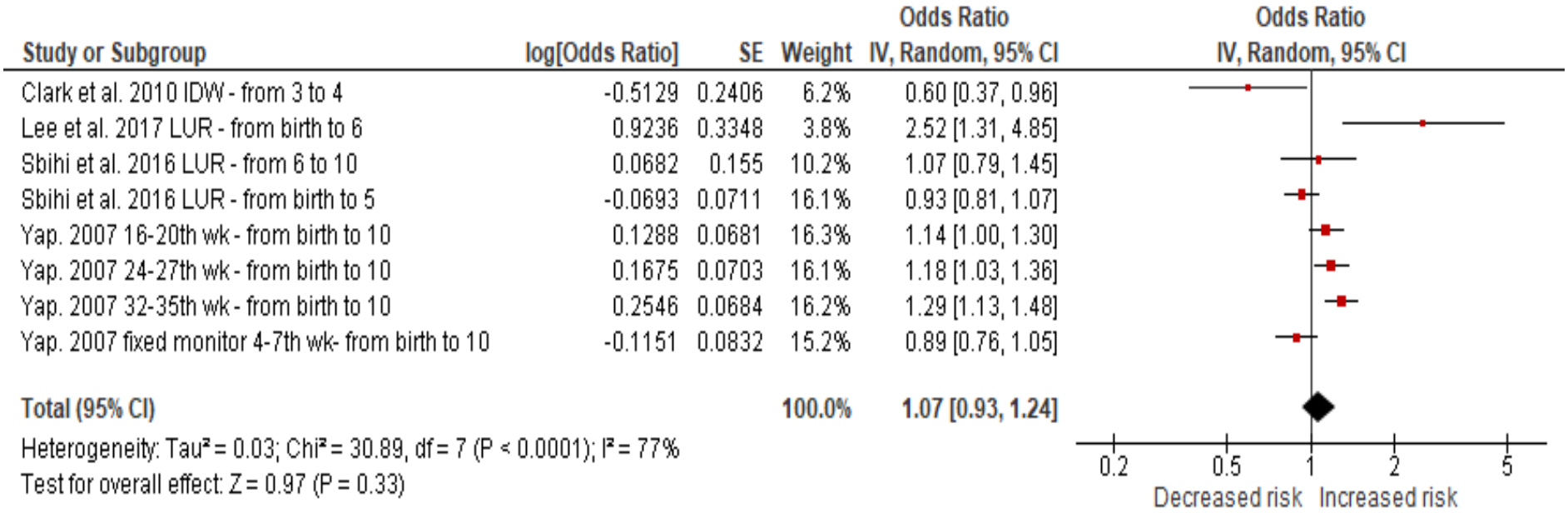

Figure S2-1. Sensitivity analysis of the association between prenatal $PM_{2.5}$ exposure (per 10 μg/m^3) and asthma

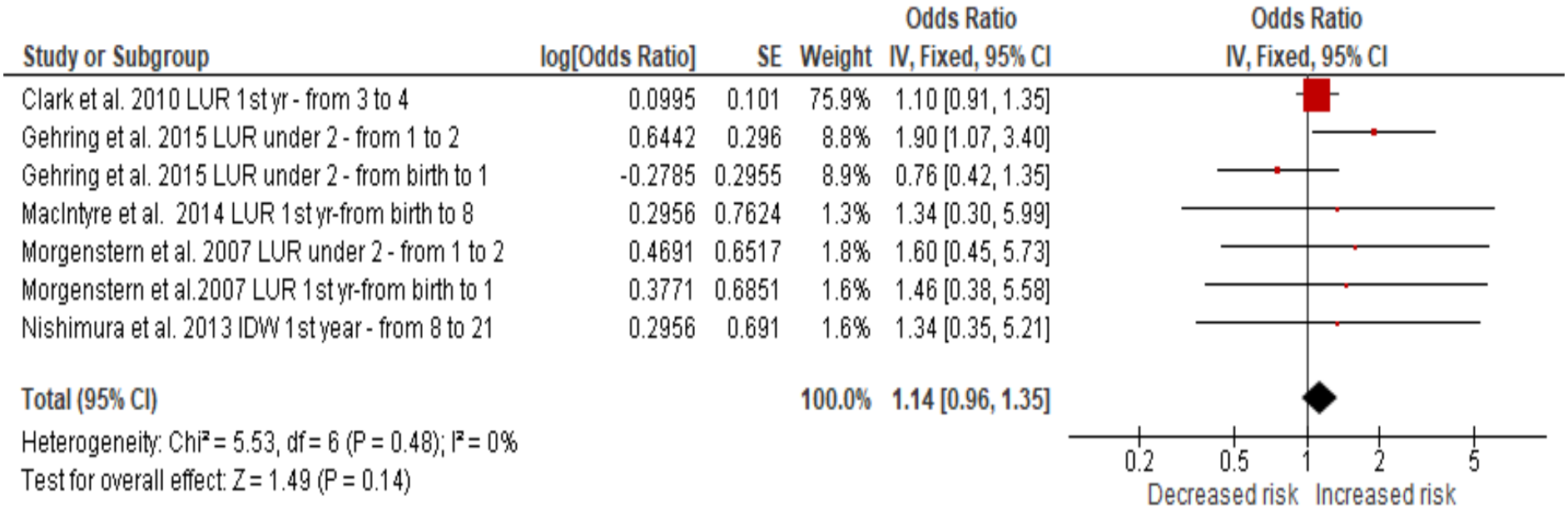

Figure S2-2. Fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and asthma

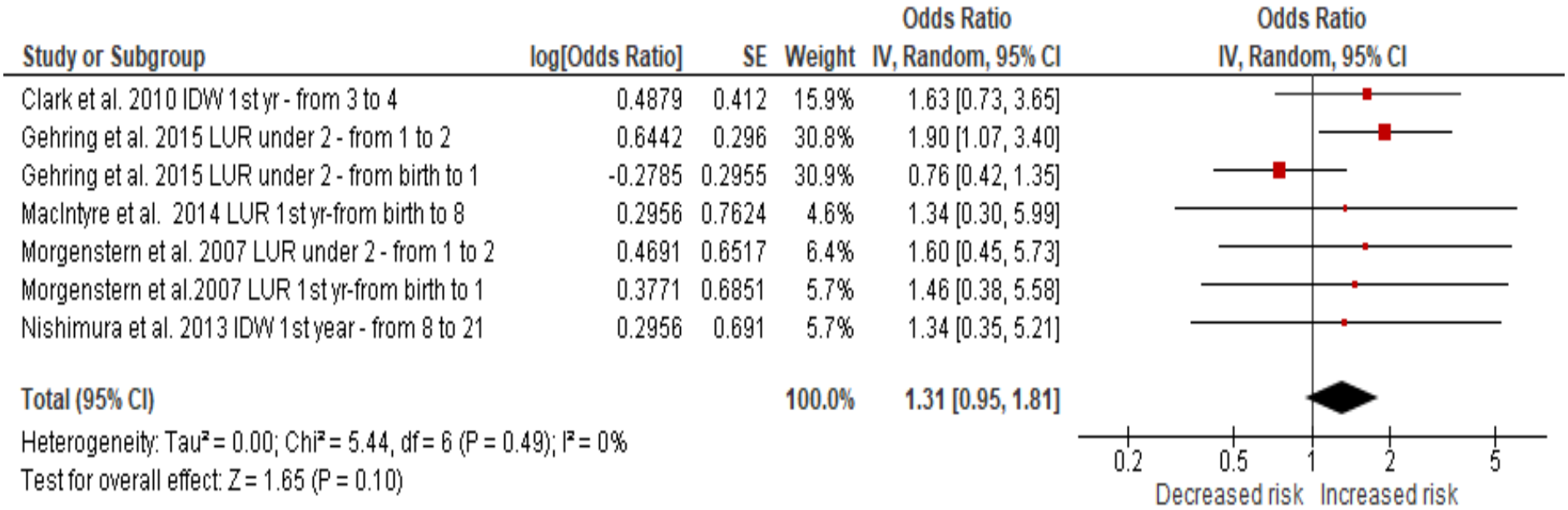

Figure S2-3. Sensitivity analysis of the association between infant $PM_{2.5}$ exposure (per 10 μg/m^3) and asthma

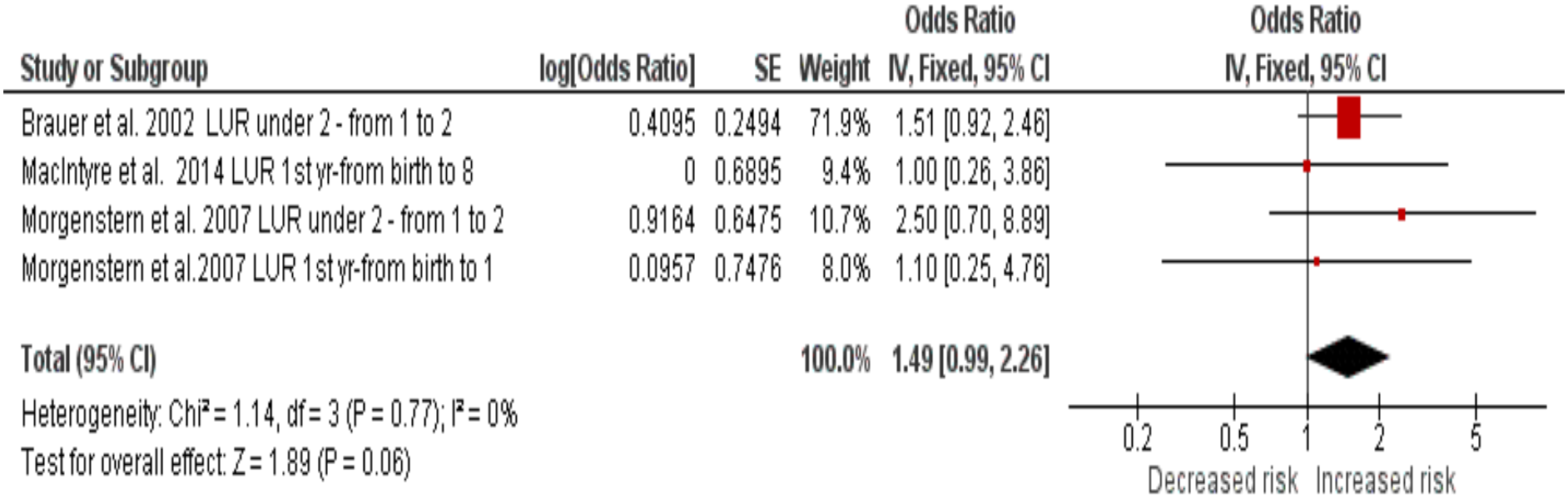

Figure S2-4. Fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure (per 10 $\mu g/m^3$) and wheezing

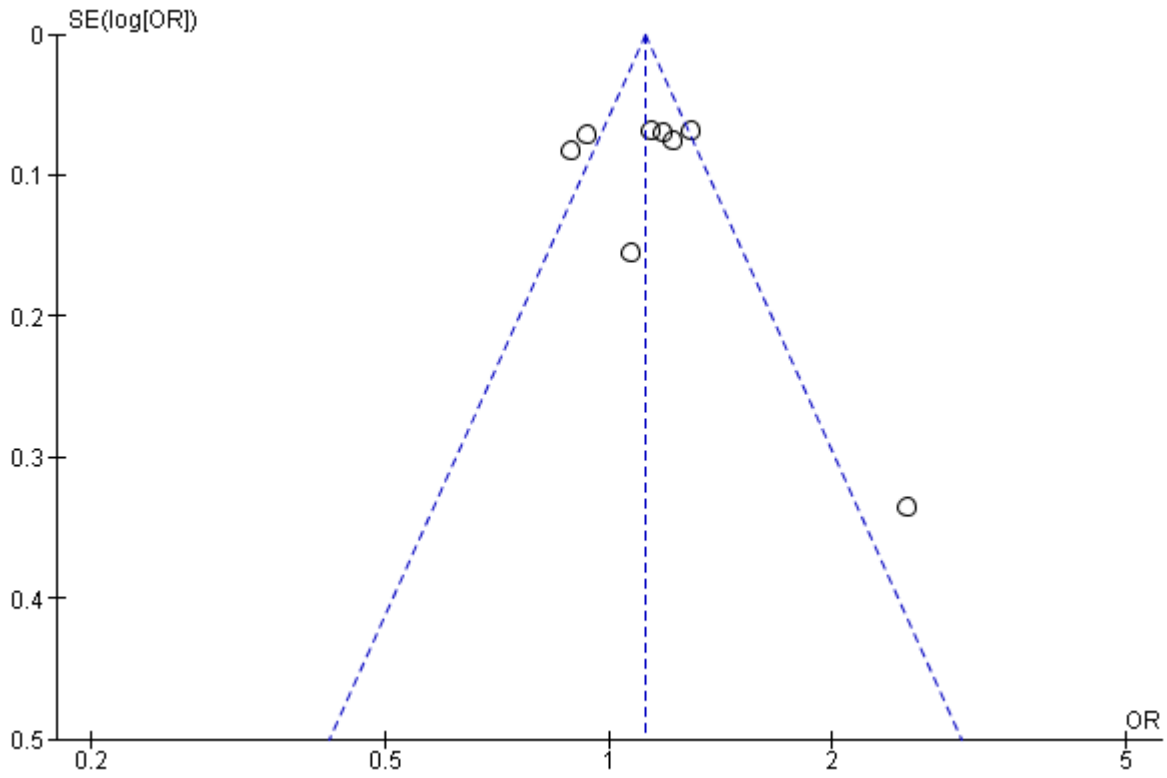

Figure S2-5. Funnel plot – fixed-effects meta-analysis of the association between prenatal $PM_{2.5}$ exposure and asthma

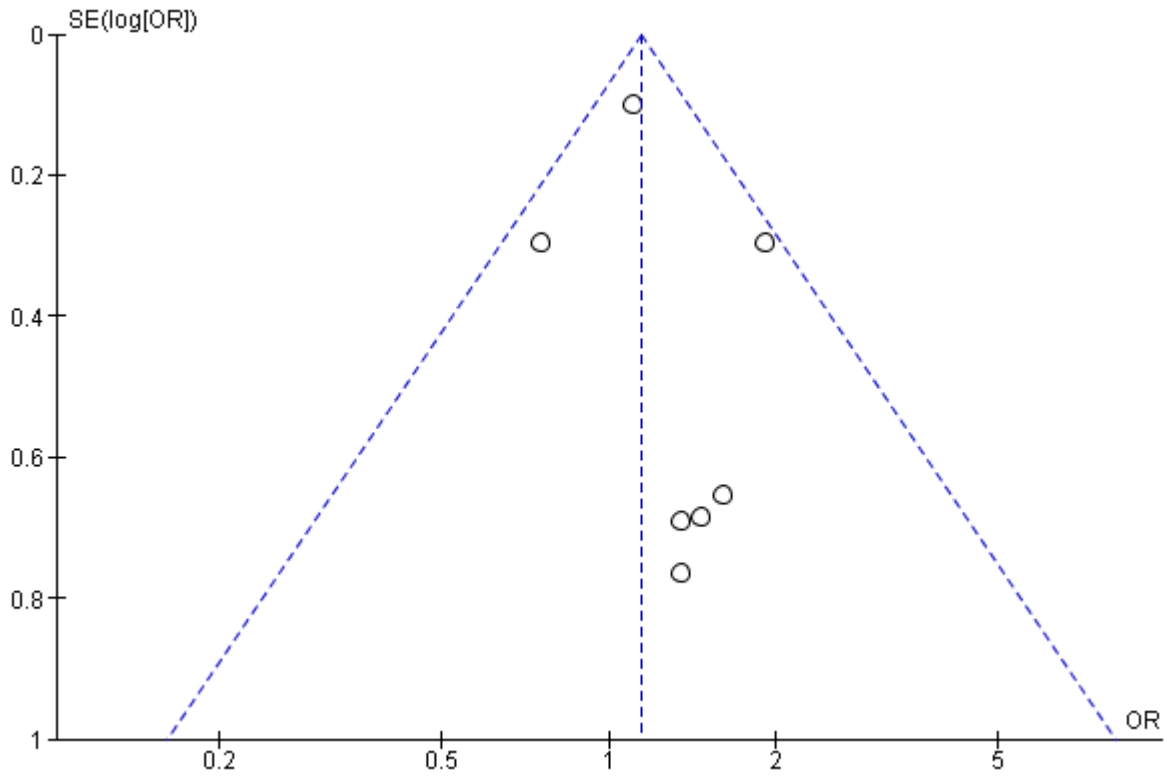

Figure S2-6. Funnel plot – fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure and asthma

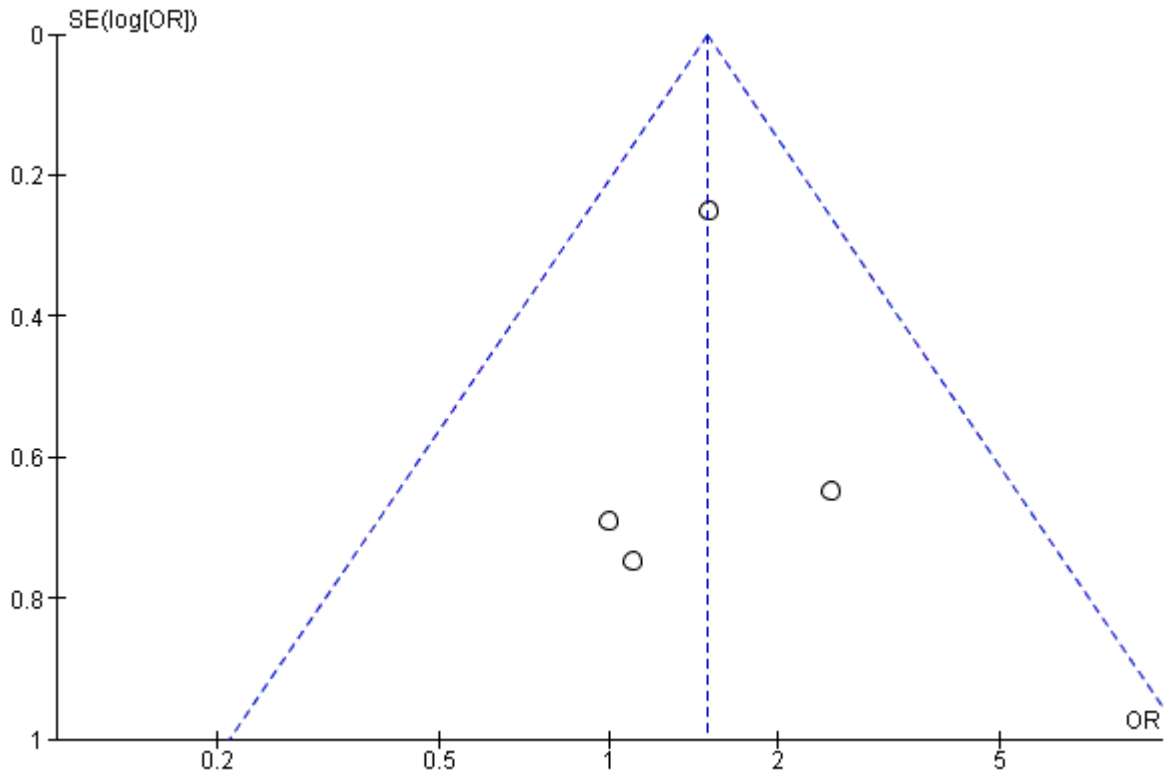

Figure S2-7. Funnel plot – fixed-effects meta-analysis of the association between infant $PM_{2.5}$ exposure and wheezing

Table S2-1. Summary of studies included in the systematic review

Study reference	**Study design**	**Study location**	**Sample size**	**$PM_{2.5}$ source; exposure estimate**	**Exposure period**	**$PM_{2.5}$ levels ($\mu g \cdot m^{-3}$)**	**Outcome definition**	**No. (%) of cases**	**Ages for outcome (years)**	**Confounding factors**
Lee[76] (Study 12)	Cohort (ACCESS)	Boston, USA	736	Traffic and other source; satellite-based LUR model according to residential history	Prenatal	Median [IQR]: 11.2 [10.2-11.9]	Maternal report of doctor-diagnosed asthma	110 (14.9%)	0-6	Gender, race/ethnicity, maternal age, prepregnancy obesity, maternal education, maternal prenatal and postnatal smoking
Sbihi[77] (Study 13)	Nested case-control	Vancouver, Canada	6,948 preschool cases and 34,621 controls or 1,711	Traffic; GIS-based LUR model according to residential history	Prenatal	Mean±SD: Preschool age: 4.09±1.6 for cases, 4.06±1.7 for	Asthma: ≥2 primary care physician diagnoses/≥1 hospital admission in a rolling 12 months according to ICD-9 code 493 and ICD-10 J45 from	Preshool age: 6948 (16.7%); school age: 1711 (16.6%)	0-5; 6-10	Gender, birth month and year, birthweight, gestational age, parity, breastfeeding, maternal education, area level income

			school cases and 8,577 controls from 68,195 births			controls; school age: 4.1±1.6 for cases, 4.0±0.7 for controls	medical records			
Clark[84] (Study 20)	Nested case-control	Southwestern British Columbia, Canada	LUR: 3,254 cases and 16,270 controls; IDW: 3,355 cases and 16,775 controls from 37,401 births	Traffic, woodsmoke and industry; GIS-based LUR model for traffic-related and woodsmoke sources & IDW approach for industrial source	Prenatal; first year of life	Mean±SD: Prenatal: 4.8±2.5 for cases, 4.7±2.5 for controls (LUR), 4.7±1.2 for cases and controls (IDW); first year of life:	Asthma: ≥2 primary care physician diagnoses in a rolling 12 months/≥1 hospital admission for asthma according to ICD-9 code 493 from medical records	LUR: 3254 (16.7%); IDW: 3355 (16.7%)	3-4	Age, gender, birthweight, gestational age, parity, breastfeeding, maternal education and neighbourhood level income in the final model; native status, maternal age and maternal smoking were also considered

				according to residential history		4.6±2.4 for cases, 4.5±2.5 for controls (LUR), 5.6±0.6 for cases and controls (IDW)				
Chiu[85] (Study 21)	Cohort (ACCESS)	Boston, USA	708	Outdoor source (not specified); satellite-based LUR model according to residential history	Prenatal	Median [IQR]: 11.2 [10.3-11.9]	Maternal reported repeated wheeze: ≥2 episodes	87 (12.3%)	0-2	Gender, race/ethnicity, season of birth, maternal education, maternal atopy, cockroach exposure, prenatal community violence
Hsu[86] (Study 22)	Cohort (ACCESS)	Boston, USA	736	Traffic and other source;	Prenatal	Median [IQR]: 11.2	Maternal report of doctor-diagnosed asthma	110 (14.9%)	0-6	Gender, race/ethnicity, maternal age, prepregnancy

				satellite-based LUR model according to residential history		[10.2-11.8]				obesity, maternal education, maternal prenatal and postnatal smoking, prenatal stress
Nishimura[87] (Study 23)	Case-control (GALA II and SAGE II)	Chicago, Bronx, Houston, San Francisco Bay Area, and Puerto Rico, USA	514 cases and 434 controls	Outdoor source (not specified); IDW approach	First year of life	Mean±SD: 11.8±3.6	Parental report of asthma: doctor-diagnosed asthma plus ≥2 symptoms of coughing, wheezing or shortness of breath in the 2 years before recruitment	514 (54.2%)	8-21	Age, ethnicity, region, SES and income in final model; maternal smoking during pregnancy, ETS exposure during the first 2 years of life, maternal language of preference were also considered
Pennington[88] (Study 24)	Cohort (KAPPA)	Atlanta, USA	19,951 for prenatal exposure; 23,100 for first year of life	Traffic; research Line-source dispersion model for near-surface	Prenatal; first year of life	Median: prenatal: 1.5; first year of life: 1.4	Asthma: ≥1 asthma diagnosis according to ICD-9 493.XX and 1 asthma-related medication dispensing (steroid/non-steroid	Prenatal: 1854 (32%); first year of life: 2149	1-6	Gender, race, city region, birth year, maternal asthma, neighbourhood SES in final model; ethnicity, maternal age, marital status, and parental education were also considered

			exposure	releases according to residential history			asthma controllers and relievers) from medical records	(32%)		
Carlsten[89] (Study 25)	Cohort (CAPPS)	Vancouver, Canada	184 high-risk children*	Traffic; GIS-based LUR model according to birth address	During the year of birth	Mean±SD: 5.6±2.6	Asthma diagnosed by a blinded paediatric allergist: ≥2 distinct cough (each ≥2 weeks), ≥2 distinct wheeze (each ≥1 week), and ≥1 of the following: nocturnal cough (≥once a week) without a cold, hyperpnoea-induced cough/wheeze, or response to β-agonist and/or anti-inflammatory drugs	23 (12.5%)	7	Gender, ethnicity, intervention status, maternal education, family history of asthma, atopy at 1 year
Jedrychowski[9]	Cohort	Krakow,	465	Indoor and	Prenatal:	Mean	Maternal reported	125	0-2	Gender, gestational age, parity,

[0] (Study 26)	(Krakow study)	Poland		outdoor sources (not specified); PEMS	*2nd trimester*	[range]: 36.1 [10.3-294.9]	duration of wheezing/whistling of the chest irrespective of respiratory infection	(26.9%)		fish consumption during pregnancy, maternal atopy, mold at home and postnatal ETS exposure in the final model; breastfeeding and maternal education were also considered
Jedrychowski[9] [1] (Study 27)	Cohort (Krakow study)	Krakow, Poland	465	Indoor and outdoor sources (not specified); PEMS	Prenatal*: 2nd trimester*	Mean [range]: 36.1 [10.3-294.9]	Maternal reported duration of wheezing/whistling of the chest irrespective of respiratory infection	125 (26.9%)	0-2	Gender, parity, maternal education, maternal atopy, postnatal ETS exposure, mold at home
Jedrychowski[9] [2] (Study 28)	Cohort (Krakow study)	Krakow, Poland	339	Indoor and outdoor sources (not specified); PEMS	Prenatal*: 2nd trimester*	Median [range]: 35.4 [10.3-294.9]	Maternal reported duration of wheezing/whistling in the chest irrespective of respiratory infection	139 (41.0%)	0-4	Gender, parity, maternal age, maternal education, maternal atopy, cord blood polycyclic aromatic hydrocarbon - adducts, dampness/mold at home, presence of wheeze during first 2 years (only in the

										3-4 years model) in the final model; prenatal ETS exposure was also considered
Gehring[49] (Study 29)	2 cohorts: GINI and LISA	Munich, Germany	1,517 for wheezing; 1,510 for asthma	Traffic; GIS-based LUR model according to birth address	First 2 years of life	Mean [range]: 13.4 [11.9-21.9]	Parental report of wheezing and doctor-diagnosed asthmatic/spastic/obstructive bronchitis	Wheezing: age 1: 258 (15.0%); age 2: 416 (25.6%); asthma: age 1: 196 (11.3%). Age 2: 303 (8.8%)	0-2	Gender, study arm, parity, maternal education, parental atopy, smoking at home, gas cooking, dampness/mold/pets at home
Morgenstern[50] (Study 30)	2 cohorts: GINI and	Munich metropolitan	2,882 for wheezing;	Traffic; GIS-based	First 2 years of	Mean [range]: 12.8	Parental report of wheezing and doctor-	Wheezing: age 1:	0-2	Gender, parity, maternal education, parental atopy, ETS

	LISA	area, Germany	2,861 for asthma	LUR model according to birth address	life	[6.8-15.3]	diagnosed asthmatic/spastic/obstructive bronchitis	471 (15.5%); age 2: 746 (25.9%); asthma: age 1: 356 (11.6%). Age 2: 555 (19.4%)		at home, gas cooking, dampness/mold/pets at home
Brauer[48] (Study 31)	Cohort (PIAMA)	Northern, western and central parts of The Netherlands	2,989 for asthma; 2,991 for wheezing	Traffic; GIS-based LUR model according to birth address	First 2 years of life	Mean [range]: 16.9 [13.5-25.2]	Parental report of doctor-diagnosed asthma and wheezing/whistling of the chest in the past 12 months	Asthma: 176 (4.8%); wheezing: 697 (18.8%)	1-2	Gender, ethnicity, study arm, maternal age, parity, breastfeeding, parental education, parental allergic status, maternal smoking during pregnancy, smoking at home, mattress cover, gas

										cooking, unvented gas water heater, any mold/pets at home
Yap[93] (Study 32)	Cohort (the Czech Republic project)	Teplice and Prachatice, Czech Republic	1,133	Outdoor source (not specified); fixed central monitoring sites	Prenatal: 4-7th, 16-20th, 24-27th, and 32-35th weeks of gestation	N.A.	Asthma: first diagnosis of asthma according to ICD-10 J45 from pediatric records	N.A.	0-10	District, birth season, parental allergy in the final model; maternal smoking during pregnancy was also considered
Rosa[94] (Study 33)	Cohort (PROGRESS)	Mexico City, Mexico	552	Outdoor source (not specified); satellite-based LUR model according to residential history	Prenatal	Median [IQR]: 1st trimester: 22.0 [18.9-25.7]; 2nd trimester: 21.1 [18.8-25.6], 3rd trimester: 22.5 [19.0-27.3]	Caregivers' report of ever wheeze and current wheeze (wheezing/whistling of the chest in the past 12 months)	Ever wheeze: 136 (24.6%); current wheeze: 66 (12.0%)	0-4	Gender, maternal age, maternal asthma, prenatal/postnatal ETS exposure, $PM_{2.5}$ exposure during other trimesters and 1 year in the final model; maternal stress and SES were also considered

Gehring[95] (Study 34)	Pooled analysis of 4 cohorts (MeDALL study): BAMSE, GINIplus, LISAplus, PIAMA	Stockholm, Sweden; Munich and Wesel area, Germany; northern, western and central part of The Netherlands	14,126	Outdoor source (mainly traffic); GIS-based LUR model according to birth address (BAMSE: dispersion model)	First 2 years of life	Mean±SD: 7.8±1.2 for BAMSE; 17.4±0.7 for GINI/LISA North; 13.4±1.0 for GINI/LISA South; 16.4±0.7 for PIAMA	Parental report of asthma: ≥2 of the following: doctor-diagnosed asthma, wheezing/whistling of the chest in the past 12 months or prescribed asthma medication during the past 12 months	N.A.	0-2	Native nationality, cohort, parity, breastfeeding, parental education, parental asthma or hay fever, maternal smoking during pregnancy, parental smoking at home, gas cooking, dampness/mold/pets at home, daycare attendance and municipality (BAMSE) in final model; gender and SES were also considered
MacIntyre[96] (Study 35)	Pooled analysis of 4 cohorts (TAG study): LISA, GINI, PIAMA,	Munich, Germany; northern, western and central parts of The Netherlands; Vancouver,	2,755 (CAPPS only included high-risk children)	Traffic; GIS-based LUR model according to birth address	First year of life	Mean±SD: 15.2±3.4	Parental report of doctor-diagnosed asthma and wheeze symptoms; asthma was also confirmed by a pediatric allergist in CAPPS	N.A.	0-8	Gender, study, intervention status, city, birthweight, maternal age, parental allergy, maternal smoking during pregnancy, ETS at home, NO2 exposure during first year of life

	CAPPS	Canada								

Abbreviations: PIAMA, the Prevention and Incidence of Asthma and Mite Allergy study; GIS, geographic information system; LUR, land use regression; CAPPS, the Canadian Asthma Primary Prevention study; SD, standard deviation; ACCESS, the Asthma Coalition on Community, Environment, and Social Stress project; IQR, interquartile range; IDW, inverse distance weighted; ICD, International Classification of Disease; GINI, German Infant Nutrition Intervention Programme; LISA; Influences of Lifestyle Related Factors on the Immune System and Development of Allergies in Children; MeDALL, Mechanisms of the Development of Allergy project; BAMSE, Barn (children), Allergy, Milieu, Stockholm, an Epidemiology project; GINIplus, German Infant study on the influence of Nutrition Intervention plus air pollution and genetics on allergy development; LISAplus, Life style Immune System Allergy plus air pollution and genetics; N.A., not applicable; SES, socioeconomic status; PEMS, personal environmental monitoring sampler; ETS, environmental tobacco smoke; TAG, the Traffic, Asthma and Genetics study; NO_2, nitrogen dioxide; GALA II, the Genes-environments and Admixture in Latino Americans; SAGE II, the Study of African Americans, Asthma, Genes and Environments; KAPPA, the Kaiser Air Pollution and Pediatric Asthma study; PROGESS, the Programming Research in Obesity, Growth, Environment and Social Stressors study; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm. *, Having ≥ 1 first-degree asthmatic relative or ≥2 first-degree relatives with other IgE-mediated allergic disease

Table S2-2. Risk of bias assessment for cohort studies according to the CASP checklist

Study reference	1	2	3	4	5	6a	6b	9	10	11	Notes	Quality
Lee[76] (Study 12)	Yes	No-low recruitment rate (78.1%); although not significant, non-participants were slightly less likely to be ethnic minorities or to have a low education level and slightly more likely to report a low income level than the participants	Yes	No-maternal reported outcomes	No-not adjusted for heredity	Yes	Yes-from birth to 6	Yes	No-see comments in Column 3	Yes	Maternal reported outcome: reporting/recall bias; not adjusted for heredity; not accounted for other pollutants; not generalisable to the overall US population	Moderate
Chiu[85] (Study 21)	Yes	Yes	Yes	No-maternal reported outcomes	No-not adjusted for ETS exposure	Yes	No-from birth to 2	Yes	Yes	Yes	Not adjusted for ETS exposure; maternal reported outcome: reporting/recall bias; small sample size	High
Hsu[86] (Study 22)	Yes	No-low recruitment rate (78.1%);although not	Yes	No-maternal	Yes	Yes	Yes-from birth to 6	Yes	No-see comments in	Yes	Maternal reported outcomes: recall/reporting bias; not	High

		significant, non-participants were slightly less likely to be ethnic minorities or to have a low education level and slightly more likely to report a low income level than the participants		reported outcomes					Column 3		generalisable to the overall US population	
Pennington[88] (Study 24)	Yes	Yes	Yes	Yes	No-not adjusted for ETS exposure	Yes	Yes-from birth to 6	Yes	Yes	Yes	Not adjusted for ETS; lack of detailed data on individual-level SES, high correlation between prenatal and postnatal exposure: unable to determine the relative importance of exposure during different periods; outcome misclassification in early life asthma; KPGA population: urban population with high asthma rates, large African American population,	High

											and high SES: not generalisable to distinctly different populations	
Carlsten[89] (Study 25)	Yes	No-a small high-risk population*	Yes	Yes	No-not adjusted for ETS exposure	No-37% loss of follow-up	Yes-from birth to 7	Yes	No-a small high-risk group	Yes	A small high risk group; not adjusted for ETS exposure; modest sample size: limiting the precision in effect estimates; extrapolation of the LUR based estimates over time	Moderate
Jedrychowski[90] (Study 26)	Yes	Can't tell	Yes	No-maternal reported outcomes	Yes	Yes	No-from birth to 2	Yes	Can't tell	Yes	Non-smoking mothers; unable to distinguish the effect of prenatal exposure from that of the postnatal exposure; maternal reported outcomes: reporting/recall bias	Moderate
Jedrychowski[91] (Study 27)	Yes	Can't tell	Yes	No-maternal reported outcomes	Yes	Yes	No-from birth to 2	Yes	Can't tell	Yes	Non-smoking mothers; unable to distinguish the effect of prenatal exposure from that of the postnatal exposure; maternal reported outcomes: reporting/recall bias	Moderate
Jedrychowski[92] (Study 28)	Yes	Can't tell	Yes	No-maternal	Yes	No-33% loss to	No-from birth to 4	Yes	Can't tell	Yes	Non-smoking mothers; unable to distinguish effect of prenatal	Moderate

				reported outcomes		follow-up & incomplete data					exposure from that of the postnatal exposure; maternal reported outcomes: reporting/recall bias	
Gehring[49] (Study 29)	Yes	No-a higher rate of participants with an atopic and a well-educated (>10 years) parent compared with the original cohort reported by Fuertes et al.[101]	Yes	No-parental reported outcomes	Yes	Yes	No-from birth to 2	Yes	No-likely to exclude children with non-atopic and less-educated parents	Yes	Unable to distinguish between long-term and short-term effects: exposure and health data collected on an annual basis instead of a daily basis; questionnaire data: recall/reporting bias; excluding preterm births and low birth weight infants in LISA might bias the results towards the null; young age for accurate diagnosis; short follow-up duration	Moderate
Morgenstern[50] (Study 30)	Yes	No- a higher rate of participants with an atopic and a well-educated (>10 years)	Yes	No-Parental reported outcomes	Yes	Yes	No-from birth to 2	Yes	No-likely to exclude children with non-atopic	Yes	No validated exposure measurements for suburbs; questionnaire data: reporting/recall bias; high rates of well-educated	Moderate

		parent compared with the original cohort [101]							and less-educated parents		and non-atopic parents; excluding preterm birth/low birth weight infants in LISA may bias the results towards the null; young age for accurate diagnosis; short follow-up duration	
Brauer[48] (Study 31)	Yes	No-low recruitment rate (53%) according to Koopman et al.[102]	Yes	No-parental reported outcomes	Yes	Yes	No-from birth to 2	Yes	Can't tell	Yes	Questionnaire data: recall/reporting bias; misclassification of asthma for infants and very young children; short follow-up duration	Moderate
Yap[93] (Study 32)	Yes	No-low recruitment rate (17%); more full term, normal birth weight children sampled from the POS	Yes	Yes	No-not adjusted for SES	Yes	Yes-from birth to 10	Yes	No-more full term, normal birth weight children sampled from the POS	Yes	More full term, normal birth weight children than the local population; not adjusted for SES; exposure measurements relied on daily average pollution at one central location for each districts: misclassification for individuals	High
Rosa[94] (Study 33)	Yes	Can't tell	Yes	No-caregiver	Yes	No-32% loss to	no-from birth to 4	Yes	Can't tell	Yes	Caregiver reported outcomes: reporting/recall bias	Moderate

				reported outcomes		follow-up & incomplete data						
Gehring[95] (Study 34)	Yes	No-BAMSE: low recruitment rate (75%); less smoking parents in the cohorts than the local population according to Wickman et al.[103]; GINIplus and LISAplus: a higher rate of participants with an atopic and well-educated parent compared with the original cohort [101]; PIAMA: 53% recruitment rate; including more well-	Yes	No-parental reported outcomes	Yes	Yes	No-from birth to 2 (further follow-up data were not included in this review)	Yes	No-see comments in Column 3	Yes	Questionnaire data: reporting/recall bias; not generalisable to local population: children with well-educated parents were over-represented; exposure models based on air pollution measurement campaigns from 2008-2010 to assess exposure for the entire duration of follow-up & based on birth addresses without accounting for locations other than home or time-activity patterns and long term trends	Moderate

		educated native-speakers compared with general population in The Netherlands [104]										
MacIntyre[96] (Study 35)	Yes	No-fewer infants with low birth weight, more older mothers, more atopic parents and fewer mothers smoking during pregnancy compared with the total recruited population for each cohort; CAPPS only included a small high-risk population[a]	Yes	No-parental reported outcomes, except CAPPS being confirmed by pediatric allergists	No-not adjusted for SES	No-46% loss to follow-up & incomplete data	Yes-from birth to 8	Yes	No-fewer infants with low birth weight, more older mothers, more atopic parents and fewer mothers smoking during pregnancy in the cohorts than in the	Yes	Not adjusted for SES; fewer infants with low birth weight, more older mothers, more atopic parents and fewer mothers smoking during pregnancy in the cohort compared with total recruited population for each cohort: selection bias; parental reported outcomes: recall/reporting bias	Moderate

									local population			

Abbreviations: ETS, environmental tobacco smoke; LUR, land use regression; LISA, Influences of Lifestyle Related Factors on the Immune System and Development of Allergies in Children; BMASE, Barn (children), Allergy, Milieu, Stockholm, an Epidemiology project; GINI, German Infant Nutrition Intervention Programme; PIAMA, the Prevention and Incidence of Asthma and Mite Allergy study; CAPPS, the Canadian Asthma Primary Prevention study; SES, socioeconomic status; KPGA, Kaiser Permanente Georgia; POS, the Pregnancy Outcome Study. *, Having ≥ 1 first-degree asthmatic relative or ≥2 first-degree relatives with other IgE-mediated allergic disease

Table S2-3. Risk of bias assessment for case-control studies according to the CASP checklist

Study reference	**1**	**2**	**3**	**4**	**5**	**6**	**9**	**10**	**11**	**Notes**	**Quality**
Sbihi[77] (Study 13)	Yes	Yes-cohort better	Yes	Yes	Yes	No-not adjusted for heredity and ETS exposure	Yes	Yes	Yes	Administrative data were not collected for research purposes and lacked individual-level information (e.g. SES measures); exposure misclassification: exposures in microenvironments other than the homes during pregnancy were not considered; no formal comparison of pregnancy and post-natal exposures was conducted in the absence of linked residential histories throughout the follow-up period; not adjusted for heredity and ETS exposure	High
Clark[84]	Yes	Yes-cohort	Yes	Yes	Yes	No-not	Yes	Yes	Yes	Administrative data were not	High

(Study 20)		better				adjusted for heredity and ETS exposure				collected for research purposes and lacked individual-level information (e.g. SES measures); exposure misclassification: exposures in microenvironments other than the homes during pregnancy were not considered; no formal comparison of pregnancy and postnatal exposures was conducted in the absence of linked residential histories throughout the follow-up period; not adjusted for heredity and ETS exposure	
Nishimura[87] (Study 23)	Yes	Yes-cohort better	No-an ethnic minority population; self/parents reported	no-an ethnic minority population; matched cases/controls	Yes	Yes	Yes	No-an ethnic minority children	Yes	An ethnic minority population: Latino and African American races; case definition based on self/parent-reported information; less complete regional monitoring of $PM_{2.5}$;	Moderate

			outcomes	by geographical area/recruitment centre						reduced accuracy in exposure estimates: Puerto Rico has only 2 monitoring stations; no personal air sampling; no measurement of indoor or prenatal air pollution; case-control matched by geographical region/area	

Abbreviations: ETS, environmental tobacco smoke; SES, socioeconomic status; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm

Notes for CASP quality assessment of all included studies

The Critical Appraisal Skills Programme (CASP) checklists provided 12 and 11 questions for cohort[80] and case control studies,[81] respectively. It evaluated the internal and external validity of the studies including selection bias, classification, measurement or recall bias for exposure and outcome assessment, adjustment for important confounding factors, the completion and length of follow-up and other characteristics regarding the relevance and generalisation of the results. Important confounding factors were maternal smoking during pregnancy or environmental tobacco smoke (ETS) exposure, heredity and socio-economic status (SES).[16] Any follow-up of children who were less than 6 years of age was considered insufficient as asthma diagnosis for preschool children is challenging.[97]

Due to the fact that there is no scoring system for CASP checklist, we defined articles as having high quality if there were ≥ 7 positive answers to the questions in the CASP checklists, moderate quality if there were ≥ 4 positive answers to the questions, and poor quality if there were ≤ 3 positive answers to the questions.

All studies clearly stated their focused issues on the associations between prenatal and infant $PM_{2.5}$ exposure and the subsequent development of wheezing or asthma. There were no information on recruitment method and comparisons between the cohorts and general population in 5 studies.[48, 90-92, 94] More than half of the studies (n = 10) were considered with potential for selection bias because of low recruitment rates (< 80%),[48, 76, 86, 93, 95] only including a small high-risk population [89], inappropriate matching method in a case-control study and different characteristics between participants and non-participants (i.e. ethnicity, maternal age, SES, parental smoking status, heredity, perinatal outcomes).[49-50, 76, 86-87, 93, 95-96] The differences between participants and non-participants may affect the generalisability of the results in those studies. $PM_{2.5}$ was objectively measured in all studies despite potential exposure

misclassifications acknowledged in 7 studies,[50, 77, 84, 87, 89, 93, 95] while wheezing or asthma status was defined based on parental or self-reports in most studies (n = 13), which might lead to information bias. There were 5 studies without adjustment for maternal smoking or ETS exposure,[77, 84-85, 88-89] 3 studies without adjustment for heredity[76-77, 84] and 2 studies without adjustment for SES.[93, 96] The overall follow-up was complete among most studies except 4 with ≥ 30% loss to follow-up,[89, 92, 94, 96] whilst the follow-up period was generally short with only 6 studies following the participants for over 6 years.[76, 86, 88-89, 93, 96]

Overall, all the included studies had fairly good qualities for assessing the association between prenatal and infant $PM_{2.5}$ exposure and wheezing or asthma development.

Table S2-4. Original risk estimates of the 18 studies investigating prenatal and infant $PM_{2.5}$ exposure and wheezing/asthma development

Study Reference	**$PM_{2.5}$ increment ($\mu g/m^3$)**	**Original risk estimates (adjusted OR/RR/HR/RD, 95%CI)**
Lee[76] (Study 12)	Prenatal exposure: 1.7	Asthma from birth to age 6: **1.17 (1.04 to 1.30)** **Stratified analyses:** Prenatal maternal stress: high prenatal stress group: **1.15 (1.03 to 1.26)** low prenatal stress group: not significant (no data) gender & prenatal maternal stress: males born to mothers experiencing high stress: **1.28 (1.15 to 1.41)** other groups: not significant (no data)
Sbihi[77] (Study 13)	Prenatal exposure: preschool age asthma: 1.45; school age asthma: 1.46	Asthma from birth to 6 (preschool): 0.99 (0.97 to 1.01) Asthma from 6 t o10 (school age): 1.01 (0.97 to 1.06) **Stratified analyses (preschool asthma):** gender: stronger effects in females than in males (no data) birthweight: stronger effects in children with birthweight < 2500 g than those with birthweight ≥ 2500 g (no data) gestational age: similar effects in both groups (no data) maternal age: stronger effects in children with old mothers than those with young mothers

		(no data) parity: similar effects in both groups (no data) SES: similar effects in both groups (no data)
Clark[84] (Study 20)	Prenatal and infant exposure: 1	Prenatal exposure: asthma from age 3 to 4: IDW: 0.95 (0.91 to 1.00); LUR: 1.02 (1.00 to 1.03) Infant exposure: asthma from age 3 to 4: IDW: 1.05 (0.97 to 1.14); LUR: 1.01 (0.99 to 1.03) **Stratified analyses:** gender: prenatal exposure: males: IDW: 0.94 (0.88 to 1.00); LUR: 1.01 (0.99 to 1.03) females: IDW: 0.98 (0.91 to 1.05); LUR: 1.03 (1.00 to 1.06) infant exposure: males: IDW: 1.02 (0.92 to 1.13); LUR: 1.00 (0.98 to 1.02) females: IDW: 1.10 (0.96 to 1.26); LUR: 1.03 (1.00 to 1.06)
Chiu[85] (Study 21)	Prenatal exposure: high/low exposure (> 11.22 vs ≤ 11.22); low exposure as reference	Repeated wheeze from birth to 2: **2.02 (1.20 to 3.40)** Wheezing category (0-1, 2,or ≥ 3) from birth to 2: multinominal logit models: 2 vs 0-1: **2.01 (1.04 to 3.88)**, ≥ 3 vs 0-1: 2.03 (0.98 to 4.41); adjacent-categories logit models: 2 vs 0-1: **1.55 (1.10 to 2.19)**, ≥ 3 vs 0-1: **2.40 (1.20 to 4.79)** Wheezing category (0-1, 2-3, or ≥ 4) from birth to 2: multinominal logit models: 2-3 vs 0-1: **1.46 (1.02 to 2.10)**, ≥ 4 vs 0-1: **15.5 (2.61 to 92.5)**; adjacent-categories logit models: 2-3 vs 0-1: **2.09 (1.33 to 3.27)**, ≥ 4 vs 0-1: **4.36 (1.77 to 10.69)**
Hsu[86]	Prenatal exposure: 10	No data

(Study 22)		**Stratified analyses:** gender: associations were stronger in males than in females (**interaction p = 0.01**)
Nishimura[87] (Study 23)	Infant exposure: 1	Asthma from age 8 to 21: 1.03 (0.90 to 1.18) **Stratified analyses:** gender: males (280 cases + 212 controls): 0.92 (0.73 to 1.16) females (218 cases + 222 controls): 1.13 (0.98 to 1.30) total IgE: > 200 IU/mL (292 cases + 200 controls): 1.06 (0.93 to 1.21) ≤ 200 IU/mL (221 cases + 235 controls): 1.00 (0.85 t o1.17) family history of asthma: yes (168 cases + 64 controls): 1.05 (0.87 to 1.26) no (262 cases + 340 controls): 0.96 (0.77 to 1.21)
Pennington[88] (Study 24)	Prenatal and infant exposure: natural log-transformed $PM_{2.5}$: 2.7-fold increase; continuous $PM_{2.5}$: 1; quintiles (quintile 1 as reference); Cox proportional hazards regression for infant $PM_{2.5}$ exposure: 2.7-fold increase	Asthma definition: 1 asthma diagnosis + 1 medication dispensing **Cumulative asthma incidence:** Prenatal exposure (natural log-transformed): age 2: **0.015 (0.003 to 0.027)** age 3: **0.018 (0.002 to 0.035)** age 4: **0.023 (0.001 to 0.044)** age 5: **0.032 (0.007 to 0.065)** age 6: **0.035 (0.006 to 0.065)** Prenatal exposure (continuous):

		age 2: 0.005 (-0.002 to 0.011) age 3: 0.004 (-0.005 to 0.013) age 4: 0.007 (-0.005 to 0.018) age 5: 0.009 (-0.005 to 0.023) age 6: 0.010 (-0.007 to 0.027) Prenatal exposure (quintiles): age 5: quintile 2: **0.048 (0.014 to 0.082)** quintile 3: 0.025 (-0.009 to 0.059) quintile 4: **0.057 (0.020 to 0.094)** quintile 5: **0.042 (0.001 to 0.083)** Infant exposure (natural log-transformed): age 2: 0.012 (0.000 to 0.023) age 3: **0.019 (0.003 to 0.034)** age 4**: 0.025 (0.004 to 0.046)** age 5: **0.041 (0.016 to 0.066)** age 6: **0.035 (0.005 to 0.064)** Infant exposure (continuous): age 2: 0.003 (-0.004 to 0.010) age 3: 0.004 (-0.005 to 0.013)

		age 4: 0.008 (-0.005 to 0.020) age 5: 0.013 (-0.002 to 0.028) age 6: 0.009 (-0.009 to 0.027) Infant exposure (quintiles): age 5: quintile 2: **0.049 (0.017 to 0.081)** quintile 3: **0.044 (0.011 to 0.077)** quintile 4: **0.064 (0.029 to 0.100)** quintile 5: **0.054 (0.014 to 0.094)** Infant exposure (Cox proportional hazards regression): age 5: **1.16 (1.07 to 1.26)** Infant exposure (different asthma definitions): age 5: 1 asthma or wheeze diagnosis: **0.037 (0.011 to 0.064)** 1 asthma diagnosis: **0.047 (0.022 to 0.072)** 2 asthma diagnoses: **0.034 (0.012 to 0.056)** 3 asthma diagnoses: **0.031 (0.009 to 0.052)** 2 asthma diagnoses OR 1 acute asthma diagnosis: **0.039 (0.016 to 0.062)** 1 asthma diagnosis OR 2 medication dispensings: **0.039 (0.012 to 0.067)**

		1 asthma diagnosis AND 2 medication dispensings: **0.042 (0.018 to 0.066)** 1 asthma diagnosis OR 1 controller dispensing: **0.048 (0.022 to 0.074)** 1 asthma diagnosis AND (2 reliever dispensings OR 1 controller dispensing): **0.040 (0.016 to 0.064)** Any of the following: a) 1 asthma diagnosis AND 1 medication dispensing in the same year, b) 1 asthma-related emergency department visit or hospitalisation, c) 3 asthma diagnoses: **0.043 (0.018 to 0.068)** **Persistent asthma by age 5 (incident asthma with evidence of asthma in the past year):** Prenatal exposure (natural log-transformed): **0.044 (0.023 to 0.064)** Prenatal exposure (quintiles): quintile 2: **0.039 (0.008 to 0.070)** quintile 3: **0.037 (0.005 to 0.068)** quintile 4: **0.059 (0.025 to 0.094)** quintile 5: **0.055 (0.017 to 0.093)** Infant exposure (natural log-transformed): **0.045 (0.023 to 0.066)** Infant exposure (quintiles): quintile 2: **0.041 (0.012 to 0.070)**

		quintile 3: **0.047 (0.017 to 0.078)** quintile 4: **0.060 (0.027 to 0.093)** quintile 5: **0.054 (0.016 to 0.092)** **Stratified analyses for infant exposure and asthma by age 5 (2.7-fold increase):** gender: males: 0.027 (-0.011 to 0.066) females: **0.047 (0.014 to 0.080)** race: white children: **0.053 (0.017 to 0.089)** black children: **0.048 (0.005 to 0.091)** maternal asthma: yes (n = 1,140): 0.027 (-0.052 to 0.107) no (n = 6,606): **0.041 (0.012 to 0.069)**
Carlsten[89] (Study 25)	Infant exposure: 4.1	Asthma diagnosed at age 7: **3.10 (1.30 to 7.40)**
Jedrychowski[90] (Study 26)	Prenatal exposure: high/low exposure (> 35.30 vs ≤ 35.30), low exposure as reference	Number of days wheezing from birth to 2: **1.36 (1.29 to 1.43)**
Jedrychowski[91] (Study 27)	Prenatal exposure: higher/medium/low exposure (> 53.40/35.30-53.40 vs ≤ 35.30), low exposure as reference	Number of days wheezing from birth to 2: higher exposure: **1.62 (1.42 to 1.86)**; medium exposure: **1.13 (1.03 to 1.23)**
Jedrychowski[92] (Study 28)	Prenatal exposure: high/low exposure (> 33.40 vs ≤ 33.40), low exposure as reference	Number of days wheezing from birth to 2: Poisson portion (IRR): **1.38 (1.25 to 1.51);** logistic portion (1/OR): 1.32 (0.84 to 2.08)

		Number of days wheezing from age 3 to 4: Poisson portion (IRR): 1.06 (0.92 to 1.22); logistic portion (1/OR): 1.03 (0.60 to 1.77)
Gehring[49] (Study 29)	Infant exposure: 1.5	Asthmatic/spastic/obstructive bronchitis from birth to 1: 0.98 (0.80 to 1.20) Asthmatic/spastic/obstructive bronchitis from age 1 to 2: 0.92 (0.78 to 1.09) Wheezing from birth to 1: 0.91 (0.76 to 1.09) Wheezing from age 1 to 2: 0.96 (0.83 to 1.12) **Stratified analyses:** gender: asthmatic/spastic/obstructive bronchitis from birth to 1: males (n = 845): 0.97 (0.76 to 1.25) females (n = 761): 0.98 (0.68 to 1.41) asthmatic/spastic/obstructive bronchitis from age 1 to 2: males (n = 791): 0.92 (0.74 to 1.14) females (n = 719): 0.91 (0.68 to 1.21) wheezing from birth to 1: males (n = 844): 0.91 (0.72 to 1.16) females (n = 753): 0.94 (0.70 to 1.27) wheezing from age 1 to 2: males (n = 801): 0.93 (0.76 to 1.14) females (n = 716): 1.04 (0.83 to 1.30)

Morgenstern[50] (Study 30)	Infant exposure: 1.04	Asthmatic/spastic/obstructive bronchitis from birth to 1: 1.04 (0.90 to 1.19) Asthmatic/spastic/obstructive bronchitis from age 1 to 2: 1.05 (0.92 to 1.20) Wheezing from birth to 1: 1.01 (0.87 to 1.18) Wheezing from age 1 to 2: 1.10 (0.96 to 1.25)
Brauer[48] (Study 31)	Infant exposure: 3.2	Asthma from age 1 to 2: 1.12 (0.84 to 1.50) Wheezing from age 1 to 2: 1.14 (0.98 to 1.34)
Yap[93] (Study 32)	Prenatal exposure: 25	Asthma from birth to 10: 4-7th gestational weeks exposure: 0.75 (0.50 to 1.13) 16-20th gestational weeks exposure: 1.38 (0.99 to 1.93) 24-27th gestational weeks exposure: **1.52 (1.08 to 2.15)** 32-35th gestational weeks exposure: **1.89 (1.35 to 2.64)**
Rosa[94] (Study 33)	Prenatal exposure: 3.8	Ever wheeze from birth to 4: not significant for any trimester exposure (no data) Current wheeze at age 4: not significant for any trimester exposure (no data) **Stratified analyses:** Prenatal stress: Ever wheeze from birth to 4: Low stress group: 1st trimester: 0.99 (0.83 to 1.18) 2nd trimester: 0.92 (0.76 to 1.12) 3rd trimester: 0.96 (0.82 to 1.13)

		High stress group: 1st trimester: 1.18 (0.97 to 1.43) 2nd trimester: 1.06 (0.85 to 1.32) 3rd trimester: 0.94 (0.78 to 1.15) Current wheeze at age 4: Low stress group: 1st trimester: 0.84 (0.61 to 1.16) 2nd trimester: 0.74 (0.54 to 1.04) 3rd trimester: 0.96 (0.74 to 1.26) High stress group: 1st trimester: **1.35 (1.00 to 1.83)** 2nd trimester: 0.99 (0.71 to 1.38) 3rd trimester: 0.83 (0.61 to 1.13)
Gehring[95] (Study 34)	Infant exposure: 5	Asthma incidence: from birth to 1: 0.87 (0.65 to 1.16) Asthma incidence: from age 1 to 2: **1.38 (1.03 to 1.84)** Asthma prevalence: from birth to 1: 0.97 (0.72 to 1.32) Asthma prevalence: from age 1 to 2: **1.38 (1.03 to 1.86)**
MacIntyre[96] (Study 35)	Infant exposure: 1	Ever asthma from birth to 8: 1.03 (0.89 to 1.20) Current asthma at age 6 to 8: **1.35 (1.07 to 1.70)**

		Ever wheeze from birth to 8: 1.00 (0.87 to 1.14) Current wheeze from birth to 8: 1.18 (0.98 to 1.43) Ever asthma and current wheeze at age 6 to 8: 1.22 (0.98 to 1.52) **Stratified analyses:** genotype: ever asthma from birth to 8: GSTP1 rs1138272: TT/TC: 1.03 (0.67 to 1.60) CC: 1.02 (0.87 to 1.21) GSTP1 rs1695: GG/GA: 0.97 (0.77 to 1.23) AA: 1.09 (0.89 to 1.33) TNF rs1800629: AA/AG: 1.07 (0.77 to 1.48) GG: 1.03 (0.86 to 1.23) current asthma at age 8: GSTP1 rs1138272: TT/TC: **2.19 (1.03 to 4.65)** CC: **1.29 (1.01 to 1.65)** GSTP1 rs1695: GG/GA: 1.19 (0.76 to 1.85) AA: **1.40 (1.06 to 1.84)** TNF rs1800629: AA/AG: 1.34 (0.87 to 2.05) GG: **1.42 (1.04 to 1.93)** ever wheeze from birth to 8:

		GSTP1 rs1138272: TT/TC: 1.14 (0.75 to 1.74) CC: 0.97 (0.83 to 1.12) GSTP1 rs1695: GG/GA: 0.98 (0.80 to 1.21) AA: 1.02 (0.84 to 1.24) TNF rs1800629: AA/AG: 1.04 (0.77 to 1.39) GG: 0.99 (0.84 to 1.16) current wheeze at age 6 to 8: GSTP1 rs1138272: TT/TC: 1.56 (0.90 to 2.72) CC: 1.15 (0.94 to 1.41) GSTP1 rs1695: GG/GA: 1.14 (0.85 to 1.54) AA: 1.20 (0.96 to 1.52) TNF rs1800629: AA/AG: 1.26 (0.86 to 1.85) GG: 1.17 (0.93 to 1.47) ever asthma plus current wheeze at age 6 to 8: GSTP1 rs1138272: TT/TC: **1.95 (1.09 to 3.50)** CC: 1.15 (0.91 to 1.46) GSTP1 rs1695: GG/GA: 1.17 (0.80 to 1.72) AA: 1.22 (0.95 to 1.56) TNF rs1800629: AA/AG: 1.32 (0.89 to 1.95)

		GG: 1.24 (0.94 to 1.63)

Abbreviations: $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 μm; OR, odds ratio; RR, risk ratio; HR, hazard ratio; RD, risk difference; 95%CI, 95% confidence interval; IRR, incidence rate ratio. Significant results were shown in bold.

Chapter 3

Early Life Exposure to Coal Mine Fire Smoke Emissions and Altered Lung Function in Young Children

Chapter 3. Early Life Exposure to Coal Mine Fire Smoke Emissions and Altered Lung Function in Young Children

3.1 Preface

Chapter 2 highlighted the limited and inconsistent evidence on the respiratory effects of $PM_{2.5}$ exposure during early life, indicating the needs for epidemiological studies to address this research gap. The Hazelwood coal mine fire provided an opportunity to evaluate children's respiratory health following early life exposure to coal mine fire emissions. The aim of Chapter 3 (Aim 2) was to evaluate the association between exposure to a six-week episode of air pollution from the coal mine fire in children aged less than two years, and their lung function three years after the fire. Lung function was measured using a non-invasive and easy to use method, the forced oscillation technique (FOT), which is particularly suitable for young children[105-106]. A modified version of this Chapter has been published in *Respirology*.

3.2 Introduction

Short-term health impacts associated with exposure to fine particulate matter, a major pollutant generated by fires[107], have been well characterised. There is consistent evidence associating short-term smoke exposure with increased physician visits, emergency department presentations and hospitalizations for respiratory diseases[4, 8]. However, the health risks from relatively short, that is days to weeks in duration, air pollution episodes have not been characterised, especially in children[4]. This represents an important gap in the available evidence because severe episodic exposure to fire smoke is likely to increase with climate change[108].

Infants and young children are more susceptible to the respiratory impacts of air pollution exposure due to their less developed airways and immune system, and faster breathing rates compared with adults[109]. The first two years of life is a critical window for lung growth[72]. There is a small but growing body of evidence suggesting that long-term exposure to air pollutants during infancy could result in measurable respiratory health impacts later in life. For example, exposure to traffic-related air pollution during the first year of life has been associated with impaired lung function in both children and adolescents[110-111]. However, evidence of the respiratory impacts from relatively short durations of air pollution exposure during infancy is extremely limited[112]. One study investigating the Great Smog in London suggested that exposure to the Great Smog during the first year of life could increase the risk of childhood asthma[113].

The Hazelwood coal mine fire was ignited by embers from wildfires in February 2014 in the Latrobe Valley, Victoria, Australia and lasted for 45 days. In this mine fire episodes of moderate to extreme air pollution were experienced in several nearby towns for days to weeks. The peak 24-hour average concentration of $PM_{2.5}$ reached 731 $\mu g/m^3$ at the closest town of Morwell, dramatically higher than the national air quality standard of 25 $\mu g/m^3$[2-3]. The health harms resulting from exposure to smoke from coal mine fires could be comparable to those from forest and peat fires. There is a similar spectrum of toxic components, especially in smouldering fires, and the temporal patterns of exposure are similar – often being time limited but severe[4].

We investigated the infants aged less than two years who were exposed to smoke from the mine fire with the aim of evaluating the association between the magnitude of smoke exposure and lung function three years after the fire.

3.3 Methods

3.3.1 Recruitment

This study was based on a prospective cohort study of 571 children who were born between 01/03/2012 and 31/12/2015 and resided in the Latrobe Valley at the time of the fire (see Figure S1, Appendix 3)[114]. At recruitment (February to September 2016), the participating parent/carer of each child completed a baseline questionnaire on sociodemographic, health and family characteristics. Only the 203 members of the cohort who were ≤2 years old at the time of the fire were old enough to attempt respiratory function testing and invited to participate in this study [Figure S3-1 in Appendix 3]

The Tasmanian Health and Medical Human Research Ethics Committee (reference H14875) approved this study. Additional approval was received from the Human Research Ethics Committees of Monash University, Monash Health, and the University of Melbourne. All parents or caregivers of the studied participants provided signed consent forms for both the baseline survey and the clinical testing.

3.3.2 Exposure estimate

Hourly $PM_{2.5}$ concentrations during the fire period from 09/02/2014 to 31/03/2014 were estimated using meteorological, dispersion and chemical transport modelling at a spatial resolution of 1×1 km[115]. The model quantitatively calculated $PM_{2.5}$ emission rates at an hourly timestep based on parameters such as how much coal was burned using maps of the area and estimated emission factors. Personal exposures were then calculated for 24-hour average and peak periods based on participants' day/night locations.

3.3.3 Clinical testing

Children's lung function was evaluated three years after the fire, (March to July 2017), using the forced oscillation technique (FOT) (TremoFlo C-100 device, Thorasys, Montreal, Quebec, Canada) according to ATS/ERS guidelines[116]. We reported standardized Z scores for baseline

Rrs and Xrs at a frequency of 5 Hz (Rrs_5 and Xrs_5), and the area under the reactance curve (AX)[117]. We excluded measurements with artefacts such as mouth or tongue movement, leakage, swallowing, glottal closure or talking and those having a coherence of <90% at one or more frequencies. Three to five acceptable measurements with a coefficient of variation <10% were obtained for each child [Appendix 3].

3.3.4 Covariates

All covariates were selected *a priori* according to the existing literature[118-123] [Appendix 3].

3.3.5 Statistical analysis

We used Pearson's Chi-square test and Welch's t test for the comparisons of characteristics between our studied participants and the full cohort. We calculated β-coefficients and 95% confidence intervals (CIs) from multiple linear regression models to evaluate the associations between infant $PM_{2.5}$ exposure and lung function with or without adjustment for *a priori* selected covariates. We chose the increments of 10 μg/m^3 for average $PM_{2.5}$ and 100 μg/m^3 for peak $PM_{2.5}$ (close to the interquartile ranges (IQRs) of average and peak $PM_{2.5}$ in this study) to enable comparison with other air quality studies suggesting decreased lung function in children after a 10 and 71 μg/m^3 increase in average and peak $PM_{2.5}$ exposure[124-125]. Five participants (6.0%) had missing values for covariates. We used multiple imputation by chained equations to estimate missing data for covariates[126][Table S3-1, Appendix 3]. We conducted sensitivity analysis by excluding the participants with imputed data. Additionally, maternal smoking status during pregnancy and gender were assessed as interaction terms in the multivariable regression models. We also conducted stratified analysis by gender to assess the different effects of fire smoke exposure on males and females. We performed statistical analyses using R 3.5.0 (the R Foundation, Vienna, Austria). A *p*-value <0.05 was considered statistically significant.

3.4 Results

3.4.1 Participants' characteristics

Of the 203 children eligible for the study, the parents/carers of 137 provided consent for later clinical follow-up. Of these, 101 children attended the clinic and 85 (84.2%) successfully completed FOT testing. We excluded one participant from data analysis due to the poor quality of the FOT measurements (Figure 3-1).

Almost one fifth (17.9%) of the participants were exposed to maternal tobacco smoking while *in utero*, and two fifths (39.3%) had mothers with ≤12 years of education, i.e. completion of secondary education or less. Nearly a quarter (23.8%) of children lived in a house with a current smoker. The mean±standard deviation (SD) age of the children at the time of FOT testing was 4.3±0.5 years. The mean annual background $PM_{2.5}$ concentration in this area (6.7 μg/m^3) was lower than the national air quality standard of 8 μg/m^3[3] (Table 3-1).

Our participants had comparable characteristics with the entire cohort (n=571) in terms of gestational age, birthweight, gender, maternal alcohol or tobacco use during pregnancy, primary carer's education level and smoking status, maternal stress and breastfeeding duration (Chi-square or t test $p>0.05$ for all comparisons; Table S3-2, Appendix 3).

3.4.2 $PM_{2.5}$ exposure during the fire period

The median [IQR] of the average and peak $PM_{2.5}$ levels during the fire period were 7.9 [6.8, 16.8] and 103.4 [60.6, 150.7] μg/m^3, respectively (Table 3-1). Children were exposed to a wide range of $PM_{2.5}$ during the fire period as indicated by the large IQRs.

3.4.3 Lung function measures and risk factors

The mean±SD Z scores of baseline Rrs_5, Xrs_5 and AX were 0.56±0.80, -0.76±0.88 and 0.72±0.92, respectively. Three of the 84 children had Z scores for $Rrs_5 \geq 2$, nine had Z scores for $Xrs_5 \leq -2$ and six had Z scores for AX ≥2.

After adjustment for $PM_{2.5}$ and other covariates, maternal smoking during pregnancy was strongly associated with impaired lung reactance, indicated by a decreased Xrs_5 (-1.15; 95%CI -1.71, -0.60; $p<0.05$) and increased AX (0.74; 95%CI 0.10, 1.38; $p<0.05$), while lower maternal education was associated with higher Xrs_5 (0.46; 95%CI 0.06, 0.86; $p<0.05$). Other covariates were not associated with any of the outcomes.

3.4.4 Infant fire $PM_{2.5}$ exposure and lung function

In univariable analysis, $PM_{2.5}$ was associated with Xrs_5 and AX, but not with Rrs_5 (Table 3-2).

Multivariable model suggested a linear relationship with no threshold for AX. Each 10 $\mu g/m^3$ increase in average $PM_{2.5}$ was associated with increased AX (0.26; 95%CI 0.02, 0.50; $p<0.05$). Similarly, for every 100 $\mu g/m^3$ increase in peak $PM_{2.5}$, we observed no association with AX (0.17; 95%CI -0.00, 0.33; $p>0.05$), consistent with reduced lung function. However, the association between average or peak $PM_{2.5}$ exposure and Xrs_5, seen in the univariable analysis, was no longer present (Table 3-3; Figure 3-2). There was no evidence that maternal smoking or gender modified the association between mine fire $PM_{2.5}$ and lung function (Table S3-3, Appendix 3).

3.4.5 Stratified analysis

Stratified analysis in girls and boys did not show statistically significant associations, although boys tended to have stronger associations than girls for measures of reactance (Table S3-4, Appendix 3).

3.4.6 Sensitivity analysis

After excluding participants with imputed data (n=5), the results were very similar, although slightly stronger (Table S3-5, Appendix 3).

3.5 Discussion

At a three-year follow-up of an infant cohort, we observed an association between elevated concentrations of $PM_{2.5}$ during the coal mine fire and worsening peripheral lung mechanics. These results suggest that exposure to mine fire smoke in early life may have influenced lung growth and development. The measured changes in lung function associated with the fire smoke exposure were small for each incremental increase in exposure but would be likely to be of clinical relevance in the most severely exposed children. Furthermore, reductions in lung function as assessed by FOT, and measured on a single occasion, do not necessarily mean that there is a clinical problem or that one might subsequently develop. A recent study suggested that infants with low lung function during the first year could recover in later childhood[127].

We are not aware of other published studies evaluating early life exposure to smoke from fires, or other short to moderate duration episodes of air pollution, and lung function in preschool aged children. The only comparable study, in terms of exposure, was conducted in monkeys but not in humans. The California fires of 2008 caused degraded air quality for a period of three weeks in a primate research facility soon after the birth of 50 rhesus macaque monkeys, and their lung function was evaluated in adolescence[52]. Unlike our results, these authors found moderate reductions in airway resistance in the exposed animals compared with the unexposed indicating better lung function. Increased lung stiffness was also observed in the exposed monkeys but only in females, while our study implied stronger effects in boys than in girls after coal mine fire smoke exposure, although the results were not statistically significant. Future

studies are needed to compare the effects of early life exposure to short duration of poor air quality on lung function by gender.

Epidemiological studies have evaluated the impact of exposure to different concentrations of constant background air pollution, as distinct from short-term pollution episodes, in early life and later lung function. One study evaluated the association between exposure to traffic-related air pollution during the first year of life and adolescent lung function using a similar approach to ours[128]. The authors reported mixed results with some associations identified between reduced lung function and exposure to oxides of nitrogen, but not particulate matter. Studies evaluating exposure to traffic-related $PM_{2.5}$ in infancy and lung function measured with spirometry at 7-10 years of age have reported both reduced[39], or unchanged[40] lung function. However, these comparisons should be considered with caution because of different populations, metrics, sources and durations of air pollution exposure that were investigated.

Consistent with current literature, we found that maternal tobacco smoking during pregnancy had negative effects on children's lung function. Many epidemiological studies have indicated an adverse effect of maternal tobacco smoking on the lung health of infants and children[120, 129-135]. Our findings further highlight the need for smoking cessation support for parents, from the pre-conception period and onwards, to improve their children's respiratory health.

The direction of associations between lower maternal education and lung function in our study was unexpected and not consistent with the weight of existing evidence regarding SES and child health[122, 136-138]. While we do not have a good explanation for these findings, it could be chance findings in the context of multiple comparisons or inaccurate measurement of maternal education. This finding should be interpreted cautiously because of the small number of children in the subgroups (e.g. n=33 for children with mothers without post-secondary qualifications).

A strength of the study was our ability to adjust for participants' activity patterns to estimate personal $PM_{2.5}$ exposure estimates. Furthermore, we were able to use a simple, non-invasive and objective method of evaluating lung function outcomes suitable for young children[116]. In addition, we conducted multiple imputation to deal with missing values, which could avoid the reduction of sample size and minimize bias[139]. Sensitivity analysis of complete cases revealed similar results to the main findings, indicating that our results were robust.

However, our study has some limitations. First, while we were able to evaluate outcomes in children exposed across a wide range of $PM_{2.5}$, we did not include a group of children with no smoke exposure at all because children in this group were too young to do the FOT testing. Second, the exposure estimates were drawn from modelled air quality data because the monitoring conducted during the fire across the Latrobe Valley was absent during the first week of the fire. Furthermore, our exposure estimates relied upon parental recall of their whereabouts during the fire period and there is therefore, a risk of exposure misclassification and recall error. While most respondents reported that they were confident of their recall of events during the fire, we were unable to test this objectively. However, there is evidence suggesting a strong correlation between confidence and accuracy of recall in eyewitness studies[140-141]. Finally, although our study population were representative of the full cohort in terms of demographic and socioeconomic characteristics, a higher proportion of children with well-educated and non-smoking parents were recruited from the local population[114], which might influence the generalisability of the study findings.

It is possible that the results were influenced by residual confounding. We adjusted for the most important factors such as maternal tobacco smoking, secondhand smoke exposure and maternal education. Education status is a widely used proxy for SES, but in our analysis, lower educational attainment was found to have an unexpected protective association. Therefore, maternal education might not have been the most appropriate marker of SES in our participants.

For this reason, our analysis also included the index of socio-economic disadvantage by region[142] for the residential location of each participant. However, inclusion of this marker did not appreciably change the results. In addition, multiple comparisons might also affect the results due to the small sample size. Further studies with large sample size are needed to confirm these findings.

It has been shown that improvements in air quality were associated with improved lung function in children[143-144]. Given that the mine fire episode was brief and air quality in the region is generally very good, the effects observed in this study might change as children grow. Therefore, it is important to continue to monitor lung function in this group, to identify if the differences persist. Further studies with a larger sample size and wider range of exposures by including the *in utero* and no exposure groups at follow-ups would be important for confirming these initial findings.

3.6 Conclusion

In conclusion, early life exposure to short-term high intensity air pollution can possibly alter lung development in children. It will be important to continue to monitor children's lung function to investigate long-term outcomes.

Table 3-1. Characteristics of the participants

Characteristics	Mean±SD	Range
Birthweight* (kg)	3.4±0.6	1.4, 5.3
Gestational age (week)	39.5±1.9	33.0, 43.0
IRSD	3.3±2.8	1.0, 10.0
Background $PM_{2.5}$ exposure ($\mu g/m^3$)	6.7±0.9	3.4, 8.3
Age at clinic visit (years)	4.3±0.5	3.4, 5.3
Height (cm)	106.8±6.2	94.5, 128.8
Weight (kg)	19.7±4.9	13.6, 48.5
	n (N)‡	%
Gender: Boys	41 (84)	48.8
Maternal alcohol use during pregnancy: yes	9 (83)	10.8
Maternal smoking during pregnancy: yes	15 (84)	17.9
Maternal education: secondary education or less	33 (84)	39.3
Maternal history of asthma: yes	22 (84)	26.2
Maternal stress during pregnancy: frequently stressed	12 (84)	14.3
Effect of coal mine fire on maternal stress: 'increased a lot'	31 (83)	37.3
Second hand smoke exposure: yes	20 (84)	23.8
Breastfeeding: ≤3 month	27 (83)	32.5
Respiratory medication use 24 hours before FOT testing: yes	11 (84)	13.1
Cold/flu-like illnesses in the past 3 weeks: yes	52 (84)	61.9
$PM_{2.5}$ concentrations during the fire period ($\mu g/m^3$)	Median	IQR
24-hour average $PM_{2.5}$	7.9	6.8, 16.8
24-hour peak $PM_{2.5}$	103.4	60.6, 150.7

Abbreviations: SD, standard deviation; IRSD, Index of Relative Socio-economic Disadvantage; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; IQR, interquartile range. *Participants with complete data for birthweight (n=82). ‡n represents number of participants with specific characteristics, while N represents number of participants with completed data for the variable.

Table 3-2. Univariable analysis of infant coal mine fire $PM_{2.5}$ exposure and other covariates on lung function

Univariable analysis (n=84)	β-coefficient (95%CI)		
	Z (Rrs_5)	Z (Xrs_5)	Z (AX)
Average $PM_{2.5}$ (10 μg/m^3 increase)	0.18 (-0.00, 0.36)	-0.21 (-0.41, -0.02) *	0.24 (0.04, 0.45)*
Peak $PM_{2.5}$ (100 μg/m^3 increase)	0.10 (-0.02, 0.23)	-0.12 (-0.26, 0.02)	0.16 (0.02, 0.31)*
Birthweight (1 kg increase)	0.06 (-0.24, 0.35)	0.03 (-0.29, 0.36)	0.10 (-0.24, 0.45)
Gestational age (per week increase)	0.04 (-0.05, 0.13)	-0.02 (-0.12, 0.08)	0.05 (-0.06, 0.15)
IRSD (per unit increase)	-0.04 (-0.10, 0.02)	0.04 (-0.02, 0.11)	-0.02 (-0.09, 0.05)
Background $PM_{2.5}$ (1 μg/m^3 increase)	-0.03 (-0.22, 0.16)	0.10 (-0.11, 0.31)	-0.05 (-0.27, 0.17)
Maternal alcohol use during pregnancy: yes	0.16 (-0.40, 0.72)	-0.38 (-0.98, 0.23)	0.35 (-0.29, 0.99)
Maternal smoking during pregnancy: yes	0.36 (-0.08, 0.80)	-0.86 (-1.32, -0.40)§	0.61 (0.11, 1.12)*
Maternal education: secondary education or less	-0.01 (-0.36, 0.34)	0.27 (-0.12, 0.65)	-0.265 (-0.67, 0.14)
Maternal history of asthma: yes	-0.16 (-0.55, 0.23)	-0.12 (-0.55, 0.31)	0.10 (-0.36, 0.55)
Maternal stress during pregnancy: frequently stressed	0.14 (-0.35, 0.63)	-0.26 (-0.80, 0.28)	0.24 (-0.33, 0.81)
Effect of coalmine fire on maternal stress: 'Increased a lot'	0.09 (-0.26, 0.45)	0.04 (-0.35, 0.43)	0.01 (-0.40, 0.42)
Second hand smoke exposure: yes	0.31 (-0.09, 0.71)	-0.19 (-0.64, 0.25)	0.14 (-0.32, 0.61)
Breastfeeding duration: ≤3 months	0.23 (-0.14, 0.60)	-0.05 (-0.46, 0.35)	0.08 (-0.35, 0.50)
Recent cold/flu-like illness: yes	0.17 (-0.18, 0.52)	0.10 (-0.29, 0.49)	0.00 (-0.41, 0.41)
Respiratory medication use: yes	-0.04 (-0.55, 0.47)	0.27 (-0.29, 0.83)	-0.23 (-0.82, 0.36)

Abbreviations: CI, confidence interval; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; IRSD, Index of Relative Socio-economic Disadvantage. *P<0.05. §P<0.001.

Table 3-3. Multivariable analysis of infant coal mine fire $PM_{2.5}$ exposure and other covariates on lung function

Multivariable analysis (n=84)	β-coefficient[‡] (95%CI)		
	Z (Rrs_5)	Z (Xrs_5)	Z (AX)
Average $PM_{2.5}$ (10 μg/m^3 increase)	0.13 (-0.09, 0.35)	-0.18 (-0.39, 0.03)	0.26 (0.02, 0.50)[*]
Peak $PM_{2.5}$ (100 μg/m^3 increase)	0.07 (-0.08, 0.22)	-0.08 (-0.23, 0.07)	0.17 (-0.00, 0.33)

Abbreviations: CI, confidence interval; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers. [‡]Models adjusted for birthweight, gestational age, IRSD, background $PM_{2.5}$ exposure, breastfeeding duration, maternal alcohol use during pregnancy, maternal smoking during pregnancy, maternal education, maternal history of asthma, maternal stress during pregnancy or during the fire, secondhand smoke exposure, cold/flu-like illnesses in the past three weeks and respiratory medication use 24 h before FOT testing. Average and peak $PM_{2.5}$ were modelled separately, and models included all covariates listed in the table. [*]$P<0.05$.

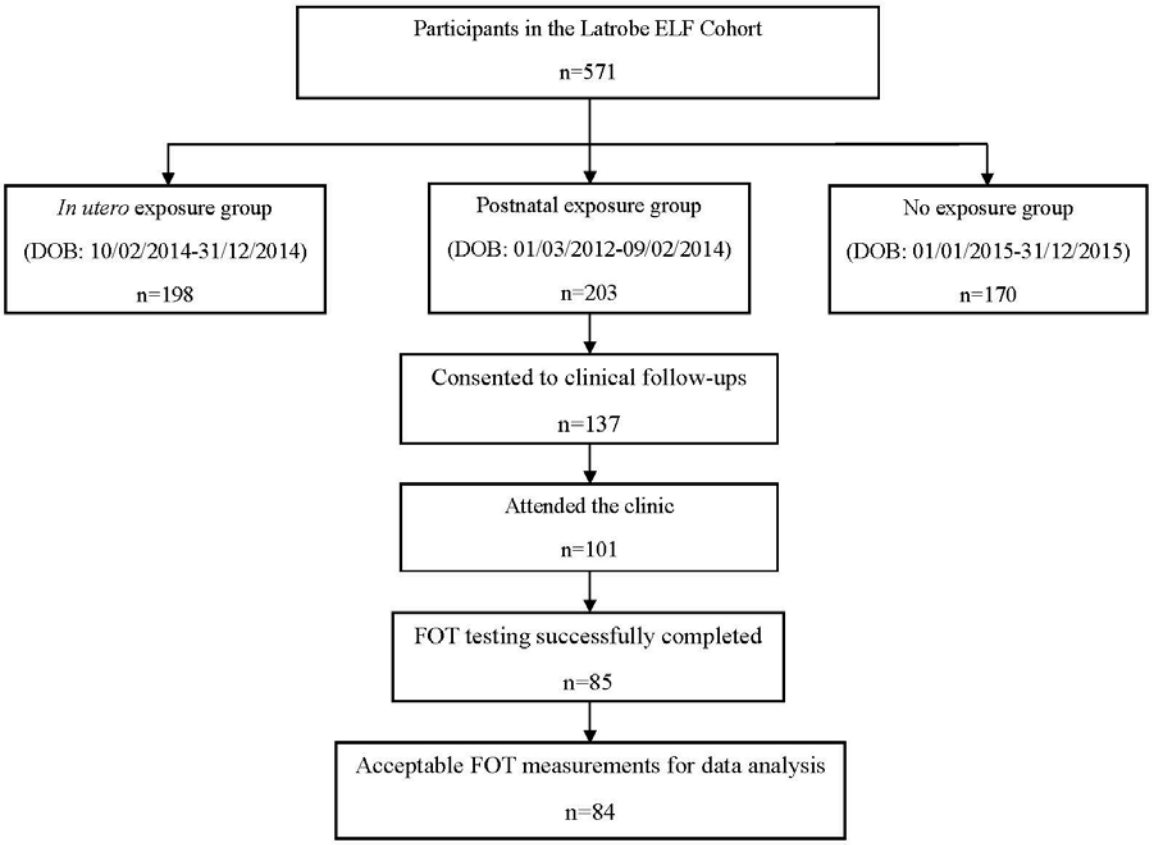

Figure 3-1. Flow chart of the children participating in this study.

DOB, date of birth; FOT, forced oscillation technique.

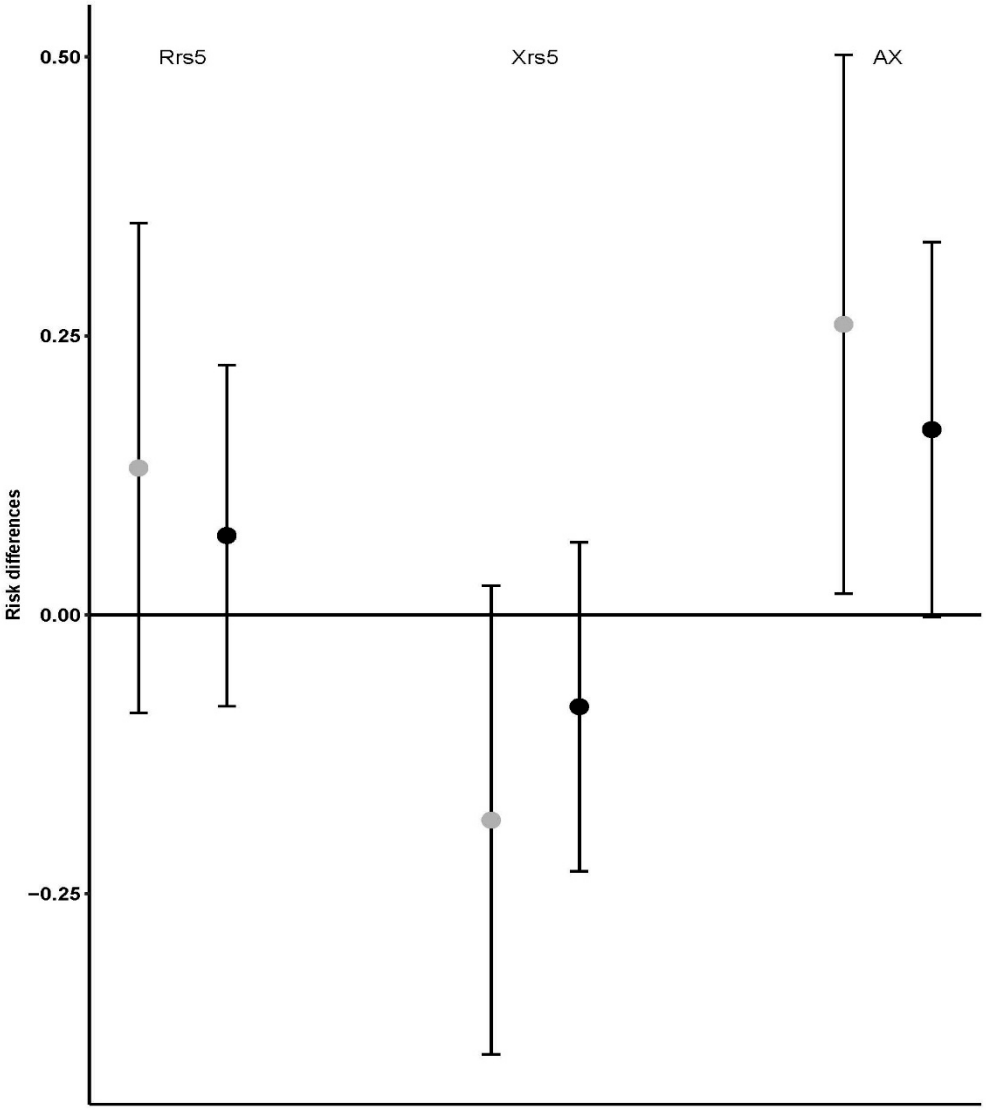

Figure 3-2. Risk differences (points) and 95%CIs (whiskers) for the associations between infant fire $PM_{2.5}$ exposure and lung function.

The risk difference presents the coefficients from multivariable linear regression analysis demonstrating the change in measures per incremental increase in exposure to fire-related $PM_{2.5}$. Rrs5, Resistance at a frequency of 5 Hz; Xrs5, Reactance at a frequency of 5 Hz; AX, the area under the reactance curve; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers.

3.7 Appendix 3

Methods: details of clinical invitation and testing, definitions of covariates and multiple imputation

Figure S3-1. Maps of the Latrobe Valley showing the approximate residential locations of the participants at the time of the fire. Locations have been randomly plotted within an area of 4 square km.

Table S3-1. Comparison of characteristics between participants with and without missing data

Table S3-2. Comparisons of socioeconomic characteristics between participants in our study and in the entire cohort

Table S3-3. Test for effect modification by maternal smoking during pregnancy and gender

Table S3-4. Infant coal mine fire $PM_{2.5}$ exposure and lung function stratified by gender

Table S3-5. Infant coal mine fire $PM_{2.5}$ exposure and other covariates on lung function without multiple imputation

Methods

Invitation for clinical testing

All invited families were sent an initial approach letter containing a unique log-in to book an appointment online. SMS reminders were used to prompt online bookings. Non-responders were followed up by telephone. Booked participants received a reminder phone call the day before the appointment. Parents/carers were asked to confirm their children had not had a recent infection which may affect the results of lung function testing. Articles in media outlets including newspaper, TV, radio and social media, and a study newsletter promoting the clinical testing were sent to all enrolled families, aimed to publicise the clinical testing and increase participation.

Clinical testing

On arrival at the clinic, parents or carers completed a survey on the clinical information of the participating children including date and time of the clinic attendance, gender, date of birth, and information about factors that might influence the results of the lung function testing (i.e. any respiratory medications used in the past 24 h and any cold or flu-like illnesses experienced in the past three weeks). In addition, we measured children's height and weight using a calibrated stadiometer and portable scales before lung function testing.

Lung function testing in preschool children is challenging as it requires a high level of cooperation[105]. The forced oscillation technique (FOT) is a non-invasive and effort independent method to evaluate the respiratory system impedance including resistance and reactance, which is particularly suitable for young children[105-106], and has been widely used in previous studies[145-148].

We invited children whose parents consented to clinical follow-ups to participate in respiratory function testing using FOT (TremoFlo C-100 device, Thorasys, Montreal, Quebec, Canada) according to ATS/ERS guidelines[116]. The device was calibrated every day before use. Children were asked to sit upright on a chair or on the lap of an adult with their head in a neutral position and a clip on their nose. The FOT equipment used a pseudo-random noise-forcing signal at frequencies from 5 to 37 Hz to measure respiratory system mechanics during children's tidal breathing with their cheeks and chin supported by a staff member to prevent signal shunting. Given that lung function was associated with age, gender, height and weight, we reported mean Z scores of all acceptable measurements for respiratory system resistance at 5 Hz (Rrs_5), reactance at 5 Hz (Xrs_5) and the area under the reactance curve (AX) adjusted for these factors using published references ranges according to the equation[117]:

$$\text{Z score} = \text{measured value} - \text{reference value}/\text{standard error of the estimate (SEE)}.$$

Covariates definition

Information on a priori covariates was obtained from the parent/carer-reported baseline questionnaires including birthweight, gestational age, total breastfeeding duration (≤3 month vs. >3 months), social economic status (SES) markers including 12 years of maternal education secondary education) or less, vs. post-secondary, Index of Relative Socio-economic Disadvantage (IRSD) deciles within Victoria[142], maternal smoking or alcohol use during pregnancy (yes vs. no), second hand smoke (SHS) exposure (yes vs no), maternal overall stress during pregnancy (frequently stressed vs. not/infrequently stressed) or during the fire (increased a lot vs. not/slightly affected), maternal history of asthma (yes vs no) and background $PM_{2.5}$ exposure. SHS exposure was defined as living in a household with a current smoker. Data on maternal stress during the fire was validated by repeating the questions at the clinic survey. Maternal history of asthma was defined as a positive answer to the question: "Has the study child's biological mother been told by a doctor that you/they have asthma?".

Background $PM_{2.5}$ exposure was assigned to the residential address of each participant from a validated satellite-based land use regression model of yearly average $PM_{2.5}$ for 2015, the most recent year for which data were available. The spatial distribution and concentrations of $PM_{2.5}$ show little variation from year to year in the region[149].

Information on respiratory medication used in the past 24 h and any cold or flu-like illnesses experienced in the past three weeks were obtained from the clinical survey.

Multiple imputation by chained equations (MICE)

MICE was performed using the "mice" package in R 3.5.0 under the assumption of missing at random (MAR)[126]. There were five participants (6.0%) having missing values on breastfeeding duration (n=1), maternal stress during the fire (n=1), birthweight (n=2) or maternal alcohol use during pregnancy (n=1). Participants with missing data were exposed to slightly higher levels of $PM_{2.5}$ during the fire period and had smaller Z scores for Rrs_5 and AX, and a less negative Z score for Xrs_5. Mothers of all the five participants with missing data did not drink alcohol or smoke tobacco during pregnancy, had no history of asthma and 80.0% had ≤12 years of education (Table S1).

We included all FOT outcome measures, average and peak $PM_{2.5}$ concentrations, all a priori selected covariates, children's age at the clinic, gender, weight and height in the imputation models to make the missing at random assumption plausible. We ran 20 imputations to reduce bias. There was evidence of deviation from normality for birthweight in our dataset (Anderson-Darling normality test p=0.044 with the estimates of skewness and kurtosis of -0.387 and 4.635, respectively). Therefore, we used predictive mean matching (PMM) to impute missing values of birthweight[150], while missing values of binary variables (i.e. breastfeeding duration, maternal stress during the fire and maternal alcohol use during pregnancy) were imputed using logistic regression models.

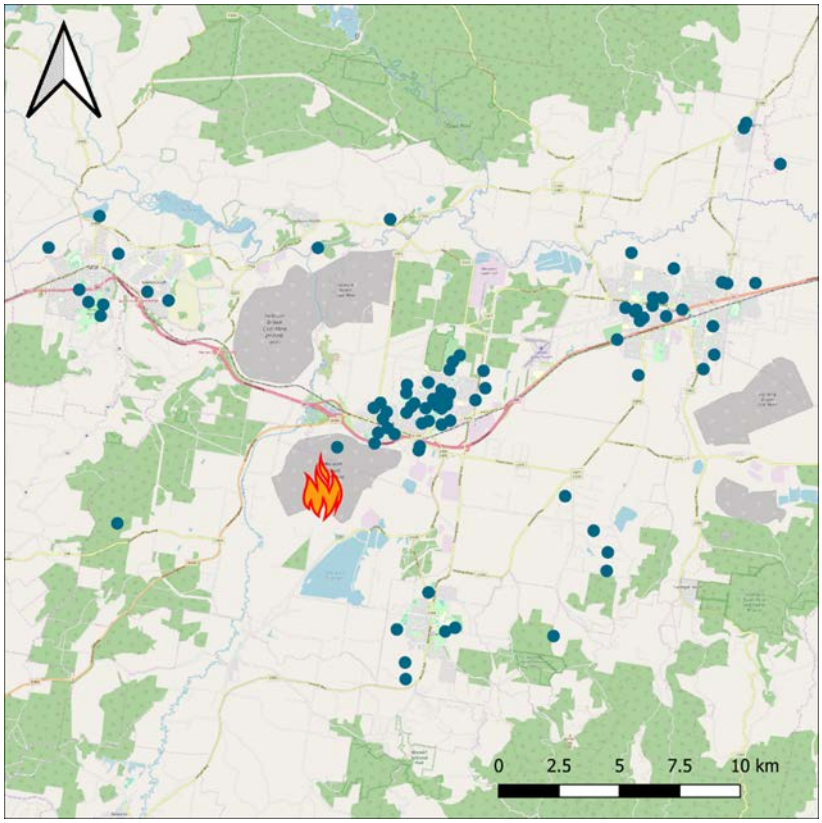

Figure S3-1. Map of the Latrobe Valley showing the approximate residential locations of the participants at the time of the fire.

Table S3-1. Comparison of characteristics between participants with and without missing data

	Complete cases (n=79)	Missing cases (n=5)
	Mean±SD	
Z(Rrs_5)	0.60±0.80	-0.03±0.64
Z(Xrs_5)	-0.77±0.90	-0.48±0.37
Z(AX)	0.74±0.94	0.43±0.49
Age at clinic (years)	4.3±0.5	4.9±0.4
Height (cm)	106.6±6.1	110.1±7.1
Weight (kg)	19.6±4.9	20.1±4.0
Gestational age (weeks)	39.5±1.9	40.2±1.3
Birthweight* (kg)	3.4±0.6	3.3±0.6
IRSD	3.4±2.9	3.0±1.6
Background $PM_{2.5}$ ($\mu g/m^3$)	6.8±0.9	6.5±1.1
	Median [IQR]	
Average $PM_{2.5}$ during the fire period ($\mu g/m^3$)	7.8 [6.9-16.8]	11.9 [6.8-14.4]
Peak $PM_{2.5}$ during the fire period ($\mu g/m^3$)	95.6 [60.1-153.3]	111.1 [89.0-115.1]
	n (%)	
Gender: boys	38 (48.1%)	3 (60.0%)
Maternal alcohol use during pregnancy*: yes	9 (11.4%)	0 (0.0%)
Maternal smoking during pregnancy: yes	15 (19.0%)	0 (0.0%)
Maternal education: secondary education or less	29 (36.7%)	4 (80.0%)
Maternal history of asthma: yes	22 (27.8%)	0 (0.0%)
Maternal stress during pregnancy: frequently stressed	11 (13.9%)	1 (20.0%)
Effect of coalmine fire on maternal stress*: 'increased a lot'	31 (39.2%)	0 (0.0%)
Second hand smoke exposure: yes	18 (22.8%)	2 (40.0%)
Breastfeeding: ≤3 months*	26 (32.9%)	1 (20.0%)
Respiratory medication use 24 hours before FOT testing: yes	11 (13.9%)	0 (0.0%)
Cold/flu-like illnesses in the past 3 weeks: yes	49 (62.0%)	3 (60.0%)

Abbreviations: SD, standard deviation; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve; IRSD, Index of Relative Socio-economic Disadvantage; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; IQR, interquartile range; FOT, forced oscillation technique. *Variables having missing values.

Table S3-2. Comparisons of socioeconomic characteristics between participants in our study and in the entire cohort

	The FOT cohort (n=84)	The entire cohort (n=571)	*P*-value
	Mean±SD		Welch t-test
Gestational age (weeks)	39.5±1.9	39.2±2.0	0.21
Birthweight (kg)	3.4±0.6	3.4±0.7	0.76
	n (%)*		Chi-square
Gender: boys	41 (48.8%)	294 (51.5%)	0.73
Maternal alcohol use during pregnancy: yes	9 (10.8%)	56 (10.1%)	0.98
Maternal smoking during pregnancy: yes	15 (17.9%)	102 (18.2%)	1.00
Primary carer' education: secondary education or less	29 (34.5%)	225 (39.5%)	0.45
Primary carer' smoking status: yes	14 (16.7%)	108 (18.9%)	0.73
Maternal stress during pregnancy: frequently stressed	12 (14.3%)	97 (17.4%)	0.58
Effect of coalmine fire on maternal stress: 'increased a lot'	31 (37.3%)	189 (34.4%)	0.69
Breastfeeding: ≤3 months	27 (32.5%)	221 (39.3%)	0.29

Abbreviations: FOT, forced oscillation technique; SD, standard deviation. *Participants with missing data were excluded.

Table S3-3. Test for effect modification by maternal smoking during pregnancy and gender

Multivariable analysis (n=84)	*P**		
	Z(Rrs_5)	Z(Xrs_5)	Z(AX)
Maternal smoking * average $PM_{2.5}$	0.13	0.15	0.19
Maternal smoking * peak $PM_{2.5}$	0.21	0.36	0.54
Gender * average $PM_{2.5}$	0.23	0.99	0.87
Gender * peak $PM_{2.5}$	0.26	0.75	0.93

Abbreviations: $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve. *Models adjusted for birthweight, gestational age, IRSD, background $PM_{2.5}$ exposure, breastfeeding duration, maternal alcohol use during pregnancy, maternal smoking during pregnancy, maternal education, maternal history of asthma, maternal stress during pregnancy or during the fire, secondhand smoke exposure, cold/flu-like illnesses in the past three weeks and respiratory medication use 24 h before FOT testing.

Table S3-4. Infant coal mine fire $PM_{2.5}$ exposure and lung function stratified by gender

Multivariable analysis (n=84)	β-coefficient* (95%CI)	
	Boys	Girls
Average $PM_{2.5}$ (10 μg/m^3 increase)		
Z (Rrs_5)	-0.04 (-0.43, 0.34)	0.05 (-0.34, 0.45)
Z (Xrs_5)	-0.23 (-0.58, 0.13)	0.02 (-0.35, 0.40)
Z (AX)	0.26 (-0.15, 0.68)	-0.00 (-0.45, 0.45)
Peak $PM_{2.5}$ (100 μg/m^3 increase)		
Z (Rrs_5)	-0.00 (-0.25, 0.24)	0.02 (-0.25, 0.30)
Z (Xrs_5)	-0.09 (-0.32, 0.14)	0.04 (-0.22, 0.30)
Z (AX)	0.21 (-0.05, 0.48)	-0.01 (-0.32, 0.30)

Abbreviations: CI, confidence interval; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve. *Models adjusted for birthweight, gestational age, IRSD, background $PM_{2.5}$ exposure, breastfeeding duration, maternal alcohol use during pregnancy, maternal smoking during pregnancy, maternal education, maternal history of asthma, maternal stress during pregnancy or during the fire, secondhand smoke exposure, cold/flu-like illnesses in the past three weeks and respiratory medication use 24 h before FOT testing.

Table S3-5. Infant coal mine fire $PM_{2.5}$ exposure and other covariates on lung function without multiple imputation

Multivariable analysis (n=79)	β-coefficient‡ (95%CI)		
	Z (Rrs_5)	Z (Xrs_5)	Z (AX)
Average $PM_{2.5}$ (10 μg/m^3 increase)	0.15 (-0.08, 0.37)	-0.20 (-0.43, 0.02)	0.29 (0.04, 0.55)*
Peak $PM_{2.5}$ (100 μg/m^3 increase)	0.08 (-0.08, 0.23)	-0.09 (-0.25, 0.07)	0.18 (0.00, 0.36)*

Abbreviations: CI, confidence interval; Z (Rrs_5), Z score for resistance at a frequency of 5 Hz; Z (Xrs_5), Z score for reactance at a frequency of 5 Hz; Z (AX), Z score for the area under the reactance curve; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers. ‡Models adjusted for birthweight, gestational age, IRSD, background $PM_{2.5}$ exposure, breastfeeding duration, maternal alcohol use during pregnancy, maternal smoking during pregnancy, maternal education, maternal history of asthma, maternal stress during pregnancy or during the fire, secondhand smoke exposure, cold/flu-like illnesses in the past three weeks and respiratory medication use 24 h before FOT testing. Average and peak $PM_{2.5}$ were modelled separately, and models included all covariates listed in the table. *$P<0.05$.

Chapter 4

Exposure to coal mine fire emissions during the First 1000 Days of Life and Subsequent Health Service and Medication Usage in Children

Chapter 4. Exposure to coal mine fire emissions during the First 1000 Days of Life and Subsequent Health Service and Medication Usage in Children

4.1 Preface

Chapter 3 used individual data to investigate the association between early life exposure to $PM_{2.5}$ from coal mine fire emissions and children's lung function. The effects of coal mine fire smoke exposure during *in utero* or infancy on respiratory and immune-related illnesses such as wheezing/asthma, allergy and infections are still unclear. In this Chapter, participants' records from the Latrobe ELF study were linked with the number of general practitioner (GP) consultations and medication dispensations collected from two national governmental databases in Australia: Medicare Benefits Schedule (MBS) and Pharmaceutical Benefits Scheme (PBS). This study aimed to evaluate the association between early life exposure (*in utero* and infant) to $PM_{2.5}$ from coal mine fire emissions and the frequency of GP attendances and dispensations of prescribed asthma inhalers, steroid skin creams, and antibiotics during the year following exposure. A modified version of this Chapter has been published in *Environmental Pollution.*

4.2 Introduction

The first thousand days of life, including the periods *in utero* and the first two years after birth, is recognised as a critical window for the development and growth of the respiratory and immune systems [151]. There is emerging evidence that air pollution exposure during this period could result in adverse immunological or respiratory outcomes later in life. For example, previous meta-analyses have demonstrated that early life exposure to traffic-related air

pollution is associated with the development of childhood asthma and allergic diseases [29, 152]. Intrauterine exposure to both $PM_{2.5}$ and second-hand smoke (SHS) has been associated with increased risk of infantile eczema [43]. Epidemiological studies have also shown significant associations between air pollution exposure *in utero* or during the first year of life and childhood pneumonia, bronchiolitis and ear infections [30, 153-154], further highlighting potential susceptibility during this period. Exposure to air pollution prompts immediate immune responses [155-156] and can modulate later immune expression [154, 157]. It is therefore plausible that short-term exposure to air pollution in the critical first 1000 days of life, from conception to age 2 years, could affect later immunological function [158]. However very few studies have evaluated this.

Smoke from outdoor landscape fires including burning forest, grass and peat makes a significant contribution to air pollution [5] and is an increasing global concern due to the rising frequency and severity of fires resulting from climate change [6]. Epidemiological studies suggest that smoke exposure is associated with short-term increases in medication usage, physician/emergency department visits, hospitalisations and death [112, 159]. However, evidence of the health outcomes following early life exposure to short-to-medium duration smoke events (i.e. weeks) is extremely limited [112, 160].

Embers from a bushfire in the Latrobe Valley region of Victoria, Australia, ignited a fire in an open cast coal mine located close to several rural towns in February 2014 that lasted for about 45 days. The episode resulted in dramatically increased concentrations of $PM_{2.5}$. The peak daily average $PM_{2.5}$ concentration reached 731 $\mu g/m^3$ in the closest town, Morwell, which is substantially higher than the national daily air quality standard of 25 $\mu g/m^3$ [2, 161]. One of the main concerns of the community during this period was the possible risks to their long-term health. As there was little existing evidence to draw on, the state government initiated a long-

term study, the Hazelwood Health Study, to investigate the health and wellbeing of adults and children affected by the smoke episode [162].

We hypothesised that exposure to air pollution from the coal mine fire during the intrauterine or infant periods would increase the risk of common allergic or infective illnesses in the year following exposure. The aim of this study was to test if exposure to smoke from the coal mine fire during the first 1000 days of life was associated with increased physician visits or dispensing of medications used to treat infections, asthma or atopic skin conditions.

4.3 Materials and Methods

4.3.1 Study design

We linked data from a cohort of children recruited to the Latrobe Early Life Follow-up (ELF) Study [162] to two national Australian administrative health datasets: the Medicare Benefits Schedule (MBS) and the Pharmaceutical Benefits Scheme (PBS). Data were extracted by the Australian Department of Human Services for the period from each child's date of birth to 31/12/2016. The MBS dataset contained de-identified information on claims to Medicare, the national insurance system, for out-of-hospital health services such as visits to GPs and specialists. The PBS dataset contained de-identified information on prescription medications dispensed to patients. It captured medication dispensations that were subsidised by the Australian government.

The Latrobe ELF cohort comprises 571 children born between 01/03/2012 and 31/12/2015, who were recruited from the Latrobe Valley, Victoria, during 2016 as part of a long-term follow-up study of the health impacts of the 2014 Hazelwood coal mine fire [163]. Details of this cohort are described elsewhere [162]. Sociodemographic, health and residential characteristics of the participants (n=571) were obtained from a baseline questionnaire completed by

parents/carers at enrolment. Parental consent for linkage with MBS and PBS datasets was obtained from 311 participants. We recruited four groups of participants according to their dates of birth and gestational age at delivery. These were: (1) the intrauterine exposure group, which comprised children whose mothers were pregnant with them during the period of the mine fire; (2) the infant exposure group which comprised children who were aged between 0-2 years during the entire fire period; (3) the mixed exposure group, who were born during the fire period; and (4) an unexposed group, who were conceived and born in the year following the fire. Children in the mixed exposure group (n=25) were not included in our primary analysis. The annual average $PM_{2.5}$ concentration during the year 2015 when most of the unexposed children were born was 6.7 μg m^{-3}[164], therefore, the unexposed children had been exposed to very low levels of environmental $PM_{2.5}$ during their perinatal periods.

The Tasmanian Health and Medical Human Research Ethics Committee (reference H14875) approved this study. Additional approval was received from the Human Research Ethics Committees of Monash University, Monash Health, and the University of Melbourne. All parents or caregivers of the studied participants provided signed consent forms for accessing data from the MBS/PBS datasets.

4.3.2 Exposure estimates

Hourly coal mine fire-specific $PM_{2.5}$ concentrations during the time of the fire (09/02/2014-31/03/2014) were estimated using meteorological, dispersion and chemical transport modelling at a spatial resolution of 1×1 km. Details of the methods used in generating the modelled exposure estimates have been previously reported [165]. The full model included $PM_{2.5}$ from natural sources, traffic, power stations, landscape fires and mine fire emissions. The differences between the model run with, and without, mine fire emissions were used to estimate the concentration of mine fire-specific $PM_{2.5}$. The magnitude of the modelled $PM_{2.5}$ matched reasonably well with the observed $PM_{2.5}$ concentrations, but the exact timing of the peak values

was less accurate on an hourly basis [165]. Therefore, we calculated individual 24-hour average and the peak hourly value of 24-hour $PM_{2.5}$ concentrations during the exposure period, based on air pollution concentrations at participants' day and night locations from baseline questionnaires. Those children conceived after the mine fire were allocated a mine fire-specific $PM_{2.5}$ concentration of zero.

We also assessed each child's exposure to annual average nitrogen dioxide (NO_2) concentrations in order to adjust for the effects from longer-term exposures to background non-fire sources of air pollution particularly from motor vehicles and power stations. Annual ambient nitrogen dioxide (NO_2) concentrations for the years 2011 to 2015 were estimated using a national satellite-based land-use regression (LUR) model [166] at 'mesh blocks', the smallest spatial unit in the Australian census (n = ~347,000 nationally) [167]. In an external validation, the LUR model explained 66% of spatial variation in NO_2 at traffic-influenced and background sites (RMSE: 2 ppb [25%]) [168]. We assigned birth year NO_2 estimates to the participants according to their home addresses at birth.

4.3.3 Outcome definition

Health outcomes of interest were decided *a priori*, including GP attendances, dispensations of prescribed asthma inhalers, steroid skin creams and antibiotics during the first year of life, or the year following the fire. We analysed all MBS claims relating to consultations with a GP and PBS records of dispensations of prescribed medications used to treat asthma, atopic dermatitis, and bacterial infections (Table S4-1, S4-2, S4-3 and Table S4-4, Appendix 4).

Evaluation of outcomes in intrauterine exposure analysis

For intrauterine exposure analysis, we included children in the intrauterine exposure group (birthdate: 01/04/2014-31/12/2014) and all children who were not exposed to mine fire smoke in their first year of life. Unexposed children included the unexposed group who were

conceived and born after the fire (birthdate: 01/01/2015-31/12/2015), and also those from the infant exposure group who were not exposed to mine fire smoke until their second year of life (birthdate: 01/03/2012-09/02/2013). Our main outcome measures for intrauterine exposure analysis were restricted to the first year of life.

Evaluation of outcomes in infant exposure analysis

This analysis included children aged 0-2 years at the time of the fire, and the unexposed group of children born during 2015. For the infant exposure group, we evaluated outcomes in the year following the fire from 01/04/2014 to 31/03/2015 and for the comparison group we evaluated outcomes in the year from 01/01/2016 to 31/12/2016.

4.3.4 Covariates

We selected a list of potential confounders and effect modifiers *a priori* using a directed acyclic graph in DAGitty [169-170]. Potential covariates were selected according to the existing literature on air pollution and child health [171-176]. We included age (months), sex, maternal tobacco smoking status during pregnancy (yes vs. no), SHS exposure (yes vs. no), maternal prenatal stress (frequently stressed vs. not/infrequently stressed), birth year nitrogen dioxide (NO_2) exposure and socio-economic status (SES) indicated by both maternal education (≤year 12 vs. post-secondary) and the Socio-economic Index (IRSD) deciles within Victoria [177]. The IRSD measures the relative socio-economic disadvantage of people and households within an area. A low score indicates greater disadvantage or lower SES. SHS exposure status was determined by whether there was a regular smoker in the child's house at baseline.

4.3.5 Statistical analysis

Intrauterine and infant exposure analysis were conducted separately. Negative binomial regression models were used to assess the associations between 10 or 100 μg/m^3 increases in average and peak $PM_{2.5}$ exposure, respectively, prenatally or postnatally, and GP attendances,

and dispensations of prescribed asthma inhalers and antibiotics, with and without adjustment for covariates. The association between mine fire $PM_{2.5}$ exposure and dispensations of steroid skin creams was assessed using logistic regression models by defining the outcome as a binary variable due to the low frequency (0.2 per child per year during the first year of life and the year following the fire) in the participants. Maternal prenatal stress was excluded from these models, as the models failed to converge because of complete or quasi-complete separation (i.e. low or no maternal prenatal stress perfectly predicted the outcomes). Possible effect modification by sex was evaluated by adding an interaction term in the multivariable models. Multiple imputation by chained equations was employed to estimate missing covariates values (n=4 for both intrauterine and infant exposure analysis) by generating 20 independent datasets [126]. Imputation models included exposure, all covariates, maternal stress during the fire and outcome variables. All statistical analyses were performed in R 3.5.3 (the R Foundation) [178] via RStudio, and a *p* value <0.05 was considered statistically significant.

4.4 Results

4.4.1 Participant characteristics

Parents/carers of 311 (54.5%) children from the full Latrobe ELF cohort (n=571) consented to be linked to the MBS/PBS datasets. There were 88 children in the intrauterine exposure group, 77 in the no exposure group, 121 in the infant exposure group, and 25 children born during the fire period. Therefore, 218 children were included in the intrauterine exposure analysis, while 198 were included in the infant exposure analysis.

In the intrauterine exposure analysis, no statistically significant differences were observed between exposed and non-exposed children for ambient NO_2 exposure, or across sex, tobacco smoke exposure, SES and maternal prenatal stress (Table 4-1; $p>0.05$ for all comparisons). In

contrast, children in the infant exposure group were, on average, older by approximately 4.6 months (Table 4-2; $p<0.05$) than those in the no exposure group. The other covariates were approximately equally distributed across different groups (Table 4-2; $p>0.05$ for all comparisons). Exposure to mine fire $PM_{2.5}$ was higher in the infant exposure group than in the intrauterine exposure group (Table 4-1, Table 4-2).

Overall, a higher proportion of well-educated (i.e. post-secondary) (67.8%) and non-smoking (87.1%) primary carers of the children were included in this study compared with the full ELF cohort (Table S4-5, Appendix 4).

4.4.2 GP visits and medication use by exposure groups

The frequencies of GP attendances, and dispensations of prescribed asthma inhalers, steroid skin creams and antibiotics were generally low among all participants (Table 4-3). No significant differences were observed between exposed and non-exposed children in the intrauterine exposure analysis (Table 4-3; $p>0.05$ for all comparisons). In the infant exposure analysis, the average rate of antibiotic prescribing was approximately double in the group exposed compared with those not exposed (1.5 vs. 0.8, $p<0.05$), but there was a lower frequency of prescribed steroid cream dispensations (0.1 vs. 0.4, $p<0.05$) in the exposed children during the one year follow up period (Table 4-3).

4.4.3 Associations between mine fire smoke exposure and health outcomes

For intrauterine exposure analysis, univariable and multivariable regression analyses did not show any significant associations between intrauterine mine fire $PM_{2.5}$ exposure and any of the outcomes (Table 4-4, Table 4-5).

For infant exposure analysis, univariable analyses suggested that mine fire $PM_{2.5}$ exposure (continuous variable) was associated with increased antibiotic dispensations during the follow-up year (Table 4-6). After adjusting for potential confounders, every 10 $\mu g/m^3$ increase in

average $PM_{2.5}$ exposure during infancy were associated with increased incidence of antibiotics being dispensed during the year following the fire: adjusted incidence rate ratio (IRR) 1.24 (95%CI, 1.02, 1.50; $p<0.05$). Every 100 μg/m^3 increase in peak $PM_{2.5}$ during infancy was also associated with an increase in antibiotic dispensations (IRR 1.14, 95%CI 1.00, 1.31; $p<0.05$). Similar associations were not found for other outcomes (Table 4-7).

There was no evidence of effect modification by sex in either the intrauterine or infant exposure analyses (Table S4-6, Appendix 4; interaction $p>0.05$ for all analyses).

4.5 Discussion

To our knowledge, this study provides the first evidence that infant exposure to increased $PM_{2.5}$ derived from coal mine fire emissions over a medium duration was associated with increased dispensations of antibiotics during the year following the fire. The association was independent of potential confounders including age of the child, tobacco smoke exposure, socio-economic status and background air pollution exposure. In contrast, we did not observe significant associations for other outcomes among infants (frequency of GP attendances or the usage of medications for asthma or allergic skin conditions), nor did we observe effects of *in utero* exposure to fire smoke with any of the outcomes during the first year of life.

Our finding of an increase in antibiotic use after mine fire smoke exposure during infancy is similar to an American study evaluating the associations between short-term increases in ambient $PM_{2.5}$ concentrations and respiratory infections in young children aged 0-2 years [179]. The authors of the American study suggested that every 10 μg/m^3 increase in $PM_{2.5}$ concentration was associated with a 15% (95% CI, 12%, 19%) greater odds of healthcare encounters for acute lower respiratory infections one month following the exposure. We are not aware of previous epidemiological studies investigating the immune effects of perinatal

exposure to fire smoke. However, animal and cell line studies have shown that perinatal exposure to particles from landscape fire emissions induces oxidative stress and inflammation, resulting in immune dysregulation and increased susceptibly to respiratory infections [52, 180-181].

Our study did not observe any significant associations between *in utero* fire smoke exposure and antibiotic usage during the first year of life. This is inconsistent with current evidence regarding intrauterine air pollution exposure and childhood respiratory infections. For example, a Polish study suggested a dose-response relationship between *in utero* $PM_{2.5}$ exposure levels and the incidence of recurrent respiratory infections (≥5 episodes of bronchitis and/or pneumonia) from birth to age 7 (OR 2.44; 95%CI, 1.12, 5.36) [182]. Another study suggested that intrauterine exposure to traffic-related air pollution, estimated by proximity to a major roadway and traffic density, was associated with increased risk of childhood respiratory infection [154]. This inconsistency might be due to the relatively short duration of exposure in our study compared with the Polish study, the different ages of the children at the time of follow-up, and the different chemical composition and toxicological properties of $PM_{2.5}$ from the fire emissions and urban sources [53]. It is also worth noting that although a proportion of our study participants were exposed to very high concentrations of mine fire $PM_{2.5}$, the average $PM_{2.5}$ concentrations in our study were much lower than the cut-off points (2.8 vs. 26.6 and 45.9 μg m^{-3}) used in the Polish study.

Our study did not observe any significant associations between either intrauterine or infant fire smoke exposures and asthma inhaler dispensations by age 3. There is very limited evidence regarding the long-term risk of childhood wheezing or asthma after perinatal exposure to severe, medium duration air pollution events. The only comparable study investigated the association between early life exposure to the Great Smog of 1952 in London and childhood asthma assessed by self-reported diagnosis from birth to age 15. That study suggested that children exposed to the Great Smog during infancy had increased risk of childhood asthma by 19.87

percent (95%CI, 3.37, 36.38) compared with those conceived before or after the event and those living beyond the affected area. *In utero* smog exposure was not associated with asthma development [113]. The inconsistent results for infant exposure between the Great Smog study and ours could be due to the different data collection methods, the challenges of asthma diagnoses in preschool children [183] and our participants who were exposed during infancy had a mean age of 2.0 years during the year of followed up. A harvesting effect might also exist due to increased deaths from the Great Smog.

Using asthma medication prescription as an indicator of asthma diagnosis might underestimate asthma incidence as many asthma inhalers can be purchased without a prescription. Further, while prescription data can be a good proxy for the diagnosis of many diseases such as asthma [184], we were not able to directly evaluate diagnoses among the cohort. It will be important to continue to monitor these outcomes in our participants to further explore any potential associations.

The observed increase in the dispensing of antibiotics might represent an increase in infections commonly managed with antibiotics, or it could reflect a lower threshold for prescribing antibiotic by doctors in the year following the fire, or an increase in parental concern associated with a greater number of requests for antibiotics. However, the large effect size, the unchanged rate of doctor attendances in the year following the fire, and the lack of association with antibiotic prescribing in the first year of life in children who were exposed *in utero*, all suggest that doctor or parental health seeking behaviour did not appreciably change and these factors are unlikely to explain the association we observed.

A strength of the study is that we estimated individual $PM_{2.5}$ exposure adjusting for residential histories and activity patterns for each participant during the fire period, and we were able to adjust for exposure to background air pollution using modelled estimates of annual non-fire

related NO_2. This could reduce exposure measurement error. A previous study reported that ignoring residential mobility when estimating traffic-related air pollution exposure caused a modest bias of the associations towards the null [185]. In addition, we used multiple imputations to minimise the bias from missing data, and loss of power associated with reductions in sample size [139].

However, we acknowledge some limitations in this study. First, our sample of 286 participants was relatively small and this limited the power of our analyses to detect significant associations, especially those of small magnitude. A small sample may also affect the generalisability of our study because it was not completely representative of the wider population. Relative to the local population, a higher proportion of children with well-educated and non-smoking parents were recruited [162] and included in our study. Our results could be an underestimate of the impacts which might be expected in a population with a higher prevalence of smoking and social disadvantage. Second, exposure misclassification and recall error may have occurred due to the subjective measurement of participants' locations during the fire period for which we relied on parental reports. However, most respondents reported that they were confident of their recall and eyewitness studies have suggested a strong correlation between measures of confidence and accuracy of recall [186]. In addition, the exposure estimate modelling we used could not capture the impact of home air conditioning systems on personal exposures. Personalized monitoring devices can be more accurate but not feasible to deploy during a public health emergency such as this coal mine fire. Third, the use of medication dispensation data from PBS datasets as indicators of childhood illnesses may introduce measurement error. The MBS/PBS datasets only recorded the histories of medical service usage and medication dispensations that were covered by the Australian government, so asthma inhalers purchased without a medical prescription are not included in this analysis [187-188]. Furthermore, seasonal variations in circulating pathogens may impact on antibiotic prescription. However, the effect

size (i.e. around 24% increase) was large enough to suggest a possible association between infant coal mine fire smoke exposure and increased childhood infections. In addition, our results might be influenced by residual confounding. However, we adjusted for the most important factors including tobacco smoke exposure, SES and background air pollution exposure.

4.6 Conclusions

In conclusion, our study suggested that infant exposure to a short-term severe air pollution event was associated with increased childhood antibiotic dispensations, which might reflect increased childhood infections. Future follow-up of the participants will be necessary to confirm these findings and evaluate long-term effects.

Table 4-1. Comparison of participant characteristics between groups in the intrauterine exposure analysis

Characteristics	Intrauterine exposure group (n=88)	No exposure group (n=130)	
	Median [IQR]		P*
IRSD deciles within Victoria	3 [1, 5]	3 [1, 7]	0.84
Birth year NO_2 exposure (ppb)	3.9 [3.4, 4.4]	4.0 [3.3, 4.6]	0.88
Average mine fire $PM_{2.5}$ ($\mu g/^{-3}$)	2.8 [1.6, 7.8]	0.0 [0.0, 0.0]	-
Peak mine fire $PM_{2.5}$ ($\mu g/m^3$)	76.7 [49.7, 162.3]	0 [0.0, 0.0]	-
	n (%)		P†
Sex: male	39 (44.3%)	64 (49.2%)	0.48
Maternal smoking during pregnancy: yes	9 (10.2%)	23 (17.7%)	0.13
Secondhand smoke exposure§: yes	16 (18.2%)	27 (20.9%)	0.62
Maternal prenatal stress‡: frequently stressed	16 (18.4%)	20 (15.5%)	0.58
Maternal education§: ≤year 12	28 (31.8%)	47 (36.4%)	0.48

Note: IQR, interquartile range; IRSD, Index of Relative Socio-economic Disadvantage; NO_2, nitrogen dioxide.

*Mann-Whitney U test. †Pearson's chi-square test. §Having missing values (n=1). ‡Having missing values (n=2).

Table 4-2. Comparison of participant characteristics between groups in the infant exposure analysis

Characteristics	Infant exposure group (n=121)	No exposure group (n=77)	
	Median [IQR]		P*
Age at the start of outcome year (months)	11.6 [7.1, 17.0]	7.0 [3.4, 9.3]	0.00
IRSD deciles within Victoria	2 [1, 5]	3 [1, 8]	0.07
Birth year NO_2 exposure (ppb)	4.3 [3.6, 4.9]	3.8 [3.2, 4.3]	0.00
Average mine fire $PM_{2.5}$ ($\mu g/m^3$)	6.8 [2.0, 13.6]	0.0 [0.0, 0.0]	-
Peak mine fire $PM_{2.5}$ ($\mu g/m^3$)	106.5 [53.1, 195.8]	0 [0.0, 0.0]	-
	n (%)		P†
Sex: male	63 (52.1%)	36 (46.8%)	0.47
Maternal smoking during pregnancy: yes	21 (17.4%)	11 (14.3%)	0.57
Secondhand smoke exposure§: yes	29 (24.2%)	14 (18.4%)	0.34
Maternal prenatal stress‡: frequently stressed	18 (14.9%)	9 (11.8%)	0.55
Maternal education‡: ≤year 12	43 (35.8%)	28 (36.4%)	0.94

Note: IQR, interquartile range; IRSD, Index of Relative Socio-economic Disadvantage; NO_2, nitrogen dioxide.

*Mann-Whitney U test. †Pearson's chi-square test. §Having missing values (n=2). ‡Having missing values (n=1).

Table 4-3. Frequency of health services and medication usage in exposed and non-exposed children

Intrauterine exposure group	Mean (per child/year)		
	Exposure group (during the first year of life)	No exposure group (during the first year of life)	P*
GP attendances	8.0	7.8	0.94
Prescribed asthma inhalers	0.4	0.3	0.60
Steroid skin creams	0.1	0.3	0.19
Antibiotics	0.8	0.7	0.25
Postnatal exposure group	Exposure group (01/04/2014-01/04/2015)	No exposure group (01/01/2016-31/12/2016)	
GP attendances	6.9	7.8	0.18
Prescribed asthma inhalers	0.7	0.4	0.20
Steroid skin creams	0.1	0.4	**0.01**
Antibiotics	1.5	0.8	**0.00**

Note: GP, general practitioner. *Mann-Whitney U test. Significant results are shown in **bold**.

Table 4-4. Univariable analysis of intrauterine mine fire $PM_{2.5}$ exposure, risk factors and health outcomes during the first year of life

Univariable analysis (n=218)	GP attendances		Dispensations of prescribed asthma inhalers		Dispensations of steroid skin creams		Dispensations of antibiotics	
	IRR (95%CI)	P	IRR (95%CI)	P	OR (95%CI)	P	IRR (95%CI)	P
Average $PM_{2.5}$ (per 10 $\mu g/m^3$ increase)	1.00 (0.85, 1.18)	1.00	0.85 (0.42, 1.69)	0.64	1.10 (0.53, 2.27)	0.80	1.20 (0.81, 1.78)	0.36
Peak $PM_{2.5}$ (per 100 $\mu g/m^3$ increase)	1.00 (0.92, 1.08)	0.93	0.99 (0.72, 1.36)	0.95	0.98 (0.67, 1.43)	0.93	1.07 (0.88,1.29)	0.50
Maternal education: ≤year 12	1.13 (0.94, 1.35)	0.20	0.99 (0.48, 2.03)	0.98	0.94 (0.40, 2.22)	0.89	**1.68 (1.08, 2.62)**	**0.02**
Maternal tobacco smoking status during pregnancy: yes	1.02 (0.80, 1.31)	0.85	1.20 (0.47, 3.07)	0.70	0.70 (0.20, 2.47)	0.58	**1.78 (1.01, 3.12)**	**0.05**
Second hand smoke exposure: yes	0.89 (0.72, 1.11)	0.32	0.75 (0.31, 1.84)	0.53	0.91 (0.32, 2.56)	0.86	1.28 (0.75, 2.19)	0.36
Maternal prenatal stress: frequently stressed	1.13 (0.90, 1.42)	0.29	1.73 (0.74, 4.04)	0.21	0.37 (0.08, 1.64)	0.19	1.52 (0.87, 2.64)	0.14
IRSD	0.97 (0.95, 1.00)	0.08	0.99 (0.88, 1.12)	0.91	1.12 (0.98, 1.27)	0.09	**0.91 (0.84, 0.98)**	**0.02**
Background NO_2 exposure	1.02 (0.97, 1.07)	0.50	0.96 (0.76, 1.20)	0.69	0.68 (0.42, 1.08)	0.11	**1.16 (1.04, 1.30)**	**0.01**

Note: GP, general practitioner; IRR, incidence rate ratio; CI, confidence interval; OR, odds ratio; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; IRSD, Index of Relative Socio-economic Disadvantage; NO_2, nitrogen dioxide. Significant results are shown in **bold**.

Table 4-5. Mine fire smoke exposure during pregnancy and health outcomes during the first year of life

Multivariable analysis (n=218)	GP attendances		Dispensations of prescribed asthma inhalers		Dispensations of steroid skin creams		Dispensations of antibiotics	
	IRR* (95%CI)	P	IRR* (95%CI)	P	OR* (95%CI)	P	IRR* (95%CI)	P
Average $PM_{2.5}$ (per 10 $\mu g/m^3$ increase)	1.00 (0.85, 1.18)	0.99	0.87 (0.45, 1.71)	0.69	1.26 (0.57, 2.77)	0.57	1.16 (0.80, 1.68)	0.43
Peak $PM_{2.5}$ (per 100 $\mu g/m^3$ increase)	1.00 (0.93, 1.08)	0.95	1.01 (0.74, 1.37)	0.97	1.00 (0.68, 1.46)	0.99	1.08 (0.90,1.31)	0.39

Note: GP, general practitioner; IRR, incidence rate ratio; CI, confidence interval; OR, odds ratio; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers. *Models adjusted for maternal education, index of relative socio-economic disadvantage, maternal smoking during pregnancy, secondhand smoke exposure, maternal prenatal stress and background nitrogen dioxide exposure.

Table 4-6. Univariable analysis of infant mine fire $PM_{2.5}$ exposure, risk factors and health outcomes during the year following the fire

Univariable analysis (n=198)	GP attendances		Dispensations of prescribed asthma inhalers		Dispensations of steroid skin creams		Dispensations of antibiotics	
	IRR (95%CI)	P	IRR (95%CI)	P	OR (95%CI)	P	IRR (95%CI)	P
Average $PM_{2.5}$ (per 10 µg/m^3 increase)	0.99 (0.89, 1.10)	0.79	1.19 (0.92, 1.54)	0.20	0.70 (0.36, 1.34)	0.28	**1.22 (1.03, 1.44)**	**0.02**
Peak $PM_{2.5}$ (per 100 µg/m^3 increase)	0.98 (0.91, 1.06)	0.58	1.12 (0.94, 1.34)	0.22	0.67 (0.40, 1.15)	0.15	**1.14 (1.01,1.28)**	**0.03**
Age (per month)	**0.98 (0.96, 0.99)**	**0.00**	1.02 (0.98, 1.06)	0.33	**0.88 (0.80, 0.98)**	**0.02**	1.01 (0.98, 1.04)	0.56
Maternal education: ≤year 12	1.11 (0.91, 1.37)	0.31	1.36 (0.80, 2.31)	0.26	0.38 (0.12, 1.19)	0.10	**1.44 (1.01, 2.05)**	**0.04**
Maternal tobacco smoking status during pregnancy: yes	0.89 (0.68, 1.17)	0.42	1.36 (0.69, 2.66)	0.38	0.24 (0.03, 1.82)	0.17	1.02 (0.63, 1.63)	0.94
Second hand smoke exposure: yes	0.82 (0.65 1.05)	0.12	0.81 (0.41, 1.63)	0.56	0.34 (0.08, 1.54)	0.16	1.21 (0.80, 1.83)	0.36
Maternal prenatal stress: frequently stressed	0.97 (0.73, 1.30)	0.84	1.48 (0.73, 3.01)	0.28	-	-	0.92 (0.55, 1.54)	0.75
IRSD	0.98 (0.95, 1.01)	0.17	0.95 (0.86, 1.03)	0.22	1.01 (0.87, 1.17)	0.91	0.98 (0.92, 1.04)	0.50
Birth year NO_2 exposure (per ppb)	1.02 (0.97, 1.08)	0.40	1.06 (0.92, 1.21)	0.42	0.99 (0.76, 1.29)	0.95	1.05 (0.95, 1.15)	0.34

Note: GP, general practitioner; IRR, incidence rate ratio; CI, confidence interval; OR, odds ratio; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers; IRSD, Index of Relative Socio-economic Disadvantage; NO_2, nitrogen dioxide. Significant results are shown in **bold**.

Table 4-7. Mine fire smoke exposure in infancy and health outcomes during a one-year period after the fire

Multivariable analysis (n=198)	GP attendances		Dispensations of prescribed asthma inhalers		Dispensations of steroid skin creams		Dispensations of antibiotics	
	IRR* (95%CI)	P	IRR* (95%CI)	P	OR† (95%CI)	P	IRR* (95%CI)	P
Average $PM_{2.5}$ (per 10 $\mu g/m^3$ increase)	0.96 (0.85, 1.09)	0.55	1.16 (0.86, 1.57)	0.34	0.66 (0.31, 1.38)	0.27	**1.24 (1.02, 1.50)**	**0.04**
Peak $PM_{2.5}$ (per 100 $\mu g/m^3$ increase)	0.96 (0.89, 1.05)	0.38	1.08 (0.88, 1.33)	0.46	0.65 (0.37, 1.14)	0.14	**1.14 (1.00,1.31)**	**0.05**

Note: GP, general practitioner; IRR, incidence rate ratio; CI, confidence interval; OR, odds ratio; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers. *Models adjusted for age, maternal education, index of relative socio-economic disadvantage, maternal smoking during pregnancy, secondhand smoke exposure, maternal prenatal stress and background nitrogen dioxide exposure. †Models adjusted for age, maternal education, index of relative socio-economic disadvantage, maternal smoking during pregnancy, secondhand smoke exposure and background nitrogen dioxide exposure. Significant results are shown in **bold**.

4.7 Appendix 4

Table S4-1. Medicare Benefit Schedule items for general practitioner attendances

Table S4-2. Pharmaceutical Benefits Scheme items for the dispensations of prescribed asthma medications

Table S4-3. Pharmaceutical Benefits Scheme items for the dispensations of steroid skin creams

Table S4-4. Pharmaceutical Benefits Scheme items for the dispensations of antibiotics

Table S4-5. Comparisons between participants in the study and the full cohort

Table S4-6. Effect modification by sex in intrauterine and infant exposure analysis

Table S4-1. Medicare Benefit Schedule items for general practitioner attendances

GP attendances	Items
A1 - General Practitioner Attendances To Which No Other Item Applies	3, 23, 24, 36, 37, 44
A2 - Other Non-Referred Attendances To Which No Other Item Applies	53, 54, 57
A11 - Urgent Attendance After Hours	597, 599
A13 - Public Health Physician Attendances To Which No Other Item Applies	411
A14 - Health Assessments	705
A15 - GP Management Plans, Team Care Arrangements, Multidisciplinary Care Plans	721, 723, 732
A22 - General Practitioner After-Hours Attendances To Which No Other Item Applies	5000, 5020, 5040
A23 - Other Non-Referred After-Hours Attendances To Which No Other Item Applies	5203, 5208

Table S4-2. Pharmaceutical Benefits Scheme items for the dispensations of prescribed asthma medications

Category	Item	Drug name
Adrenergic for systemic use	01103C	Salbutamol
Adrenergic, inhalants	02000G, 08288F	Salbutamol
Other drugs for obstructive airway diseases, inhalants	08345F, 08516F	Fluticasone
Other drugs for obstructive airway diseases, inhalants	08853Y	Ciclesonide
Other drugs for obstructive airway diseases, inhalants	08671J	Ipratropium
Other systemic drugs for obstructive airway diseases	08627C	Montelukast
Corticosteroids for systemic use, plain	01499X	Hydrocortisone
Corticosteroids for systemic use, plain	08285C	Prednisolone sodium phosphate

Table S4-3. Pharmaceutical Benefits Scheme items for the dispensations of steroid skin creams

Category	Item	Drug name
Corticosteroids, plain	01115Q, 01119X	Betamethasone dipropionate
Corticosteroids, plain	02812B, 02813C	Betamethasone valerate
Corticosteroids, plain	01913Q, 08043H, 01915T	Mometasone
Corticosteroids, plain	02117K, 02118L	Triamcinolone
Corticosteroids, plain	02881P, 02882Q, 02887Y, 02888B	Hydrocortisone acetate
Corticosteroids, plain	08054X, 08055Y, 08128T, 08618N	Methylprednisolone
Other dermatological preparations	08802G	Pimecrolimus

Table S4-4. Pharmaceutical Benefits Scheme items for the dispensations of antibiotics

Category	Item	Drug name
Sensory organs		
Antiinfectives	01440T	Framycetin sulfate
Otologicals		
antiinfectives	02480M	Ciprofloxacin
Corticosteroids and antiinfectives in combination	02781J	Framycetin sulfate+gramicidin+dexamethasone
Corticosteroids and antiinfectives in combination	02971J, 02974M	Triamcinolone+neomycin sulfate+gramicidin+nystatin
Antiinfectives for systemic use		
Beta-lactam antibacterials, penicillins	01886G, 01887H, 01888J, 01889K, 08705E	Amoxicillin
Beta-lactam antibacterials, penicillins	01892N, 08319W	Amoxicillin+clavulanic acid
Beta-lactam antibacterials, penicillins	08976K, 08977L, 09143F	Phenoxymethylpenicillin
Beta-lactam antibacterials, penicillins	09149M, 09150N	Flucloxacillin
Other beta-lactam antibacterials	02460L, 02461M	Cefaclor
Other beta-lactam antibacterials	03094W, 03095X, 03119E	Cefalexin
Other beta-lactam antibacterials	05499K	Cefuroxime
Macrolides, lincosamides and streptogramins	02424N, 02428T	Erythromycin ethylsuccinate
Macrolides, lincosamides and streptogramins	08129W	Roxithromycin
Macrolides, lincosamides and streptogramins	08201P	Azithromycin
Macrolides, lincosamides and streptogramins	09192T	Clarithromycin
Other antibacterials	01630T	Metronidazole
Sulfonamides and trimethoprim	03103H	Trimethoprim+sulfamethoxazole

Table S4-5. Comparisons between participants in the study and the full cohort

Characteristics	Participants in this study (n=311)	Full cohort (n=571)	
	Median [IQR]		P[a]
Age at 31/12/2016 (months)	31.8 [24.6, 42.3]	30.3 [22.8, 40.6]	0.14
Birthweight (kg)	3.5 [3.1, 3.8]	3.5 [3.0, 3.8]	0.74
Gestational age (weeks)	40 [38, 41]	40 [38, 41]	0.72
	n (%)		P[b]
Sex: male	152 (48.9%)	294 (51.5%)	0.46
Delivery mode: caesarean section	94 (30.2%)	172(30.4%)	0.95
Maternal smoking during pregnancy: yes	42 (13.5%)	102 (18.2%)	0.07
Primary carer smoking status: yes	40 (12.9%)	108 (18.9%)	0.02
Maternal prenatal stress: frequently stressed	44 (14.3%)	97 (17.4%)	0.24
Primary carer education: ≤year 12	100 (32.2%)	225 (39.5%)	0.03
Breastfeeding duration: ≤3 months	112 (36.4%)	221 (39.3%)	0.40

Notes: IQR, interquartile range.

[a]Mann-Whitney U test.

[b]Pearson's chi-square test.

Table S4-6. Effect modification by sex in intrauterine and infant exposure analysis

	Interaction p-value			
	GP attendances	Dispensations of prescribed asthma inhalers	Dispensations of steroid skin creams	Dispensations of antibiotics
Intrauterine exposure analysis (n=218)				
Average $PM_{2.5}$*Sex	0.13	0.31	0.13	0.29
Peak $PM_{2.5}$*Sex	0.18	0.25	0.29	0.34
Infant exposure analysis (n=198)				
Average $PM_{2.5}$*Sex	0.46	0.84	0.39	0.74
Peak $PM_{2.5}$*Sex	0.86	0.99	0.36	0.60

Note: GP, general practitioner; $PM_{2.5}$, particulate matter with an aerodynamic diameter less than 2.5 micrometers.

Chapter 5

The Pro-inflammatory Effects of Particulate Matter on Epithelial Cells are Associated with Elemental Composition

Chapter 5. The Pro-inflammatory Effects of Particulate Matter on Epithelial Cells are Associated with Elemental Composition

5.1 Preface

The previous Chapters provided epidemiological evidence to suggest that early life exposure to fire smoke-related $PM_{2.5}$ could be associated with adverse respiratory and immune health outcomes later in life, as indicated by reduced lung function and increased antibiotic usage during childhood. However, the potential mechanism of this association is not clear yet. As mentioned in Chapter 1, *in vitro* and *in vivo* studies using PM from all sources suggested that the pro-inflammaroy capacity of PM might partly explain how PM affects human respiratory and immune health. In addition, adverse health effects of PM vary with chemical composition; however, evidence regarding which elements are the most detrimental to health is limited. To address this, we exposed human lung cells to coal mine fire particulate matter collected from the roof space of homes impacted by the fire.

The roof space areas of homes provide a stable environment for outdoor PM to settle and deposit. As such, they can act as a reservoir for ambient PM. Roof space PM samples were collected from 36 different homes in the nearby area of the Hazelwood coal mine fire (the Latrobe Valley of Victoria, Australia) during the year 2015. Therefore, these roof space PM samples could be used as a proxy for residential cumulative exposure to air pollution from the mine fire smoke. In this Chapter I investigated the pro-inflammatory effects of the fire smoke-related PM samples on human lung cells. We also tested the cell responses resulting from the endotoxin and chemical composition of the PM. A modified version of this Chapter has been published in *Chemosphere*.

5.2 Introduction

The Global Burden of Disease study estimated that in 2015, 4.2 million deaths and 103.1 million disability-adjusted life-years were attributed to PM exposure [67]. Individual chemicals may play an important role in affecting the toxicity of PM [61, 189-190]. A multicentre study conducted in Europe compared 8 elements including Cu, Fe, K, Ni, S, Si, V, Zn in PM and found a small effect of Ni and S on decreased lung function [191]. Other studies from the same project (ESCAPE) suggested a significant positive relationship between long-term exposure to S and non-accidental death [192] and an increase in hospital admissions for cardiovascular disease and diabetes related to increases in Ni, As, and Cr, as well as Br and organic carbon concentrations in $PM_{2.5}$ mass [193].

Experimental studies have shed light on the potential biological plausibility of effects caused by different chemical components of PM. For example, higher total Fe content in coarse PM is associated with increases in lung inflammatory cell infiltrations and plasma creatine kinase levels in mice [194]. Similarly, dose-dependent production of interleukin (IL)-8 by BEAS-2B cells in response to traffic-related, industrial and rural PM is associated with Cu, Ni, Zn and endotoxin [195]. Additionally, polycyclic aromatic hydrocarbons (PAHs) in $PM_{10-2.5}$ and $PM_{2.5-1}$ samples from Helsinki (Finland) were negatively correlated with IL-6 secretion in a macrophage cell line (RAW 264.7) [65].

Collectively, these studies suggest that the chemical composition of PM significantly influences its inflammatory capacity. However, despite increasing evidence that this is the case [196-198], our understanding of the components of PM that contribute most to adverse health outcomes is still limited [61, 197]. This is due to the fact that most studies have focused on PM sampled from outdoor locations [199-201]. There is only one study collected PM samples from both indoors and outdoors of a single Finnish home during different seasons and compared

their chemical and biological composition, cytotoxicity and pro-inflammatory potential on mouse RAW264.7 macrophage cell line [202]. The authors found that the soil-derived chemical constituents including Ca, Na, Fe, Mg, K and Al were most abundant and all positively associated with TNF-α and MIP-2 production in mouse macrophages for both indoor and outdoor PM especially during warm seasons, indicating the role of PM from outdoor sources in determining indoor air quality. However, this study mainly focused on the comparisons between indoor/outdoor PM, seasonal variations and different size fractions of PM from one single home. Given that people spend approximately 85% of their time indoors [203], it is important to understand the health implications of local sources of PM and how the PM chemical composition affects the response. Most Australian roofs are constructed of terracotta clay tiles, which allow the outdoor dust to enter, accumulate and preserve in the roof space area due to the relatively open construction and undisturbed environment (less subject to temperature change, sunlight and microbial influence) [204-205]. Therefore, the roof space PM is an indirect matrix of individual's residential air pollution exposure in the past and present. The aim of this study was to investigate how IL-6 and IL-8 production by airway epithelial cells is influenced by the chemical composition of roof space PM samples from different homes as a proxy for residential cumulative exposure to outdoor air pollution.

5.3 Material and methods

5.3.1 Sample collection and preparation

We collected roof space PM samples during November to December in 2015 from 36 homes of non-smokers in Suburban Victoria, with varying house ages and building types. The primary sources of PM in this area include emissions from transport and industry, bushfires, and windblown dust. The mean annual PM_{10} ranged from 13.9 to 14.5 $\mu g/m^3$ in 2011-2015, which

was well below the national yearly average reporting standard of 25 µg/m^3 [206]. A minimum weight of 20 mg was collected using a HVS4 US EPA approved vacuum sampler [207]. Samples were collected into a labelled amber glass jar attached to the vacuum and stored at -20 ℃ until processing. We divided the samples into 4 sub-samples and randomly selected 2 sub-samples for processing and analyses. The samples were sieved through a 150 µm plastic sieve and agitated for 10 minutes using a mechanical shaker. The sieved samples were then milled with two agate balls for 2 minutes to homogenise the size fractions then aliquoted into glass vials and stored in the dark at room temperature.

5.3.2 Particle characterisation

We obtained the images with 1000 times magnification of all the 36 samples using a Hitachi SU-70 field emission scanning electron microscopy (SEM) at 1.5 kV accelerating voltage. Samples were attached to 12mm diameter aluminium SEM mounts using conductive carbon double sided sticky tabs (Ted Pella, Redding, USA) and coated with approximately 4 nm platinum in a Bal-Tec SCD 050 sputter coater. We measured the sizes of all particles for each image to calculate the mean size.

Endotoxin levels in 57 µg/mL PM suspension were assessed once for each sample prior to exposing the cells using a chromogenic limulus amebocyte lysate (LAL) assay kit (GenScript, Piscataway, NJ) according to the manufacturer's instructions. The measurable concentration range of this kit is 0.005 to 1 EU/mL. Results are reported as endotoxin units (EU) per milligram (mg) of particle.

We analysed 32 PAHs including 16 US EPA priority PAHs and 16 alkylated PAHs [Table S5-1, Appendix 5] using gas chromatography mass spectrometry (GC-MS) [208]. We also quantified 22 common elements by inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES) based on US EPA

Method 200.8 including Li, Be, V, Cr, Mn, Co, Ni, Cu, Zn, As, Se, Mo, Sb, Ba, Pb, Na, Mg, Ca, K, Fe, Al and S.

5.3.3 Cell culture

Human bronchial epithelial BEAS-2B cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA), grown in bronchial epithelial cell basal medium (BEBM) supplemented with bronchial epithelial cell growth medium (BEGM) (Lonza, Walkersville, MD, USA) and stored at +37°C in a humidified incubator with 5% CO_2.

5.3.4 Particle exposure

BEAS-2B cells were seeded in 96-well plates (100 µL/well) at a density of 2×10^5 cells/mL for cytotoxicity testing and in 12-well plates (1 mL/well) at a density of 4×10^5 cells/mL for cytokine detection. PM was suspended in Hank's balanced salt solution (HBSS) (Sigma-aldrich, St. Louis, MO, USA) and mixed thoroughly for 15 seconds by vortex prior to being added. Cells were exposed to either media alone, 5.7 or 57 µg/mL of PM suspension for 4 h or 24 h [these doses were selected on the basis of a pre-experimental pilot study; see Appendix 5 for further details]. We conducted a minimum of 6 independent experiments for each PM sample of the two doses and two time points to allow statistical comparisons.

5.3.5 Cytotoxicity test (MTS)

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure cytotoxicity after 24 h of particle exposure.

5.3.6 Enzyme-linked immunosorbent assay (ELISA)

IL-6 and IL-8 levels in the supernatants were measured using human IL-6 and human CXCL8/IL-8 DuoSet® ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

5.3.7 Statistical analysis

Sign tests and Kruskal-Wallis tests with post hoc Kruskal Nemenyi tests were used to assess the overall cellular response to PM exposures. We conducted a principal component analysis (PCA) for the elements (> 0.1% of the total concentration) and total PAH (32 PAHs) content. Prior to the PCA, power transformations were applied to the data where appropriate to ensure normal distribution of the error terms. We identified 4 factors that explained 68.33% of the variance using the screeplot and orthogonal rotation. Based on the factor loadings, a score was assigned to each PM sample. We assessed the association between cytokine production and particle characteristics including size, endotoxin and chemical factor scores using linear regression analyses. All statistical analyses were performed in R 3.2.3 and Stata 14.2. All plots were created using SigmaPlot 12.5 (Systat, Erkrath, Germany). $P < 0.05$ were considered statistically significant and data are reported as mean (SD) and range.

5.4 Results

5.4.1 Particle characteristics

Particle size, endotoxin levels, total PAH content and elemental components in the 36 PM samples are summarised in Table 5-1. The samples contained particles with similar size as indicated by the small standard deviation and range. The majority of the samples were PM_{10} (mean frequency: 84.43%, range: 73.08%-91.15%). Ca, Fe and Al were the three most common elements, although the overall chemical characteristics varied considerably between samples

as indicated by the range and standard deviation (Table 5-1). Using an approximate conversion of 10 EU/ng, cells were exposed to an average of 0.05 ng endotoxin in the high dose group.

5.4.2 Cytotoxicity of PM

Comparison of optical density between low (5.7 µg/mL) and high (57 µg/mL) exposure groups and negative control showed no statistical significance ($p>0.05$ for all comparisons), indicating no effect of particle exposure on cytotoxicity.

5.4.3 Cytokine production in response to PM

In the control group, the mean IL-6 level was 9.38 pg/mL after 4-hour and 24-hour exposure, while the mean IL-8 levels were 31.3 pg/mL and 41.0 pg/mL after 4-hour and 24-hour exposures, respectively. In the exposure group, the mean levels of IL-6 were 10.6, 26.2, 29.6 and 164.2 pg/mL after 4 h exposure to 5.7 µg/mL, 24 h exposure to 5.7 µg/mL, 4 h exposure to 57 µg/mL and 24 h exposure to 57 µg/mL of PM respectively. The corresponding values for IL-8 were higher, ranging from 31.6, 65.2, 40.0 to 217.7 pg/mL respectively. Exposure to PM caused significant dose (IL-6, $p<0.05$ for all comparisons; IL-8, $p<0.05$ for comparisons after 24 h exposure) and time (IL-6, $p<0.05$ for all comparisons; IL-8, $p<0.05$ for all comparisons) dependent increases in cytokine production that was evident 4 and 24 h post-exposure with the exception of IL-8 production 4 h post exposure to 5.7 µg/mL PM which was not elevated above control levels ($p>0.05$) (Figure 5-1). Importantly, large standard deviations were observed between individual PM samples (Figure 5-1).

5.4.4 Principal component factor analysis

Using a factor loading cutoff of |0.60|, Factor 1 was primarily characterised by +Fe, +Al and +Mn, while Factor 2 was characterised by +total PAH, +Pb and -Ca. Factor 3 was loaded on +S, +Mg, +Na and +Ba while Factor 4 was loaded on +Zn, +Cu and –K [Table S5-2, Appendix 5].

5.4.5 Particle characteristics and cytokine production

Since the most significant increases in cytokine production were observed after 24 h of treatment with 57 µg/mL of particles, we only assessed the associations between the particle characteristics and cytokine concentrations for this dose and timepoint.

Size was not associated with cytokine production in the linear regression analyses with and without adjustment for endotoxin (Table 5-2). As expected, there was a positive association between the endotoxin content and both the IL-6 ($p<0.05$) and IL-8 ($p<0.05$) concentrations (Table 5-2, Figure S5-3, Appendix 5). Similarly, Factor 1 score was positively associated with IL-6 ($p<0.05$) and IL-8 production ($p<0.05$) (Table 5-2; Figure 5-2A, Figure 5-3A). Importantly, these associations were still evident after adjusting for particle sizes or the endotoxin content (Table 5-2). In contrast, while Factor 2 score was negatively associated with IL-6 ($p<0.05$) and IL-8 ($p<0.05$) production (Table 5-2, Figure 5-2B, Figure 5-3B). These associations were still evident after adjusting for particle size, but no longer evident after adjusting for the endotoxin content. There was no association between either the Factor 3 or Factor 4 score and the cytokine production (Table 5-2, Figure 5-2, Figure 5-3). Finally, we further adjusted for both particle size and endotoxin levels and the results were consistent with the endotoxin-adjusted models (Table 5-2).

5.5 Discussion

In this study, we found a clear dose- and time-dependent relationship between PM exposure and cytokine release, and these effects were positively associated with Fe, Al and Mn content of the PM. Our findings indicate that particles from sources that generate high levels of Fe, Al and Mn may be the most detrimental to respiratory health according to the metrics that we assessed.

Our roof space PM samples were very different in terms of the levels of endotoxin, elements and PAHs compared with outdoor PM samples in other studies. For example, the PM_{10} samples from Mexico City contained much higher endotoxin levels than our samples, ranging from 29.00 to 94.00 EU/mg [209]. The Ca, Al and Fe levels in our samples were much lower than urban $PM_{2.5\text{-}10}$ samples collected in Beijing, China (Ca: 97740 ng mg^{-1}; Al: 54910 ng mg^{-1}; Fe: 32720 ng mg^{-1}) [210]. In contrast, the coarse PM collected from the backyard area of a Finnish house during summer was also abundant in soil-derived elements including Ca, Al, Na and Fe. However, the Na and Al concentration were approximately 4 and 2 times higher than our samples, while the concentration of Ca and Fe were a bit higher and lower than our samples, respectively (Ca: 38000 ng mg^{-1} vs 30030 ng mg^{-1}; Fe: 21000 ng mg^{-1} vs 25260 ng mg^{-1}) [202]. The sum of 16 PAHs in Mexico PM_{10} samples collected during rainy-warm season were remarkably higher than the sum of 32 PAHs in our samples (41.7 ng mg^{-1} vs 6.07 ng mg^{-1}) [211]. Clearly, the chemical composition of PM varies geographically with size and solubility so it is important that investigations consider local PM sources and physical characteristics when attempting to assess the respiratory health effects of PM inhalation. However, comparisons should be treated with caution since the different collection and extraction methods between studies may affect the physico-chemical properties of the particles.

Our PM samples had no effect on cytotoxicity after 24 h of exposure. This is similar to other toxicological studies of outdoor PM samples using human BEAS-2B cells. No significant decrease in cell metabolic activity was observed after 12 to 72 h of exposure to 50 μg/mL of $PM_{2.5}$ from six Chinese cities using the MTT assay. Significant decreases were only shown in those cells exposed to ≥ 100 μg/mL $PM_{2.5}$ [212]. In contrast, Wu et al.[213] suggested significant decreased cell viability and increased LDH activity in BEAS-2B cells exposed to 50 μg/mL $PM_{2.5}$ for 24 h compared with control. These results could be explained by the findings of another study that low levels of PAHs in $PM_{2.5}$ (1-50 μg/cm^2) had anti-apoptotic effects on

human lung cells after 24 h exposure [214]. The stronger cytotoxic effects of Wu and colleagues' sample might be attributed to higher levels of PAHs compared with ours (464.73 ng mg^{-1} vs 6.07 ng mg^{-1}) [215].

The pro-inflammatory potential of our PM samples is comparable to other studies using the same cell line. In the present study, the mean levels of IL-6 were approximately 2.79 and 17.50-fold higher compared with the control group after 24 h exposure to 5.7 μg/mL and 57 μg/mL of PM respectively. The corresponding values for IL-8 were much lower, ranging from 1.59 to 5.32-fold respectively. $PM_{2.5}$ samples from windblown dust or traffic emissions in the western US had less than 8-fold increases in IL-6 production after 24 h exposure at doses of 25 to 400 μg/mL, while IL-8 production was relatively higher than our results for $PM_{2.5}$ samples from a sparsely vegetated site and a rural grazing site (approximately 7-fold upregulation after 24 h exposure at 50 μg/mL), but lower for $PM_{2.5}$ samples from a high elevation site (approximately 2-fold increase) [216]. In contrast, Van Den Heuvel[195] suggested weaker effects of PM (doses: 12.5, 25, 50 and 100 μg/mL) from urban, rural and industrial locations in Flanders (Belgium) than our PM samples. BEAS-2B cells produced IL-8 in a dose-dependent way after PM exposure and there was an average 2.16-fold increase in IL-8 after 24 h of PM exposure at the highest dose (i.e. 100 μg/mL). Other *in vitro* studies have found different pro-inflammatory potentials of PM samples from various sources using different cell lines [209, 211, 217-220]. All those differences, together with the diverse PM from various sources in inducing cellular inflammation identified in the literature and the large standard deviations of cytokine production in our samples all suggest that the pro-inflammatory potential of PM is strongly related to its physico-chemical characteristics.

Our PCA identified four factors which explained most of the variance. Factor 1 (+Fe, +Al, +Mn) is likely to represent soil-derived sources. Factor 2 was characterised by +total PAH, +Pb and –Ca. Given that the Pb content is likely to be due to paint in the older houses it is

possible that the association with PAH, which is probably combustion related, is linked to proximity to roads, use of wood fires and quality of the roof. Factor 3 (+S, +Mg, +Na and +Ba) and Factor 4 (+Zn, +Cu and –K) are likely to represent combustion sources and tin roofing, respectively. A study of the roof space PM samples suggested that Ba and Mg are correlated closely with mine fire airborne PM emissions[221].

In the present study, the Fe, Al and Mn levels in the PM were positively associated with cytokine production after correcting for the endotoxin content and particle size. While IL-6 and IL-8 production were negatively associated with the Pb and PAH content, and positively associated with the Ca content (Factor 2), this relationship was not evident after adjusting for the endotoxin content and particle size. Particles with high Pb and PAH content, and low Ca content, tended to have low levels of endotoxin resulting in a negative association with cytokine production. We found no association between any of the other elements in our analysis and cytokine production. These findings compare well with one previous *in vitro* study assessing 12 metals in urban PM samples collected from Helsinki [65]. Mouse macrophage RAW264.7 cells were exposed to urban PM samples at a dose of 150 µg/mL for 24 h. Using correlation analyses for each element measured, the authors found that IL-6 levels were positively associated with the Fe, Al and Mn content of the $PM_{10–2.5}$ and $PM_{2.5–1}$ whereas there was no association with the Zn or Cu content. Likewise, a study using a similar analytical approach to ours found that IL-6 secretion in mouse monocytes/macrophages (J774A.1) and human monocytes (THP-1) in response to urban PM from Mexico City was positively associated with the Si, Sr, Mg, Ca, Al, Fe and Mn content but not Zn, S, Sb, Ni, Cu and Pb [209]. This consistency suggests that our observations are relevant to other cell types that are important in the innate response to PM. In contrast, another study in Flanders, Belgium demonstrated that the Cu and Zn content of PM_{10} were significantly associated with IL-8 production in BEAS-2B cells [195]; however, it should be noted in this study that multiple linear regression revealed that the only

characteristics of the particles that were associated with cytokine production were the endotoxin levels. The mechanisms of how variations in chemical composition cause adverse health outcomes are not clear yet because of their high heterogeneity between samples and complex biochemical interactions that are likely to occur when cells are exposed to real-world samples [222]. Further toxicological studies are needed in this field to identify the PM sources that are most detrimental to respiratory health.

The absence of an association between the PAH content, after correcting for endotoxin and particle size, is noteworthy given the substantial body of information on the toxicity of PAHs. PAHs is known to have immunosuppressive effects by reducing cytokine production, as reflected by the same or reduced cytokine production compared with negative controls. This is consistent with a previous study showing a negative association between PAH levels and IL-6 production [65] and another study showing no association [209]. Of course, the potential adverse health effects induced by PAHs may be related to other cellular outcomes including DNA damage and oxidative stress [223-227].

Our study has many strengths compared with previous studies. Firstly, we used a new, cheaper and faster method of collecting PM samples for exposure studies than in previous practice. Secondly, we analysed a wide range of chemical components including 32 PAHs and 22 elements. We employed the PCA method to account for any associations between different components of the PM. In addition, we evaluated and adjusted for the impact of biological materials such as endotoxin in the linear regression models. We chose this approach, as interventions to remove endotoxin were likely to alter the physicochemical characteristics of the PM. In addition to the strengths, there are a number of limitations that should be acknowledged. We did not evaluate other biological composition (e.g. fungi) of the PM which may also play an important role in the response. Additionally, the milling process might change the PM properties such as size. Despite our regression analyses showing non-significant

associations between cytokine production and particle size, it is possible that some of the roof space particles may not have been respirable prior to the milling process. It is also not clear where in the respiratory tract these particles are likely to have deposited. Finally, other aspects of PM-related effects such as DNA damage and oxidative stress were not assessed.

5.6 Conclusions

Our study provided novel insight into the Fe, Al and Mn content of roof space PM as the strongest determinants of the inflammatory response in bronchial epithelial cells. While we were not able to directly apportion these particles to a particular source, these elements are commonly associated with soil and combustion derived PM suggesting that there is a risk of ongoing exposure to PM from these sources in suburban homes. Future toxicological studies should explore the biochemical and molecular mechanisms by which the chemical composition of PM influences the response.

Table 5-1. Summary of physical, biological and chemical characteristics in the 36 particle samples.

	Mean (SD)	**Range**
Size (μm)	5.98 (0.94)	3.47
Endotoxin (EU/mg)	8.35 (6.56)	22.98
Total 32 PAH* (ng mg^{-1})	6.07 (8.66)	52.7
Elements (ng mg^{-1})		
Ca	30030 (11250)	48110
Fe	25260 (8357)	37330
Al	17400 (3123)	12520
S	10260 (4013)	18030
Mg	8136 (3654)	21760
Na	5745 (2255)	8864
Zn	5343 (9041)	38000
K	2881(724.9)	2920
Pb	454.8 (596.1)	2801
Mn	345.6 (120.8)	502.1
Ba	156.4 (62.09)	308.6
Cu	100.1 (143.2)	763.1

*, polycyclic aromatic hydrocarbons

Table 5-2. Associations of particle characteristics with cytokine production after 24 h of exposure to 57 µg/mL of PM.

	IL*-6				IL*-8			
	Model 1	**Model 2†**	**Model 3§**	**Model 4‡**	**Model 1**	**Model 2†**	**Model 3§**	**Model 4‡**
	Coef (p)	**Coef (p)**	**Coef (p)**	**Coef (p)**	**Coef (p)**	**Coef (p)**	**Coef (p)**	**Coef (p)**
Endotoxin	**1.01 (0.00)**	-	**1.02 (0.00)**	-	**0.75 (0.01)**	-	**0.76 (0.01)**	-
Size	-0.11 (0.62)	-0.14 (0.51)	-	-	-0.08 (0.66)	-0.10 (0.57)	-	-
Factor 1	**0.60 (0.01)**	**0.46 (0.04)**	**0.64 (0.01)**	**0.50 (0.03)**	**0.56 (0.00)**	**0.46 (0.01)**	**0.59 (0.00)**	**0.49 (0.01)**
Factor 2	**-0.58 (0.01)**	-0.40 (0.07)	**-0.58 (0.01)**	-0.38 (0.09)	**-0.41 (0.02)**	-0.26 (0.15)	**-0.41 (0.02)**	-0.25 (0.18)
Factor 3	0.07 (0.74)	0.08 (0.67)	0.09 (0.69)	0.10 (0.61)	-0.04 (0.84)	-0.03 (0.87)	-0.03 (0.89)	-0.01 (0.93)
Factor 4	-0.27 (0.22)	-0.07 (0.74)	-0.26 (0.27)	-0.03 (0.91)	-0.20 (0.25)	-0.06 (0.74)	-0.20 (0.29)	-0.03 (0.89)

*, interleukin; †, adjusted for endotoxin levels; §, adjusted for particle sizes; ‡, adjusted for endotoxin levels and particle sizes; data were transformed where necessary to meet the assumptions of normality and linearity; significant associations are shown in **bold**.

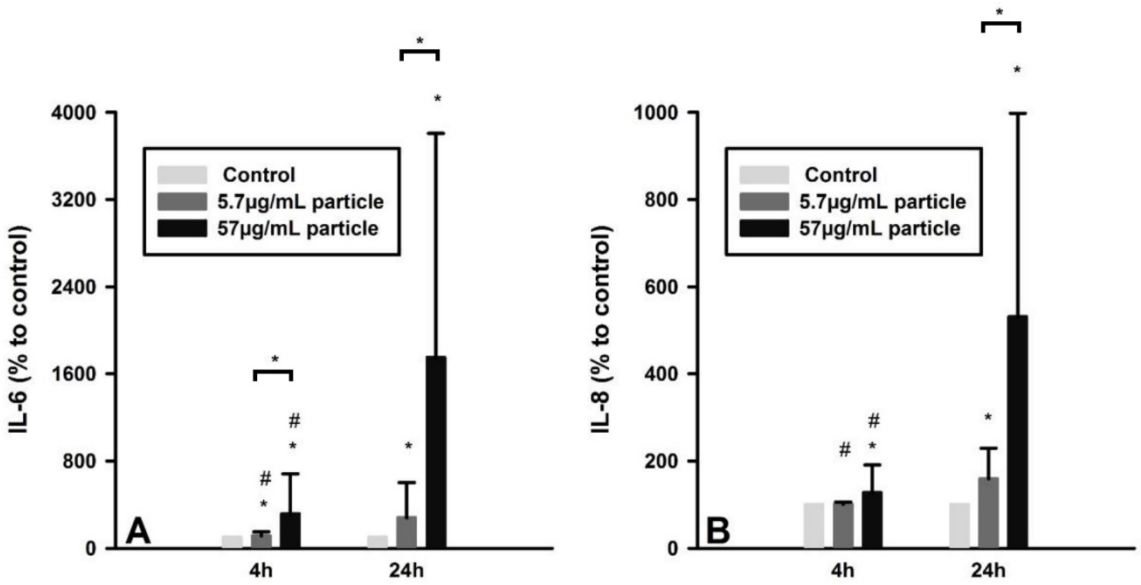

Figure 5-1. IL-6 and IL-8 production in response to roof space PM.

IL-6 (A) and IL-8 (B) production, expressed as a percentage of control levels, by BEAS-2B cells in response to 4 or 24 h exposure, at concentrations of 5.7 µg/mL or 57 µg/mL, to roof space PM sampled from 36 different homes. Data are presented as the mean (SD); * indicates $p < 0.05$ versus the control and between doses, # indicated $p < 0.05$ between times.

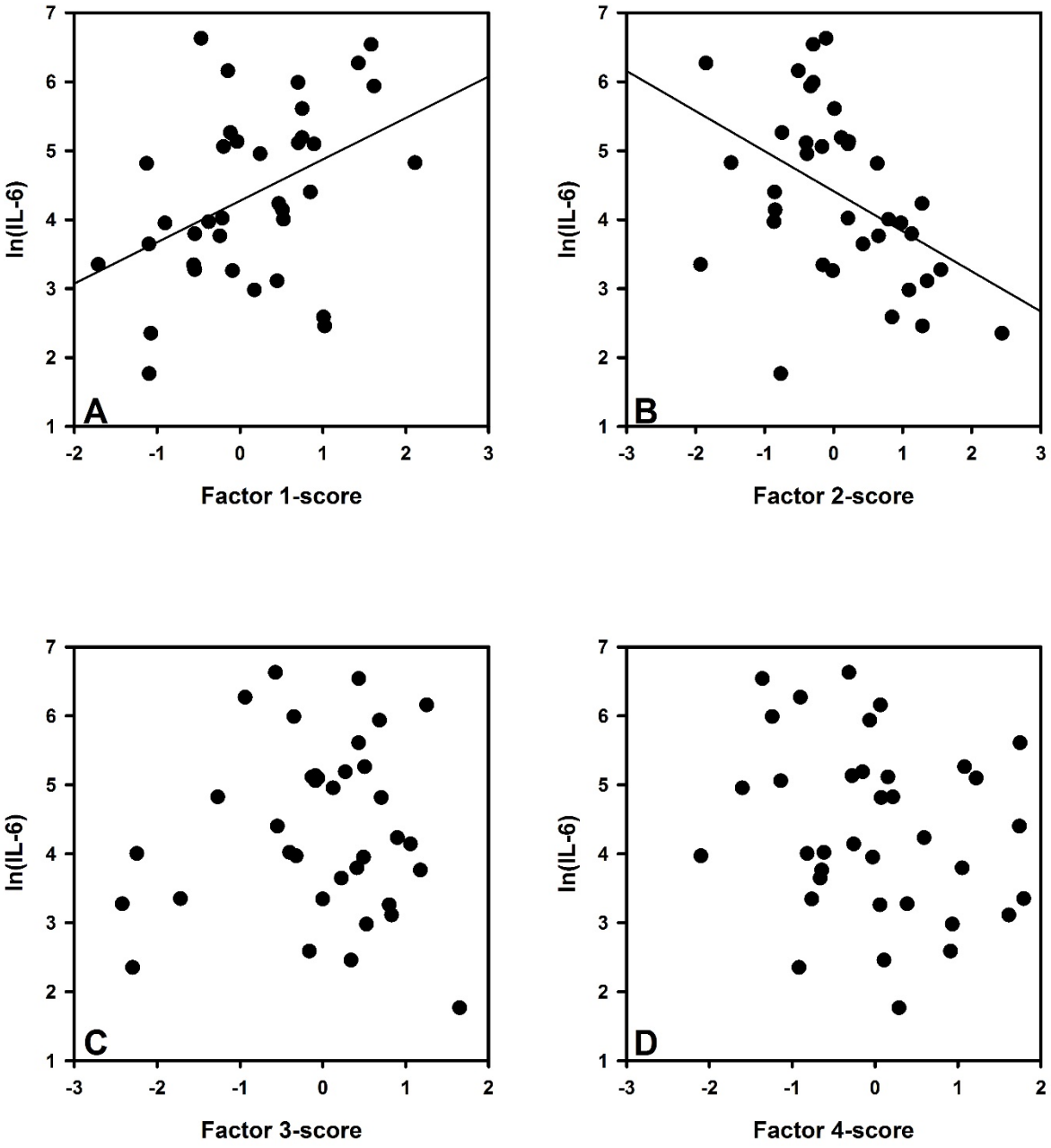

Figure 5-2. Relationship between IL-6 production and PCA factor scores.

Plots showing the relationship between IL-6 production in BEAS-2B cells, in response to 24 h of roof space PM exposure at a dose of 57 µg/mL, and the Factor 1 (A), Factor 2 (B), Factor 3 (C) and Factor 4 (D) score. Each data point represents a PM sample from an individual house and the line shows the predicted values from the linear regression.

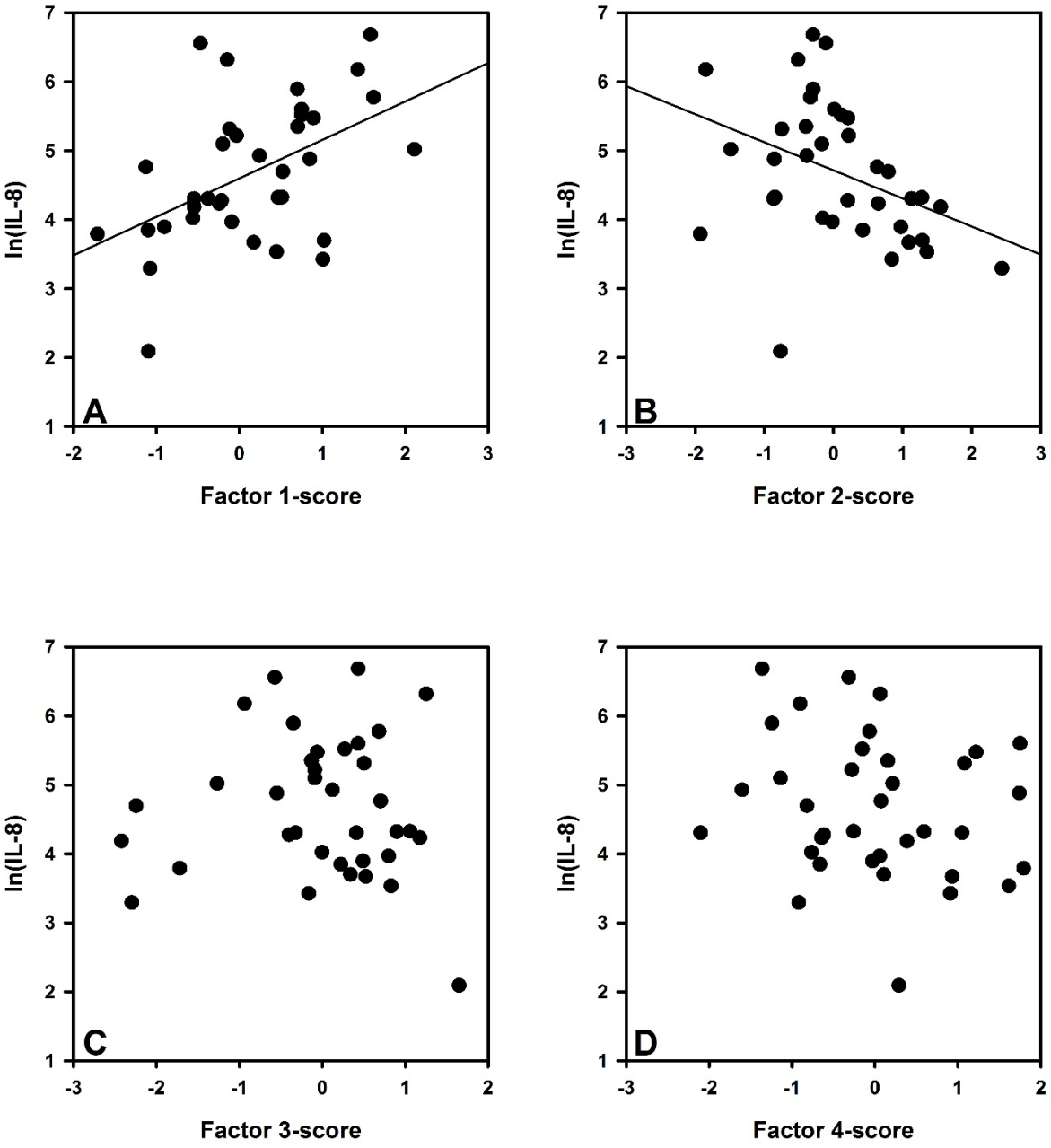

Figure 5-3. Relationship between IL-8 production and PCA factor scores.

Plots showing the relationship between IL-8 production in BEAS-2B cells, in response to 24 h of roof space PM exposure at a dose of 57 µg/mL, and the Factor 1 (A), Factor 2 (B), Factor 3 (C) and Factor 4 (D) score. Each data point represents a PM sample from an individual house and the line shows the predicted values from the linear regression.

5.7 Appendix 5

Pre-experimental dose-response trial. BEAS-2B cells were exposed to a representative PM sample at five different doses: 1.9 µg/mL, 3.8 µg/mL, 19 µg/mL, 38 µg/mL, or 57 µg/mL. We conducted six independent cell viability trials and assessed cytotoxicity along with (IL) -1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) by ELISA.

Figure S5-1: Cytotoxicity and PM dose.

Figure S5-2: Cytokine production and PM dose.

Figure S5-3: Relationship between cytokine production and endotoxin levels.

Table S5-1: Summary of 32 PAHs in the 36 particle samples.

Table S5-2: Summary of loadings from the principal component analysis for total PAH and key elements in the PM.

Pre-experimental dose-response trial.

In order to identify a particle concentration that was able to induce cellular pro-inflammatory responses without impacting on cell viability we selected a representative PM sample and exposed BEAS-2B cells to a range of doses for 24 h before assessing cytotoxicity and cytokine production.

Material and Methods

Cell culture and particle preparation

We used a human bronchial epithelial BEAS-2B cell line purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured according to the protocol described in main paper. Particles were irradiated under ultraviolet for 2 hours before use to remove bacterial content.

Particle exposure

BEAS-2B cells were seeded at 2×10^4 in 96-well plates (100 µL/well) for cytotoxicity test, while in 12-well plates (1 mL/well) we used 2×10^5 cells per well for cytokine detection. After 24 h of adherence, five different doses of the particle suspensions were tested, ranging from 1.9 µg/mL to 57 µg/mL (i.e. 1.9, 3.8, 19, 38, 57 µg/mL). We assessed the outcomes (outlined below) after 4 or 24 h of PM exposure.

Cytotoxicity

Cytotoxicity was measured by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The absorbance values in each group were read by a spectrophotometer (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

Cytokines

The levels of IL-6 and IL-8 were analysed in the same way as described in the main paper. IL-1β and TNF-α were measured using human TNF-alpha and human IL-1 beta/IL-1F2 DuoSet® ELISA kits purchased from the same company (R&D systems, Minneapolis, MN, USA). All ELISA measurements were conducted following the manufacturer's instructions.

Statistical analysis

All the responses were assessed as the percentage response relative to the control group. All statistical analyses were performed using R statistical software 3.2.3 and bar plots were created using SigmaPlot 12.5 (Systat, Erkrath, Germany). One-way analysis of variance (ANOVA) and post hoc Turkey's Honestly Significant Difference (HSD) tests were used to compare between-group differences. P values less than 0.05 were considered statistically significant and data were reported as mean (SD).

Results

Cytotoxicity

PM exposure at the doses used had no observable cytotoxic effect (Figure S5-1; $p > 0.05$).

Cytokine production

Exposure to PM at 57 µg/mL for 24 h caused an increase in IL-6 ($p = 0.04$) and IL-8 ($p = 0.00$) production. There was no effect at lower doses or at the 4 h timepoint for these cytokines ($p > 0.05$ for all comparisons). PM exposure had no effect on IL-1β or TNF-α levels ($p > 0.05$ for all comparisons) (Figure S5-2).

Conclusion

Based on these data we chose 5.7 µg/mL and 57 µg/mL for the main study.

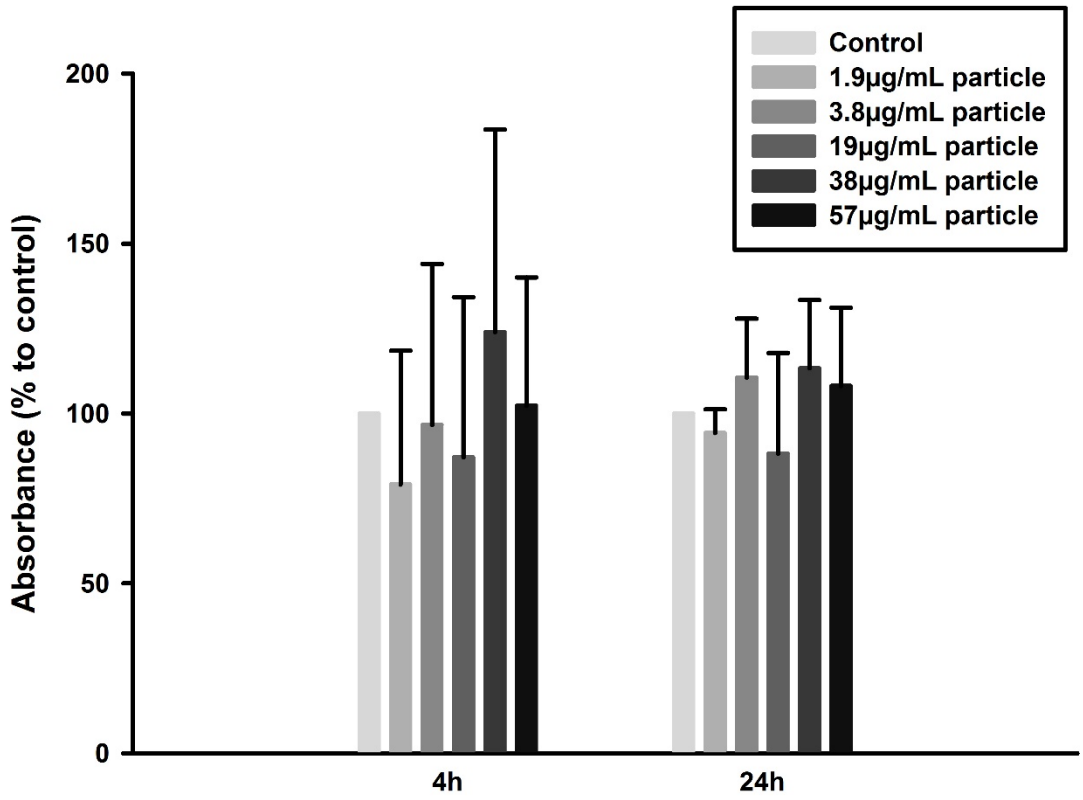

Figure S5-1. Cytotoxicity and PM dose. BEAS-2B cells were exposed to a range of doses of PM from the roof space of a representative house. Cytotoxicity was assessed after 4 or 24 h of exposure. Data are presented as the mean (SD) and expressed as a percentage of the control response. PM exposure had no observable cytotoxic effects.

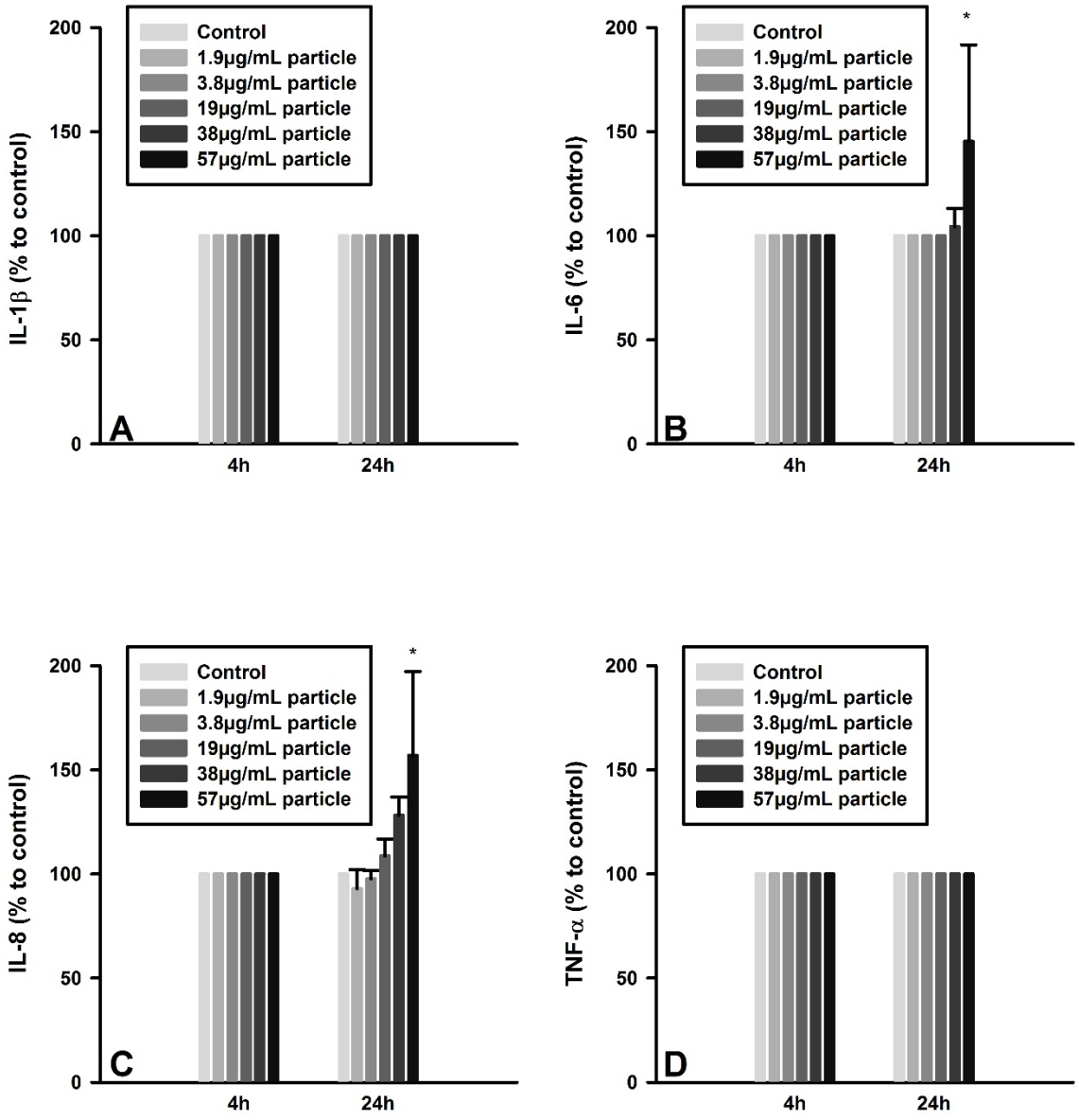

Figure S5-2. Cytokine production and PM dose. BEAS-2B cells were exposed to a range of doses of PM from the roof space of a representative house. IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D) were assessed after 4 or 24 h of exposure. Data are presented as the mean (SD) and expressed as a percentage of the control response. * indicated $p < 0.05$ versus the control.

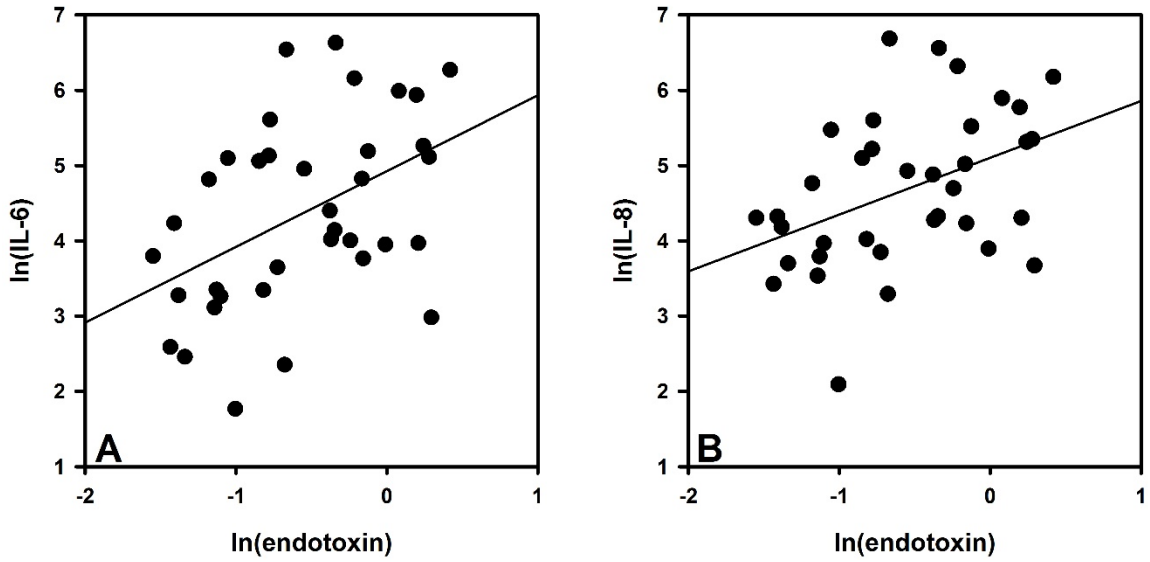

Figure S5-3. Relationship between cytokine production and endotoxin levels. Plots showing the relationship between IL-6 (A) and IL-8 (B) production in BEAS-2B cells, in response to 24 h of roof space PM exposure at a dose of 57 µg/mL, and PM endotoxin content. Each data point represents a PM sample from an individual house and the line shows the predicted values from the linear regression.

Table S5-1. Summary of 32 PAHs in the 36 particle samples

	Mean (SD)	**Range**
US EPA priority PAHs		
Phenanthrene	0.70 (1.36)	8.29
Naphthalene	0.66 (0.36)	1.53
Fluoranthene	0.62 (1.50)	9.05
Pyrene	0.60 (1.54)	9.31
Chrysene	0.28 (0.60)	3.60
Benzo(b)fluoranthene	0.24 (0.52)	3.14
Benzo(a)pyrene	0.22 (0.55)	3.29
Indeno[1,2,3-cd]pyrene	0.20 (0.47)	2.63
Benzo(k)fluoranthene/Benzo(e)pyrene	0.18 (0.41)	2.50
Benzo(g,h,i)perylene	0.17 (0.38)	2.20
Benzo(a)anthracene	0.16 (0.48)	2.82
Dibenz(a,h)anthracene	0.04 (0.11)	0.63
Anthracene	0.04 (0.08)	0.46
Fluorene	0.04 (0.04)	0.19
Acenaphthylene	0.03 (0.03)	0.16
Acenaphthene	0.02 (0.01)	0.07
Alkylated PAHs		
2-Methylnaphthalene	0.63 (0.39)	2.09
1-Methylnaphthalene	0.33 (0.22)	1.23
Retene	0.18 (0.20)	1.23
3-Methylphenanthrene	0.11 (0.19)	0.98

1-Methylphenanthrene	0.09 (0.15)	0.83
2-Methylphenanthrene	0.08 (0.14)	0.73
1-Methylanthracene	0.08 (0.13)	0.65
2,3,5-Trimethylnaphthalene	0.08 (0.07)	0.41
2-Phenylnaphthalene	0.07 (0.09)	0.43
Biphenyl	0.07 (0.09)	0.37
Perylene	0.06 (0.14)	0.85
1-Methylpyrene	0.04 (0.06)	0.35
2,3-Dimethylnaphthalene	0.03 (0.05)	0.21
2-Methylchrysene	0.02 (0.06)	0.33
1-Methylfluorene	0.02 (0.02)	0.10
1-Methylfluoranthene	0.00 (0.00)	0.00
Total PAH	6.07 (8.66)	52.70

Table S5-2. Summary of loadings from the principal component analysis for total PAH and key elements in the PM.

	Factor 1	**Factor 2**	**Factor 3**	**Factor 4**
Total PAH*	-0.43	**0.74**	0.09	-0.04
Elements				
Fe	**0.88**	-0.12	0.14	-0.03
Al	**0.81**	-0.10	-0.28	-0.05
Mn	**0.77**	-0.04	-0.03	-0.38
Pb	0.46	**0.65**	0.15	0.22
Mg	0.39	-0.04	**0.69**	-0.34
Na	-0.36	-0.14	**0.68**	-0.02
Cu	-0.21	-0.16	-0.19	**0.65**
Ca	0.12	**-0.86**	0.21	0.10
K	0.11	0.20	-0.12	**-0.68**
S	-0.09	-0.04	**0.78**	0.26
Zn	-0.09	0.30	0.11	**0.66**
Ba	-0.03	0.54	**0.60**	0.03

*, polycyclic aromatic hydrocarbons; elements whose loadings are higher than |0.60| are shown in bold.

Chapter 6

General Discussion

Chapter 6. General Discussion

6.1 Summary

Exposure to fire smoke emissions could be harmful to respiratory and immune health among the general population[8]. The *in utero* and early post-natal periods are critical for the development of human respiratory and immune systems[22], and exposure to air pollution during this period could result in adverse health outcomes in later life[29, 51]. However, evidence regarding the respiratory and immune health effects from fire smoke exposure during early development is extremely limited. As mentioned above, the current literature mostly focuses on $PM_{2.5}$ from traffic emissions. We know that fire smoke-related $PM_{2.5}$ is different from traffic-related $PM_{2.5}$ in chemical composition and toxicological characteristics. The Hazelwood coal mine fire, which resulted in remarkably increased concentrations of $PM_{2.5}$ in the nearby area, provided an opportunity to address this research gap. Therefore, this Thesis evaluated the associations between coal mine fire smoke-related $PM_{2.5}$ exposure *in utero* or during the first two years of life and respiratory and immune health as reflected by lung function, and the usage of medication and medical service during childhood. This Thesis also investigated the cytotoxic and pro-inflammatory capacity of $PM_{2.5}$ and the effect of its chemical components on the response using human bronchial epithelial cells (BEAS-2B) to explore how fire smoke-related $PM_{2.5}$ may affect human respiratory health.

Chapter 2 provided a synthesis of data on the impacts of intrauterine and infant $PM_{2.5}$ exposure and the development of wheezing or asthma. Meta-analyses of the associations between early life $PM_{2.5}$ exposure and wheezing/asthma showed positive associations, but the associations were not statistically significant. While meta-analysis of intrauterine exposure and wheezing was not possible, all studies found strong positive associations with wheezing by age 2. High

heterogeneity was present among studies of intrauterine exposure and asthma, while others showed low heterogeneity. Overall, the limited available evidence is suggestive of an association between intrauterine or infant $PM_{2.5}$ exposure and the later development of wheezing or asthma.

In Chapter 3 and Chapter 4, children's respiratory and immune health after early life exposure to the Hazelwood coal mine fire smoke was evaluated. In Chapter 3, children's respiratory system resistance and reactance three years after the fire was assessed using FOT. There was modest evidence for an association between infant exposure to elevated $PM_{2.5}$ during the six-week coal mine fire and reduced respiratory system reactance. The magnitude of the association was small, but of potential clinical importance in the most severely exposed children. In Chapter 4 the frequency of GP attendances and dispensations of medications including prescribed asthma inhalers, steroid skin creams and antibiotics during the year following intrauterine or infant exposure to coal mine fire $PM_{2.5}$ was investigated. Exposure to coal mine fire smoke during the first two years of life was significantly associated with increased likelihood of antibiotic dispensations in children, which might reflect increased infections after coal mine fire smoke exposure. No other significant associations were found in these two studies. Chapter 3 and 4 provide the first epidemiological evidence that exposure to a short-term severe air pollution event during the first two years of life could be associated with reduced lung function and increased risk of childhood infections.

In Chapter 5, the effect of chemical composition on the pro-inflammatory effects of roof space PM samples collected from 36 different homes in the Latrobe Valley, Victoria, Australia on human bronchial epithelial cells (BEAS-2B) was assessed. Roof space PM caused increased IL-6/IL-8 production in BEAS-2B cells. Higher concentrations of Fe, Al and Mn, which are commonly associated with soil and combustion derived PM, were positively associated with

increased IL-6/IL-8 production suggesting that PM from these sources poses the greatest health risk.

In summary, the series of related studies presented in this Thesis provide novel evidence that infant exposure to a short-term, severe air pollution event could cause adverse respiratory and immune health outcomes in later life, as indicated by reduced lung function and increased antibiotic dispensations during childhood. This may be a result of the pro-inflammatory response induced by PM when inhaled due to specific chemical components of fire smoke-related PM.

6.2 Implication and future directions

Chapter 2 highlighted the limited evidence on the respiratory health effects of early life $PM_{2.5}$ exposure, which emphasised the need for further epidemiological studies to assist policy makers in improving public health when events such as the Hazelwood coal mine fire occur. The Latrobe ELF Study (Chapter 3 and Chapter 4) was established after the Hazelwood coal mine fire to address this research gap. Findings outlined in these Chapters suggest significant associations between infant exposure to fire smoke and adverse respiratory and immune health outcomes, as indicated by worse lung reactance and increased antibiotic usage during childhood. To my knowledge, this is the first study to evaluate the health effects of early life exposure to air pollution from a coal mine fire, which is a severe and short-to-medium duration air pollution event. Fetuses and infants are susceptible to air pollution exposures due to their rapidly developing immune and respiratory systems and their faster breathing rates, compared with adults[25]. Therefore, it is important to understand the potential effects of air pollution exposure from the Hazelwood coal mine fire smoke in order to guide future public health responses. Policy makers and relevant departments should focus limited resources on

susceptible populations, especially young children with severe exposure. The relevant health services in Latrobe Valley may want to review existing services and strategies to protect children's health including support for tobacco cessation, maternal and child health services and health promotion. In addition, the possibility of detrimental impacts on the respiratory and immune health of children who were exposed to coal mine fire emissions during infancy will likely generate community concern. Therefore, relevant departments should keep up good communications with the community and provide professional guidance to protect people, especially infants, from air pollution exposure for future events such as a coal mine fire.

The key finding outlined in this Thesis is that exposure to fire smoke during early life could result in adverse respiratory and immune health effects later in life. It should be noted that antibiotic dispensation might also be affected by the changed habits of GPs or parental requests for antibiotics in the year following the fire, or by seasonal variations in circulating pathogens. However, the unchanged rate of GP attendances, the lack of association in the intrauterine exposure analysis and the large effect size all suggest that these factors are unlikely to explain the observed association. Poorer lung reactance, which was observed in children exposed to coal mine fire smoke during infancy, is thought to reflect altered peripheral lung mechanics and can be indicative of stiffer or smaller lungs[228-229]. This is in line with the fact that $PM_{2.5}$ could travel and deposit in the lower respiratory tract[54]. However, as mentioned in Chapter 3, the reductions in lung function measured on a single occasion do not necessarily mean that there is a clinical problem or that one might subsequently develop. The exposure duration is short (i.e. six weeks) and the air quality of the study area is generally very good. The annual average $PM_{2.5}$ concentration was 6.7 $\mu g/m^3$ in Victoria during the year 2015[164]. Thus, it is not clear whether the adverse health outcomes that have been identified could recover as the children grow. There is a study suggesting that infants with low lung function during the first year could recover in later childhood[127]. Similarly, whether the increased prescriptions of

antibiotics (as an indicator of increased infections) are correlated with later adverse health outcomes needs to be determined. For example, increased respiratory infections in early life are associated with an increased risk of persistent asthma and reduced lung function later in life[146, 230-231]. Furthermore, the major shortcoming of Chapter 3 and 4 is the small sample size included in the two studies. As mentioned in each chapter, a small sample size may limit the power of our analyses to detect significant associations and affect the generalisability of our study. Relative to the local population, a higher proportion of children with well-educated and non-smoking parents were included in our study. Therefore, our results could be an underestimate of the impacts which might be expected in a population with a higher prevalence of smoking and social disadvantage. Given the limited evidence in this field, further monitoring of this cohort and further epidemiological studies with large sample sizes are needed to confirm these findings.

Outcomes of the cell line study from Chapter 5 are in line with the epidemiological evidence in Chapter 3 and 4, suggesting that the adverse health effects of fire smoke exposure are biologically plausible. Roof space particulate matter samples collected from the houses near the coal mine fire are likely to reflect cumulative exposures to outdoor air pollutants [204-205]. The increased IL-6 and IL-8 production observed in exposed human BEAS-2B cells indicated that the fire smoke-related PM might affect human respiratory health by inducing cellular inflammation. In addition, this Chapter highlighted the importance of Fe, Al and Mn laden PM in driving the response. These data suggest that the pro-inflammatory effects of coal mine fire related PM might be a mechanism causing the respiratory and immunological effects observed in the earlier Chapters. However, it is not clear whether there are other mechanisms involved such as gene damage and oxidative stress, which are widely evaluated in studies of particulate matter from other sources[55, 58, 232] but were not assessed in these studies. Additionally, evidence on the comparison of toxicity of PM from different sources is still limited and

inconsistent. For example, one study suggested that PM from biomass combustion was the most toxic to lung health compared with those from traffic, industry, dust and coal combustion using a source apportionment method [233], while other studies found that PM_{10} from vehicle exhaust was significantly associated with emergency department visits for both respiratory and cardiovascular diseases compared with regional burning of wood and coal[234-235]. Therefore, future toxicological studies should further explore the sources of PM and the biochemical and molecular mechanisms by which the chemical composition of PM influences the response with a particular focus on early life exposure which would require the use of *in vivo* exposure models.

6.3 Conclusions

This Thesis aimed to evaluate the respiratory and immune health effects of early life exposure to $PM_{2.5}$ from coal mine fire smoke. Current evidence on the associations between early life $PM_{2.5}$ exposure and adverse respiratory outcomes during childhood is limited. My analyses provided novel findings of significant associations between infant exposure to $PM_{2.5}$ from coal mine fire emissions and adverse immune and respiratory health outcomes in later life, including worse lung reactance, and increased use of antibiotics during childhood. The underlying mechanisms might be the pro-inflammatory capacity of $PM_{2.5}$ on human lung cells. These findings have important implications for the public health response to short-term severe pollution events. Further follow-up studies are needed to confirm these findings, to investigate whether these effects persist as children grow and to further explore potential mechanisms.

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