Assessment of the biological consequences of controlling indoor solid fuel smoke emissions in Nepal

By

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UNITED KINGDOM · CHINA · MALAYSIA

This thesis is dedicated to my Parents, and my wife

Abstract

Household air pollution (HAP) exposure and its consequences for human health have been a topic of interest in the medical and engineering research world for many years. Large populations in low and middle-income countries (LMICs) are exposed to HAP as a result of the combustion of solid biomass fuel, especially for household cooking. Short-term exposure to HAP may increase respiratory symptoms including cough and breathlessness, which on more prolonged exposure may cause serious respiratory damage and lead to premature death. Airway inflammation following exposure to inhaled pollutants is likely to be a key step in this process, and little is known about the mechanisms underlying responses to biomass smoke in human lung. The main aims of this thesis were to (i) quantify 'real-life' exposure to particulate matter of aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO) measured during cooking on stoves in rural areas of Nepal in different geographical settings, (ii) assess the potential effect of biomass smoke extract generated in real life cooking on inflammatory responses in human lung tissue, and (iii) investigate the consequences of controlling biomass smoke exposure using improved cook stoves (with a flue to vent smoke out of the room) (ICS) on inflammatory responses in human lung tissue.

Using eight different types of biomass fuels, it was identified that $PM_{2.5}$ and CO emissions were higher with agricultural residue and *cow dung*

compared to fuel wood. The real-life exposure was measured during cooking on a range of stoves in 103 households in 4 different Nepalese villages situated at altitudes between ~100 m to 4000 m above sea level. It was found that in a range of settings in rural Nepal from villages at all altitudes high levels of personal exposures to indoor pollutants occur and that the exposures are higher in villages at higher altitude. I demonstrated that the biomass smoke samples collected in a real-life environment from rural Nepal have pro-inflammatory effects in both human lung tissue and HBEC. An elevated level of pro-inflammatory cytokines and chemokines measured in the cell and tissue culture supernatant following biomass smoke exposure suggests that high levels of indoor exposures are likely to produce lung inflammation. The use of ICS was effective in reducing the overall indoor exposure, and the exposure reduction was about 51%. However, the use of ICS does not prevent pro-inflammatory responses in human lung as a similar pattern of inflammatory mediators was observed with the samples from ICS cooking. Overall, this study supports the need to reduce exposures in order to improve respiratory health in this setting. Furthermore, it suggests that additional methods other than those currently being trialled may be needed to reduce exposures to levels which will prevent lung inflammation from occurring in real-life settings.

Publications

Binaya KC, Parth Sarathi Mahapatra, Dhruma Thakkar, Amanda Henry, Charlotte Billington, Ian Sayers, Siva Praveen Puppala, Ian Hall. Pro-inflammatory effects in human lung of respirable smoke extracts from indoor cooking in Nepal. Am J RespirCrit Care Med 2019 (Under review, submitted on 3 September 2019)

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Binaya KC, Parth Sarathi Mahapatra,Charlotte K Billington, Dhruma Thakker, Amanda P Henry, Siva Praveen Puppala, Bim P Shrestha, Ian P Hall. **Household air pollution (HAP) from solid biomass fuel and lung inflammation: An exposure study in rural high altitude households of Nepal.** ISEE-ISES AC, 17-19 October 2019, Daegu, Korea (Accepted for Oral Presentation)

Binaya KC, Parth Sarathi Mahapatra,Charlotte K Billington, Dhruma Thakker, Amanda P Henry, Siva Praveen Puppala, Bim P Shrestha, Ian P Hall. **Pro-inflammatory effects in human lung of respirable smoke extracts from indoor cooking in Nepal**. ICIMOD workshop 13-14 June 2019, Kathmandu Nepal (Accepted for Oral Presentation).

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Binaya KC, et al. **Association between indoor cooking with different cooking fuel and changes in lung function, blood pressure and oxygen saturation in female non-smoker at Chitwan, Nepal**. Air pollution, climate and health in Southern Asia and the HKH science-polity dialogue workshop, November 27-30, 2017, Kathmandu, Nepal (accepted for poster presentation).

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Contents

Abbreviations

ALRI	Acute lower respiratory infection
AM	Alveolar macrophage
ANOVA	Analysis of variance
ATB	Arden Tissue Bank
BALF	Bronchoalveolar lavage fluid
BEBM	Bronchial epithelial basal media
BEGM	Bronchial epithelial growth media
BMI	Body mass index
CBS	Central Bureau of Statistics
CNP	Chitwan National Park
CO	Carbon monoxide
CO2	Carbon dioxide
COHb	Carboxyhemoglobin
COPD	Chronic obstructive pulmonary disease
CRT/N	Centre for Rural Technology Nepal
CSE	Cigarette smoke extract
CTGF	Connective tissue growth factor
DALYs	Disability-adjusted life years
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
EF	Emission factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FEV1	Forced expiratory volume in first 1 second
FVC	Force vital capacity
GACC	Global Alliance for Clean Cookstoves
G-CSF	Granulocyte-colony stimulating factor
GDP	Gross domestic product
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GOLD	Global Alliance for Chronic Obstructive Lung Disease
HAP	Household air pollution
HBECs	Human bronchial epithelial cells
HP	Hypersensitive pneumonitis
IAP	Indoor air pollution
IAQ	Indoor air quality
ICS	Improved cook stove
IHC	Immunohistochemistry
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
IWA	International Workshop Agreement
LAL	Lumulusamebocyte lysate
LEMS	Laboratory emissions monitoring system
LMICs	Low and middle income countries
LPG	Liquefied petroleum gas
LPS	Lipopolysaccharide
LTB	Leukotriene
masl	meter above sea level
MCP	Monocyte chemoattractant protein
MIP	Macrophage Inflammatory protein
MMP	Matrix Metalloproteinase
NE	Neutrophil Elastase
NICE	National Institute for health and Care Excellence
NLRs	Nod-like receptors
PAHs	Polycyclic aromatic hydrocarbons
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PEF	Peak expiratory flow
PH	Pulmonary hypertension
PHRTB	Papworth Hospital Tissue Bank
PM	Particulate matter
Poly(I:C)	Polyinosinic:polycytidylic acid
PRRs	Particle recognition receptors
RH	Relative Humidity

RLRs	RIG-I-like receptors
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
TCS	Traditional cook stove
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRP	Transient potential receptor
TSP	Total suspended particles
VDC	Village development committee
VOC	Volatile organic compounds
WBT	Water boiling test
WHO	World Health Organization
WSE	Wood smoke extract

Chapter 1: Introduction

1 General Introduction

Air pollution and particulate matter (PM) exposure and associated human health effects have been a subject of common interest to many researchers for many years. Long term exposure to particulate matter with the aerodynamic diameter less than 10 and 2.5 micrometres (PM_{10} and $PM_{2.5}$) has been shown in many studies to be associated with increased risk of pulmonary and cardiovascular mortality and morbidity [1-4]. Household air pollution (HAP) resulting from the use of solid biomass fuel for domestic cooking is the major source of fine particulate matter, especially in low and middle-income countries (LMICs). A substantial part of the population around the world are exposed to air pollution in their homes, resulting in an estimated 3.8 million annual number of premature deaths worldwide [5]. Exposure to higher concentrations of a wide range of health damaging pollutants from biomass combustion is thought to be responsible for a wide range of adverse health outcomes in both adults and children, especially cardiovascular and respiratory disease. An extensive body of scientific evidence shows an increased risk of lung cancer [6], cardiovascular disease [7, 8], chronic obstructive pulmonary disease [9, 10] and acute respiratory tract infection [11] with HAP exposure.

Available epidemiological study data is sufficient to link the association of HAP exposure with adverse health effects, however, the potential biological mechanisms are yet to be fully elucidated. Inflammation is

likely to be a key step in the development of many pulmonary and cardiovascular diseases. The inflammatory effect analysis of inhaled pollutants in human cell and tissue model can provide a potential mechanistic link between HAP exposure and the adverse health outcomes. Afew studies have shown in *in vitro* experiment that PM generated from the combustion of biomass fuel is pro-inflammatory [12-14]. However, these studies lack assessment of the inflammatory effects of short term inhalation of combined products of biomass combustion in a real-life cooking environment. In addition, none of these studies analysed the potential inflammatory effects of biomass smoke on the pulmonary system using human lung explants model. It has already been shown in the previous study that an *ex vivo* human lung tissue explants model is responsive to inflammatory signals and can be used to study airway inflammation [15]. Hence this thesis further investigates the inflammatory effect of exposures to biomass smoke in real-life cooking environments using human *ex vivo* lung tissue model. In addition to this, the inflammatory effect of short term exposure was also evaluated using the human bronchial epithelial cell model system.

Traditional biomass fuels like fuel-wood, agricultural residue and animal dung have been a primary source of residential energy for cooking and heating in LMICs for many years. Around the world for domestic cooking and household heating, traditional biomass fuels are combusted indoors in a traditional stove or open hearths [16] resulting in incomplete combustion. The incomplete combustion can generate a higher

concentration of particulate matter, carbon monoxide and other health damaging pollutants like methane, polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) [17]. The emissions of fine particulate matter, for instance, in poorly ventilated dwellings using inefficient stoves can reach up to 100 fold higher than the air quality standard set by the World Health Organization [18]. Two recent studies conducted in two different districts of Nepal have found a daily indoor $PM_{2.5}$ concentration of 1376 $\mu g/m^3$ [19] and a 48-hour average $PM_{2.5}$ concentration of 417.6 $\mu g/m^3$ [20] in the household using biomass fuel in Sarlahi and Janakpur respectively. Consistently higher concentration of indoor air pollutant in households using biomass fuel was reported in many similar studies around the world. However, these studies were generally used gravimetric sampling, and few studies have monitored real-life personal exposures to HAP resulting from cooking using biomass fuel in developing countries. The concentration and properties of indoor air pollutant vary considerably among various sources and locations with diverse social and cultural behaviour. Hence in this thesis, real-life personal exposure to indoor pollutants in the household of four different rural villages of Nepal was monitored and compared with the inflammatory response seen with the samples from those households.

It is well understood that the reduction in HAP is likely to bring health benefits to all individuals who are dependent on biomass for cooking and heating. Replacement of traditional cook stoves (TCS) with a more efficient improved cook stove (ICS) is likely to be an effective solution to

reduce HAP. Developments made around the world in the design and dissemination of improved cook stove has shown its effectiveness in reducing emissions. The previous studies worldwide have shown that considerable amount of emission reduction occurred with ICS use. However, despite the reduction levels were still several folds higher than the safe limit recommended by WHO. Though the exposure level with ICS is still higher than the threshold limit, the reduction level in exposure with ICS is still likely to improve lung function. However, in one study done by Joshi et al. there was no significant difference in the lung function in terms of measured Forced Vital Capacity (FVC), Forced expiratory Volume in first second (FEV_1) and Peak Expiratory Flow (PEF) of ICS and TCS users [21]. More health impact studies on the effect of reduction of indoor air pollutant exposure after installation of ICS should be carried out, especially given that some of the previous studies have reported no significant health effects of controlling indoor pollutant exposure [22-24]. In addition, more experimental *in vivo* or *ex vivo* studies are required to look into changes in cellular mechanisms in the pulmonary system with use of ICS, given the lack of those analyses in the previous studies. This thesis hence also investigates the potential benefits of using ICS on real-life exposure reduction and associated potential inflammatory effects on pulmonary systems.

1.1 **Ambient Air pollution**

Various natural and anthropogenic activities release a complex mixture of gases and particulates in ambient air. Those substances if present in ambient air at a sufficiently high level to produce harmful effects on humans, animals and environment, are known as ambient air pollution. Worldwide air pollution is the single most environmental risk factor associated with adverse impact on human health and climate change. While hundreds of different chemical compounds are present in the ambient air, fine particulate matter is considered to have the greatest health impact [25]. Around the world exposure to fine particulate matter of aerodynamic diameter less than or equal to 2.5 micrometres ($PM_{2.5}$) is responsible for millions of premature death annually, contributed to 4.2 million deaths in 2016, and it is ranked as the 6^{th} highest risk factor for early death (Figure 1.1) [26]. The deaths attributed to ambient air pollution are mainly from cardiovascular and respiratory diseases including stroke, COPD, lung cancer and acute respiratory infections [25]. In the year 2016, an estimated 16% of total lung cancer deaths, 25% of total COPD deaths, 17% of total ischaemic heart disease death and about 26% of total respiratory infection deaths worldwide were attributed to ambient air pollution [27].

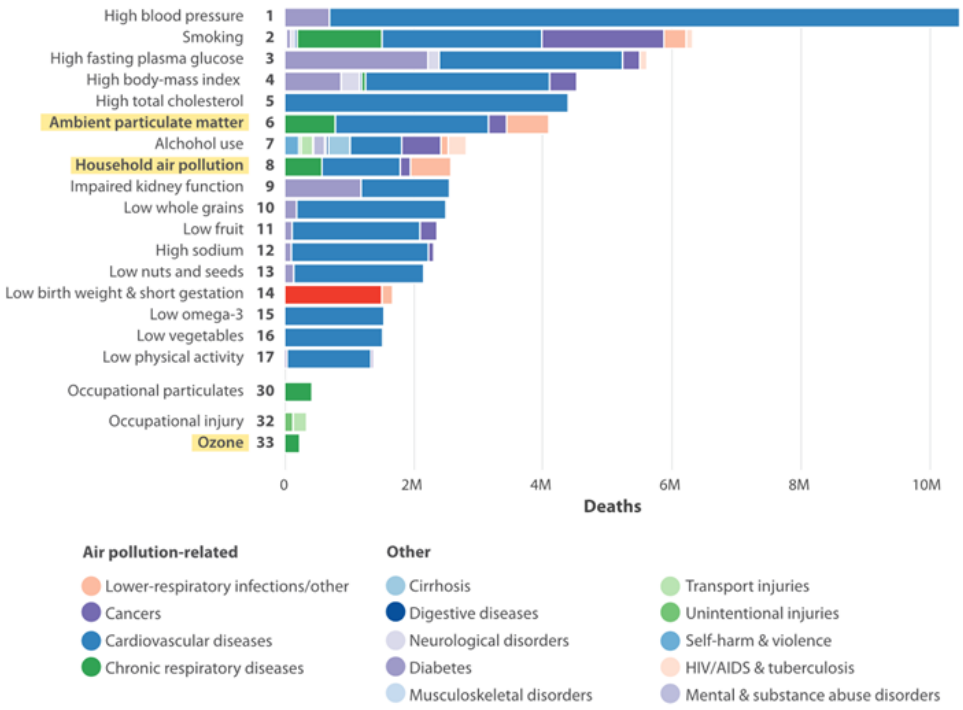


Figure 1.1 Global ranking of risk factors for a total number of deaths in 2016 (taken from the state of global air report 2018, health Effects Institute).

Worldwide ambient $PM_{2.5}$ concentrations continue to increase due to a rapid increase in the world's population and developments made around industries and transport sectors. About 92% of the world's population breath air with the annual average $PM_{2.5}$ concentration exceeding the Air Quality Guidelines set by WHO at 10 $\mu g/m^3$. Based on evidence of health effects of long term exposure to $PM_{2.5}$ and for the highest polluted regions of the world, WHO has also suggested three interim targets set at 35 $\mu g/m^3$, 25 $\mu g/m^3$ and 15 $\mu g/m^3$, and termed as WHO Interim Target 1 (WHO IT-1), Interim Target 2 (WHO IT-2) and Interim Target 3 (WHO IT-3) respectively. Figure 1.2 shows the population-weighted annual average $PM_{2.5}$ concentration in various regions of the world. It shows that about 54% population live in areas with $PM_{2.5}$ concentrations above IT-1; 67%

population lived in areas above IT-2 and 82% lived in areas exceeding IT-3 (figure 1.3).

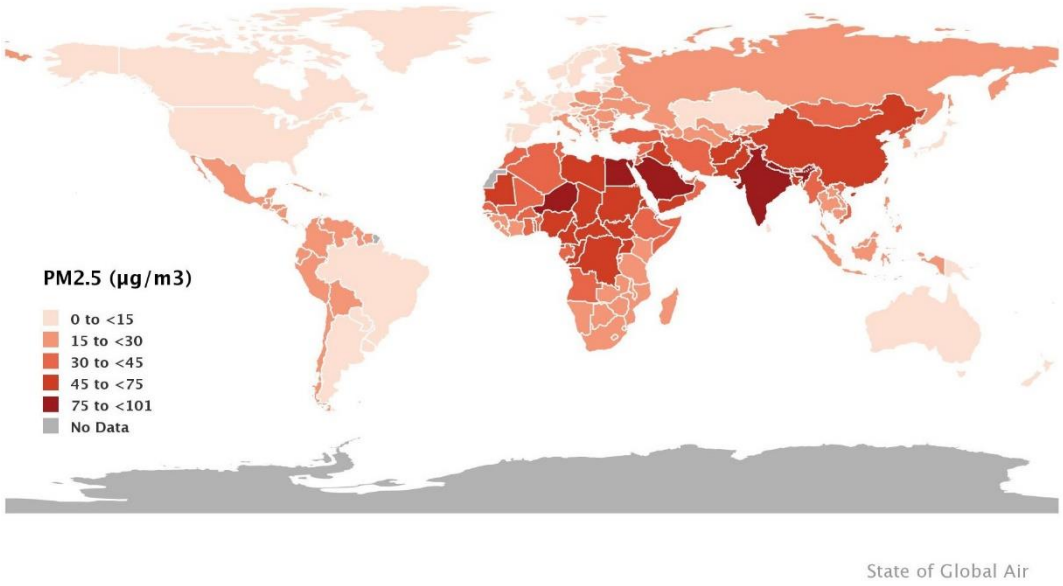


Figure 1.2 Population-weighted annual average $PM_{2.5}$ exposure around the world in 2017 (taken from state of global air report 2019).

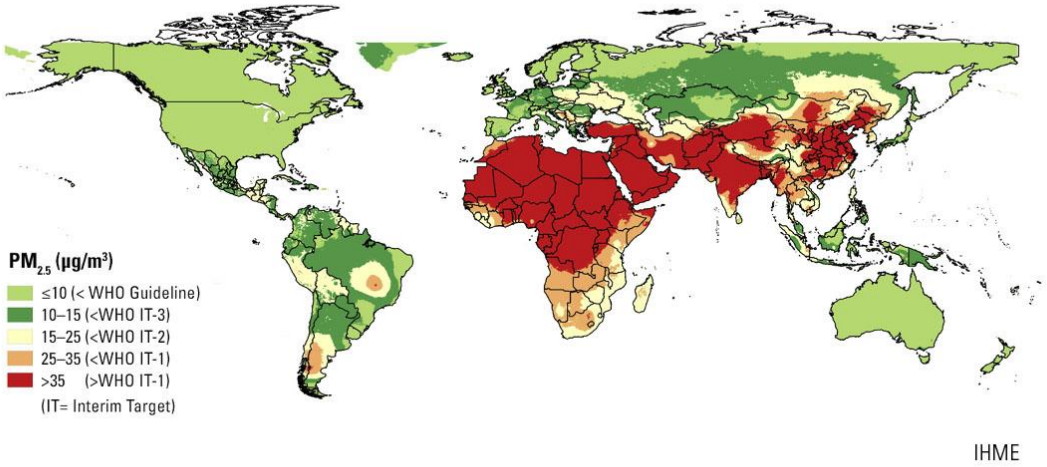


Figure 1.3 Global map comparing annual average $PM_{2.5}$ concentrations to the WHO air quality guidelines and interim targets (taken from state of global air report 2019).

Various emission sources contribute to ambient air pollution in urban and rural cities, emissions from traffic and industry being the major contributors in urban areas [28]. However, solid biomass burning for domestic cooking is the main source of fine particulate matter in many rural villages around the world. The contribution to ambient $PM_{2.5}$ from household solid fuel burning is disproportionately distributed among high-income and LMICs. A study showed that HAP contributed 12% of ambient $PM_{2.5}$ globally in 2010 whilst the contribution was nil in high-income regions but was 37% in Southern Sub-Saharan Africa [29]. Similarly, the contribution of household use of solid biomass on total emissions of $PM_{2.5}$ was 59% in Southeast Asia, 78% in Africa and 64% and 48% in India and China respectively in 2015 [30].

1.2 **Household Air pollution (HAP)**

Household air pollution (HAP) can be defined as conditions where harmful substances are present in indoor air in concentrations sufficient to produce adverse human health effects. The possible sources of HAP include indoor tobacco smoking, construction materials used in building houses, fuel used for cooking, heating and lighting, use of incense and various forms of mosquito repellents, use of pesticides and chemicals for cleaning at home, and use of artificial fragrances [31]. Use of solid biomass fuel for cooking and heating remain the most important source among those, particularly in LMICs. Approximately half of the world's population, and more than 90% of households in rural parts of developing

countries, primarily use solid biomass fuels for daily cooking and heating purposes [32]. Biomass fuel is typically combusted in low efficient stoves resulting in incomplete combustion which produces health damaging pollutants in a concentration high enough to have a significant health impact. A large proportion of the population around the world is exposed to HAP from burning solid biomass fuels for cooking (figure 1.4).

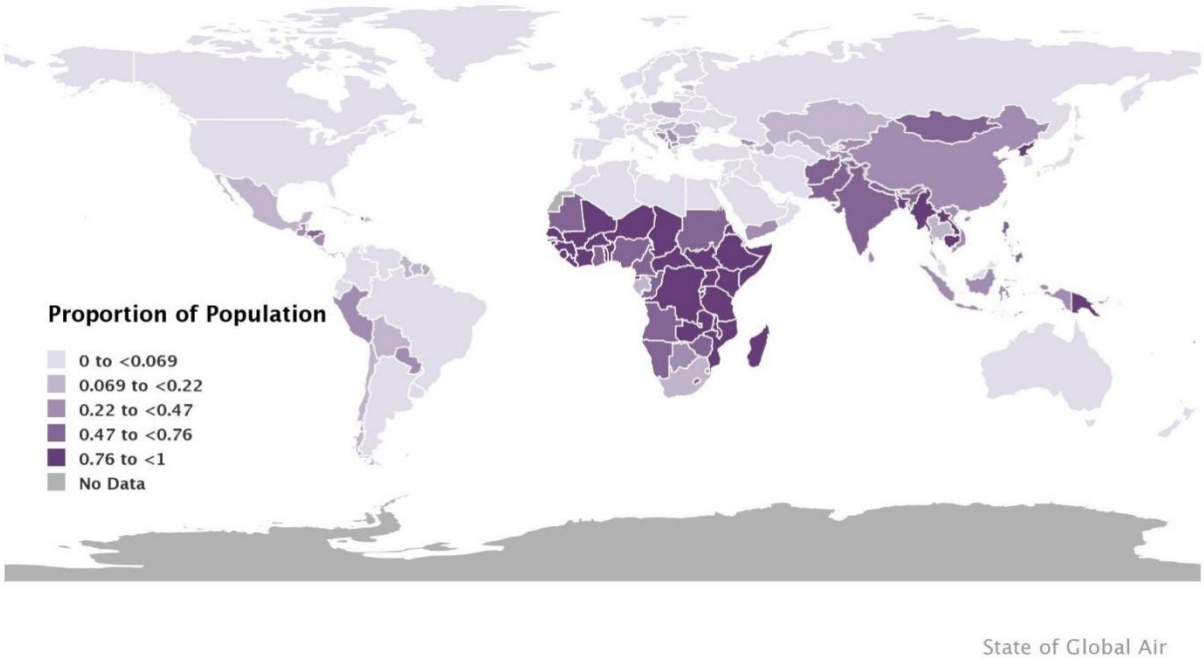


Figure 1.4 Proportion of population worldwide exposed to HAP from using solid biomass fuel for cooking in 2017 (taken from state of global air 2019 report).

HAP resulting from the combustion of solid biomass fuel is a major global burden of morbidity and mortality. In 2015, an estimated 2.8 million premature deaths and 85.6 million disability-adjusted life years (DALYs) were attributed to HAP [33]. In 2016, HAP exposure was estimated to be responsible for 7.7% of total global mortality, disproportionately distributed among high-income countries and LMICs [34]. In LMICs, HAP

contributed 10% of total mortality while only 0.2% of deaths in high-income countries were due to HAP exposure [34]. HAP is therefore considered as the most significant environmental contributor to ill health and is important mainly as a public health threat in LMICs. Given an estimation of 1.8 billion people will still rely on biomass fuel by 2040 [30], HAP will still be a significant threat to public health and will be a high risk for developing adverse health outcomes.

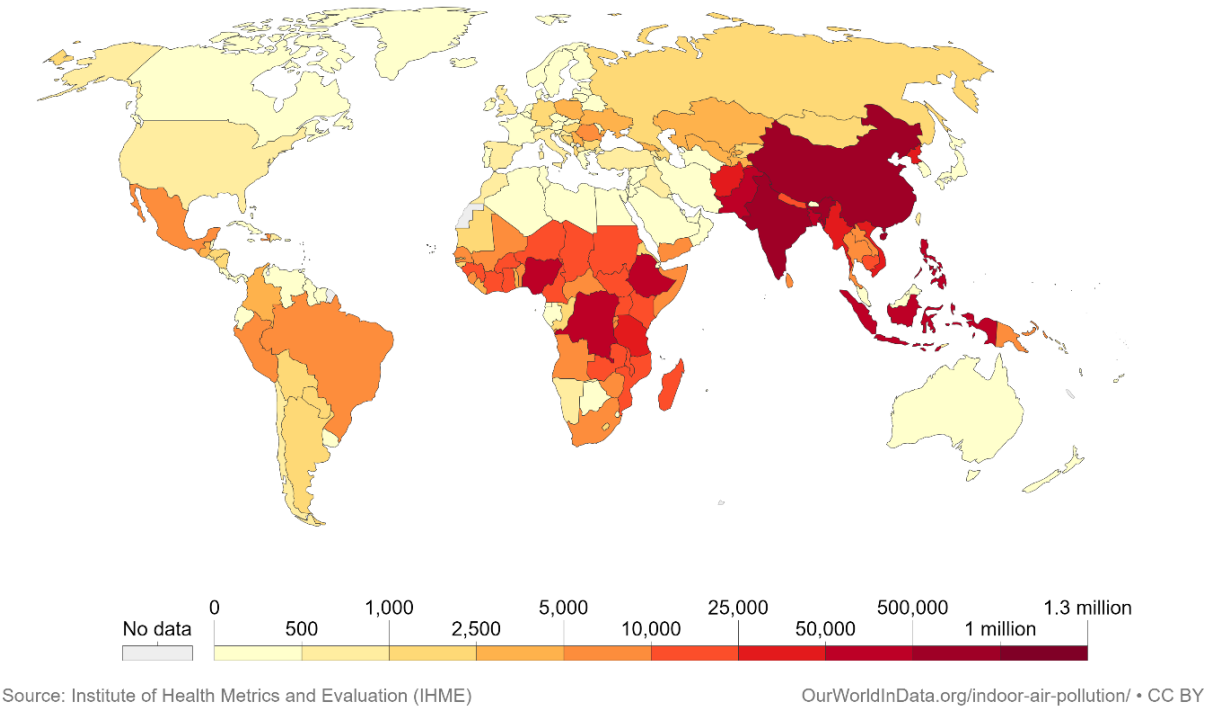


Figure 1.5 Absolute number of premature deaths attributed to an illness as a result of HAP exposure from the use of solid fuels for cooking and heating (taken from Institute of health metrics and evaluation).

1.2.1 Health effects of HAP

Growing scientific evidence links the exposure to HAP with acute lower respiratory infections in children under five, and ischaemic heart disease, stroke, COPD and lung cancer in adults [35]. The population using biomass fuel and inefficient cooking devices are also at a higher risk of burns, poisonings, musculoskeletal injuries and accidents. Studies have also shown association of low birth weight and stillbirth with prolonged use of biomass fuel for cooking [36, 37]. Lung function disorder and frequent respiratory symptoms in the population using solid biomass fuel for cooking has been reported in several studies. In rural Mexico, women using biomass fuel for cooking have been found to have frequent respiratory symptoms with a decrease in Forced Expiratory Volume in one second and Forced Vital Capacity (FEV_1/FVC) ratio [38]. Similarly, significant reductions in ventilatory function and increased airflow obstruction were reported among biomass users compared with liquefied petroleum gas (LPG) users for cooking in a study done in rural Nepal [39]. Further, a recent cross sectional study had shown a high prevalence of COPD in rural residences of Bangladesh [40] where the population using solid fuel exceeds 95% [41]. Also, Biomass smoke exposure has been found to be a major cause of COPD in most countries of sub-Saharan Africa, where 90% of rural households depend on biomass fuel for daily cooking and heating purposes [42]. In addition to this, one study has also shown the association of increased blood pressure with the exposure to

black carbon resulting from the combustion of biomass fuel for cooking [43].

Also, an increase infrequency of hospital admissions due to various health outcomes as a result of exposure to PM from biomass combustion has been reported in the previous studies. A recent study in Kaohsiung, Taiwan by Cheng MH et al. recorded a 3% (95% CI 1%-5%) increase in COPD admissions, a 4% (95% CI 1%-7%) rise in asthma admissions and a 3% (95% CI 2%-4%) increase in pneumonia admissions on cooler days (< 25°C) with a 10μg/m^3 advancement in $PM_{2.5}$ concentrations [44]. The study concluded that the observed increase in frequency of hospital admissions for respiratory diseases on cool days was due to the exposure of higher levels of PM [44]. This study was supported by results presented by Kloog et al. who showed 2.2% and 0.7% increase in hospital admission due to respiratory disease and cardiovascular disease with every 10 μg/m^3 increase in short term exposure to $PM_{2.5}$ [45]. This was similar to the association between $PM_{2.5}$ exposure and acute exacerbations of COPD shown by Ni et al. who found that activation of both pulmonary and systemic oxidative stress and inflammation was a result of $PM_{2.5}$ inhalation [46].

PM exposure from HAP is strongly associated with acute respiratory tract infections such as pneumonia and bronchiolitis, which are a leading cause of child mortality in the world [11]. The WHO estimated that globally in 2010, about 1.4 million children under the age of 5 years died from acute

lower respiratory infection (ALRI) [47] of which more than 50% deaths was attributed to HAP from solid fuel use [48]. A case control study done in Nepal by Bates et al. suggested that children younger than 36 months living in houses using solid fuel are at high risk of developing ALRI [49]. Solid fuel smoke exposure is also found to be consistently associated with an increased risk of lung cancer in both males and females [6]. Exposure to carcinogens from HAP contributes to approximately 17% of annual premature deaths from lung cancer in adults [48]. It was estimated that in LMICs about 22% and 17% of all deaths in women and men due to lung cancer were accounted for solid fuel smoke exposure [50].

A growing body of scientific evidence correlates solid biomass fuel smoke exposure with an increased risk of developing COPD [9, 10, 51, 52]. The prevalence of COPD due to solid fuel smoke exposure was found to be high in developing countries where the dependency of biomass fuel for daily cooking and heating is high [53]. Several cross-sectional, case control, longitudinal and meta-analysis studies consistently showed a direct relationship between HAP and an increased rate of COPD. For example, the meta-analysis done by Kurmi et al. showed a strong association between biomass smoke exposure and developing COPD (OR = 2.80 CI 1.85 to 4.0) and chronic bronchitis (OR 2.32, 95% CI 1.92 to 2.80) [10]. In addition to this, Orozco-Levi et al. showed a strong relationship between the exposures to wood or charcoal smoke with COPD [54]. Moreover, a meta-analysis performed by Hu et al. showed a strong association of biomass smoke exposure with the development of COPD in

both women (OR, 2.73 95% CI, 2.28-3.28) and men (OR 4.30, 95% CI 1.85-10.01) [51]. A cross sectional study in five cities of Colombia (a prevalence study of COPD) elucidated that the use of biomass stoves for more than 10 years was associated with COPD (OR 1.5, 95% CI, 1.22-1.86) [55]. Several other studies in different parts of the world also suggested that daily exposure of biomass fuel smoke increases the prevalence of chronic bronchitis and COPD [56, 57]. However, despite the evidence of this association, very little work has been done to date looking at the mechanisms underlying the relationship between biomass smoke exposure and COPD. An issue of absence of causal evidence showing a direct association of biomass smoke exposure with COPD was pointed out by Fanny et al.[58].

1.3 **Chronic Obstructive Pulmonary disease (COPD)**

1.3.1 Prevalence and Importance

COPD is a common disease and an increasingly important contribution to world public health problems [59]. Worldwide, COPD is the fourth leading cause of death and is expected to rise to the third leading cause by 2030 [60]. In 2012, above 3 million people died due to COPD accounting for 6% of all deaths globally [61]. Currently, around 210 million people are suffering from COPD globally [62]. The prevalence of COPD is even more alarming in LMICs. WHO estimated that above 90% of COPD deaths in the world occur in LMICs [61]. In Nepal, for example, non-communicable diseases are estimated to account for 60% of total deaths of which

chronic respiratory disease contributed 13% [63]. According to the WHO country specific statistical profiles, COPD was a leading cause of death in Nepal in 2012, killing about 17,000 people. The current trend of increasing prevalence of COPD has become a serious social and economic burden for the world.

Smoking is known to be a leading cause of developing COPD throughout the world [58, 60]. Higher prevalence of lung function abnormalities and a higher COPD mortality rate has been recorded in cigarette smokers than non-smokers [64]. However, various studies show evidence that smoking is not the only cause of COPD. A study conducted by Salvi et al. pointed out that an estimated 25-40% of patients with COPD are a non-smoker.[65]. Recent data from NHANES III shows 25% of COPD cases in the United States occur in lifelong non-smokers [66], similar to this 22.9% in the United Kingdom [67] and 23.4% in Spain [68]. The incidence of COPD among non-smokers in developing countries may be even higher. For instance, 26% of subjects with irreversible airflow obstruction in the PLATINO study performed in five Latin American cities were found in individuals who have never smoked [69]. Similar results were presented by Lamprecht et al. with the data analysed from 14 different countries, reporting a considerable proportion of non-smokers population with COPD [70].

A reduction in the ability to produce sufficient serum levels of Alpha-1-Antitrypsin, commonly known as alpha-1 antitrypsin deficiency, is

considered as one rare genetic factor responsible for developing COPD [71]. However, the percentage of all individuals with COPD due to alpha-1 antitrypsin deficiency syndrome is very low. For example, Hall et al. found that only 1-2 % of all individuals with COPD have severe alpha-1-antitrypsin deficiency [72].

As discussed above, long term exposure of solid fuel smoke is another possible cause of developing COPD, particularly in the rural developing world where solid fuels are being combusted every day for cooking. Accor ding to WHO, 22 % of the total deaths attributed to HAP in 2012 were due to COPD. Exposure to HAP is responsible for over one-third of premature deaths from COPD in LMICs [5]. This indicates that although cigarette smoke is the leading cause of COPD in developed countries, solid fuel smoke exposure is the leading cause of developing COPD in LMICs. Assad et al. stated in a recent study that biomass smoke exposure is directly associated with both phenotypes (Chronic bronchitis and emphysema) of COPD [73].

1.3.2 COPD: Definition

COPD is defined as a lung function disorder characterized by persistent obstruction of airflow in the lungs that is usually progressive over time and not fully reversible. COPD is an inflammatory disease. The pathogenesis of COPD normally involves small airway fibrosis and alveolar destruction [36]. The term chronic bronchitis and emphysema was previously used to describe small airways inflammation (Figure 1.6) and

lung parenchyma destruction (Figure 1.7) as two separate disease processes. However, it is now recognised that a person with COPD may have either one or both conditions. Both disease processes cause irreversible airflow obstruction and reduce lung function. According to the GOLD guideline updated in 2019, COPD is defined as *a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases [60].* Roisin et al. mentioned that COPD patients are normally diagnosed with acute exacerbations with increased dyspnea, cough with altered sputum and fever as common signs and symptoms of exacerbations [74]. The comorbidities associated with COPD are also extensive. For example, the most common comorbidities in a study of 183,681 patients with COPD were found to be cardiovascular disease (34.8%), diabetes (22.8%), asthma (14.7%) and anaemia (14.2%) [75].

1.3.3 Pathology of COPD

Airway inflammation, mucociliary dysfunction and consequent airways and lung parenchyma structural changes are the pathological features of COPD [76]. As discussed above, structural changes in airways and lung parenchyma are best described by two separate syndromes, chronic bronchitis and emphysema, which occur to a variable extent in COPD patients.

Chronic bronchitis

Chronic bronchitis is a chronic inflammatory disorder of the airways usually caused by exposure to noxious particles and gases (mainly cigarette smoke) [62] which produces a narrowing of the airways [77]. Excess cough and mucus production in the airways are common features of chronic bronchitis [62]. Several studies have identified the presence of excessive numbers of CD8+ lymphocytes in smokers, which contributes to the inflammation in the airways [78, 79]. The excess sputum production results in obstruction in the peripheral airways. Mucous gland enlargement in the airway walls due to smoking and inflammation causes goblet cell metaplasia and leads to healthy cells being replaced by more mucus-secreting cells [80]. The mucociliary transport system responsible for clearing mucus from the airways is also affected and damaged by the inflammation, which causes excess mucus accumulation in the airways resulting in deteriorating airflow obstruction [80].

Figure 1.6 Chronic Bronchitis. Small airways with excess mucus which further narrows the airways. Adapted from http://www.tudorza.com/what-is-copd.aspxb

Emphysema

Emphysema is a syndrome that primarily causes shortness of breath and is clinically defined as an "abnormal permanent enlargement of airspaces distal to terminal bronchioles, accompanied by destruction of their walls without obvious fibrosis" [81]. The definition focuses on the destruction of lung alveolar surface reducing the gas exchanging surface area, which reduces the elastic recoil force available to drive air out of the lung. Hence emphysematous destruction of the lung surface is responsible for reduced gas exchange.

Hence, air trapping and progressive airflow limitation with excess sputum production due to these pathological changes in the lung parenchyma tissue and small airway fibrosis both contribute to COPD. Besides these common syndromes, the destruction of small airways in COPD also occurs due to the damage in normal repair and defence mechanisms of airway epithelium [82, 83].

Figure 1.7 Emphysema. Health and alveolar with emphysema. Adapted from http://www.daviddarling.info/encyclopedia/E/emphysema.html

1.4 **Biomass fuel combustion**

Inefficient combustion of solid biomass fuels can emit a large number of chemicals harmful to human health. Biomass fuels are an organic material, primarily composed of two carbon-based polymers, cellulose $(C_6H_{10}O_5)_n$ about 50-70% by weight and lignin $(C_{31}H_{34}O_{11})_n$ approximately about 30% by weight [84]. The combustion of fuels breaks down these polymers into a variety of small molecules that result in release of carbon to the atmosphere as carbon dioxide. The chemical process during the combustion follows the equation below

$$CH_2O + O_2 \rightarrow CO_2 + H_20 + Heat \ldots\ldots\ldots \text{equation 1.}$$

This equation holds good only for the case of complete combustion. However, due to various factors in the real kitchen environment, including the types of fuels, fuel moisture content, inefficient stove design and insufficient oxygen supply, in most of the cases, complete combustion may not be possible. As a result of incomplete combustion, a large amount of partially oxidized organic chemicals is generated during biomass burning. Hence residential biomass fuel burning is considered as an indoor and ambient air polluting source as it contributes to a wide range of unwanted chemicals to the environment. It is well understood from previous findings that biomass fuel burning releases a significant amount of carcinogenic pollutants like polycyclic aromatic hydrocarbons (PAHs), aldehydes, particulate matters, noxious gases and other free radicals [85-87]. The pollutants from the combustion depend on various

factors like types of biomass fuel, types of stove used, combustion rate, fuel moisture content and the way fuels are being used. The major health damaging pollutants from biomass combustion is well summarized by Naeher et al. [17], presented here in Table 1.1.

Table 1.1 Major Health damaging pollutants from biomass combustion [17].

Compound	**Examples**	**Source**	**Notes**	**Mode of Toxicity**
Inorganic gases	Carbon Monoxide (CO)	Incomplete combustion	Transported over distances	Asphyxiant
	Ozone (O_3)	Secondary reaction, product of nitrogen dioxide and hydrocarbons	Only present downwind of fire, transported over long distances	Irritant
	Nitrogen dioxide (NO_2)	High temperature oxidation of nitrogen in air, some contribution from fuel nitrogen	Reactive	Irritant
Hydrocarbons	Many Hundreds	Incomplete combustion	Some transport-also react to form organic aerosols. Species vary with biomass and combustion conditions	
	Unsaturated: 40+ e.g. 1,3-butadiene			Irritant, carcinogenic, mutagenic
	Saturated: 25+ e.g. n-hexane			Irritant, neurotoxicity
	Polycyclic aromatic (PAHs): 20+ e.g. benzo[a]pyrene			Mutagenic, carcinogenic
	Monoaromatics: 28+ e.g. benzene, styrene			Carcinogenic, mutagenic
Oxygenated organics	Hundreds	Incomplete combustion	Some transport-also react to form organic aerosols. Species vary with biomass and	
	Aldehydes: 20+ e.g. acrolein,			Irritant, Carcinogenic, mutagenic

	formaldehyde		combustion conditions	
	Organic alcohols and acids: 25+ e.g. methanol acetic acid			Irritant. teratogenic
	Phenols: 33+ e.g. catechol, cresol (methylphenols)			Irritant, Carcinogenic, mutagenic, teratogenic
	Quinines: Hydroquinone, fluorenone, anthraquinone			Irritant, allergenic, redox active, oxidative stress and inflammation
Chlorinated organics	Methylene chloride, methyl chloride, dioxin	Requires chlorine in the biomass		Central nervous system depressant (methylene chloride), possible carcinogens
Free radicals	Semiquinone type radicals	Little known about their formation		Redox active, cause oxidative stress and inflammatory response, possibly carcinogenic
Particulate matter (PM)	Inhalable particles (PM_{10})	Condensation of combustion gases, incomplete combustion, entrainment of vegetation and ash fragments	Coarse and fine particles. Coarse particles are not transported far and contain mostly soil and ash	Inflammation and oxidative stress, may be allergenic
	Respirable particles	Condensation of combustion gases, incomplete combustion	For biomass smoke, approximately equal to fine particles	
	Fine particles ($PM_{2.5}$)	Condensation of combustion gases, incomplete combustion	Transported over long distances, primary and secondary production	Inflammation and oxidative stress, may be allergenic.

PAHs are one of the major groups of pollutants produced from the incomplete combustion of fuels. Biomass fuel combustion in household use for cooking and heating is one of the potential sources of PAHs. In

2004 about 57% of total global emissions of PAHs were estimated to be sourced from biomass fuel burning [88]. Over 80% of emissions of total PAHs are from developing countries where biomass dependency for household energy is high and is compounded by using low combustion efficiency stoves [88, 89]. The main component of PAHs emitted from biomass fuels is associated with fine particulate matter. The fraction of fine PM with a size less than 2.5 μm in a total PAHs emitted from fuelwood, brushwood and bamboo were approximately 86.7%, 65% and 79.7% respectively [89].

Different fuels emit particles with varying chemical composition, which also depends on the technology used for combustion. Emissions were found to be influenced by fuel type, burning appliance and operating conditions [90]. In a recent study performed by Evtyugina et al., the emissions from three commonly used woods combusted in a domestic cookstove and fireplace were measured. The highest emissions of VOCs were noted from *Black Poplar* combusted in a fireplace, whereas *Pyrenean oak* contributed maximum emissions in the woodstove [90]. Furthermore, a study in rural China reported significantly higher emissions from the wood and coal cake combusted in a simple metal stove than those from coal briquette [91]. The study found the emission factors of 28 parent PAHs, 4 oxygenated PAHs, and 9 nitro-PAHs as 182-297, 7.8-10, 0.14-0.55 mg/kg for the wood, 14-16, 1.7-2.6, 0.64-0.83 mg/kg for the briquette and 168-223, 4.7-9.5, 0.16-2.5 mg/kg for the coal cake

respectively [91]. Similarly, the combustion of three types of Pakistani wood showed a varying concentration of total carbon emissions [92].

1.5 Household air pollutant

1.5.1 Particulate matter (PM)

PM is a complex mixture of solid particles and liquid droplets of varying size, shape and composition found in the air. The mixture includes both organic and inorganic particles such as dust, dirt, pollen, soot and smoke. Many of these particles are hazardous and vary greatly in size, composition and origin. Some of these particles like dust, dirt or smoke can be detected with the naked eye although it also contains smaller particles that can only be seen with an electron microscope. PM in the air can be generated directly from a range of different anthropogenic activities such as industry, transport, biomass combustion, solid waste burning, brick kiln and also through natural sources like desert dust, volcanic emissions, forest fires, and soil erosion. There are some particles present in the air which are formed indirectly in the atmosphere by the transformation of gaseous pollutants into liquid or solid particles through different processes. Source specific PM study is very important as the physical and chemical properties of PM emitted from different sources varies considerably. In addition to this, it is also very important to understand the different size fractions of PM. Comparative size fractions of PM can be seen in Figure 1.8.

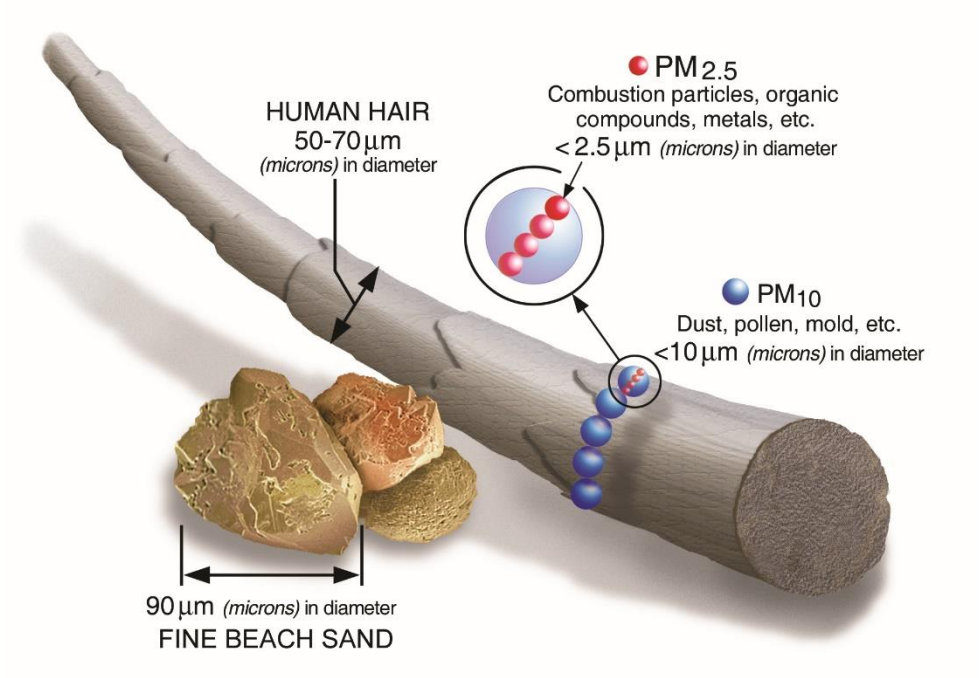


Figure 1.8 The figure represents the size comparisons for particulate matter (taken from US-EPA website (https://www.epa.gov/pm-pollution/particulate-matter-pm-basics).

The particles are normally classified by their aerodynamic properties. These properties determine the transport and deposition of the particles into the air passages of the respiratory system. Particles having same aerodynamic characteristics are normally expressed in terms of a diameter of an idealised sphere, which is referred to as aerodynamic diameter. The particles are also normally sampled based on of their aerodynamic diameter. Based on particle size, PM is often divided into a coarse fraction and fine fraction. The particles with an aerodynamic diameter ranging from 2.5 to 10 µm are considered as a coarse particle and normally expressed as PM_{10}. The fine fraction of PM contains the particles with aerodynamic diameter up to 2.5 µm which is normally expressed as $PM_{2.5}$. Also, the particles which are smaller than 0.1 µm are

known as ultrafine particles. These sizes of PM are important in relations to their ability to reach deep into the respiratory system and stimulate immune responses based on their chemical and biological characteristics.

1.5.1.1 PM deposition and clearance

A normal human airway system can be divided into three main regions (figure 1.9) [93]. The upper part of the airways (also referred to as the nasopharyngeal region) consists of nasal passages, mouth, pharynx and larynx. The main function of this region in the respiratory system is to filter large particles and transport the filtered air to the lower region of the airways. The conducting airways, also called the tracheobronchial region, includes the trachea and large bronchi. The trachea bifurcates into the two main bronchi which further divide into the sequence of small airway into the lungs. The air gets further filtered and humidified in this region and channelled to the alveolar region for gas exchange. The gas exchange takes place in small air sacs, also called alveoli, which are surrounded by pulmonary capillaries. The blood in the capillaries takes up oxygen from the inhaled air and expels carbon dioxide into the alveoli.

In a highly polluted area, a large number of particles and other gaseous pollutants can enter into the respiratory airway tract during the gas exchange process. The potential health effects of particle exposure are highly dependent on particle deposition and retention in the respiratory tract and particle clearance mechanisms. As mentioned above, the deposition and retention mechanism within the respiratory tract is highly

influenced by the particle aerodynamic properties [94]. Size depended on the deposition of particles in different regions of the respiratory tract is shown in figure 1.10 [95]. In general, a large fraction of ultrafine and coarse particles can reach up to the nasopharyngeal region and are normally removed from the region through exhalation. Smaller percentages of ultrafine and coarse particles can pass through the upper airways and are deposited in the bronchial region. Fine and ultra-fine particles can penetrate and are highly deposited into the alveolar region. The deposited ultrafine particles can be absorbed into the bloodstream and translocated into the secondary organs. Particle deposition is also dependent in part on other particle properties including shape, density and hydroscopicity [94]. The other influences on deposition are the breathing patterns and the geometry and size of the respiratory tract. The changes in air flow and particle residence time depend on a person's breathing frequency which has a major influence on deposition mechanisms. The breathing pattern may be affected in a person with lung disease due to the changes in the geometry of the bronchial and alveolar regions. Hence the particle deposition increases in people with lung disease compared to healthy people [96, 97].

The retention time and clearance of the particles from the respiratory tract are dependent on the site of deposition and particle characteristics. A large fraction of inhaled particles is exhaled through the normal respiratory mechanism before they get deposited in the airways. Most of the deposited particles in the nasopharynx are then effectively removed

through the gastrointestinal tract. Particles are also transported back to the pharynx with mucus through the action of ciliated cells in the respiratory epithelium and swallowed into the gastrointestinal tract. The clearance of deposited particles in the absence of ciliated cells from the alveolar region is through activation and uptake by alveolar macrophages. The activated alveolar macrophages can clear the particles through phagocytosis. This mechanism of particle clearance is less effective for ultrafine particles as alveolar macrophages are less efficient to phagocytose these smaller particles. Hence ultrafine particles always likely to remain in lungs for a more extended period and may, therefore, result in more serious lung injury. Also, the particles remaining in the alveolar region are likely to interact with the type 2 alveolar epithelial cells and be translocated to the blood stream and the other secondary organs [98].

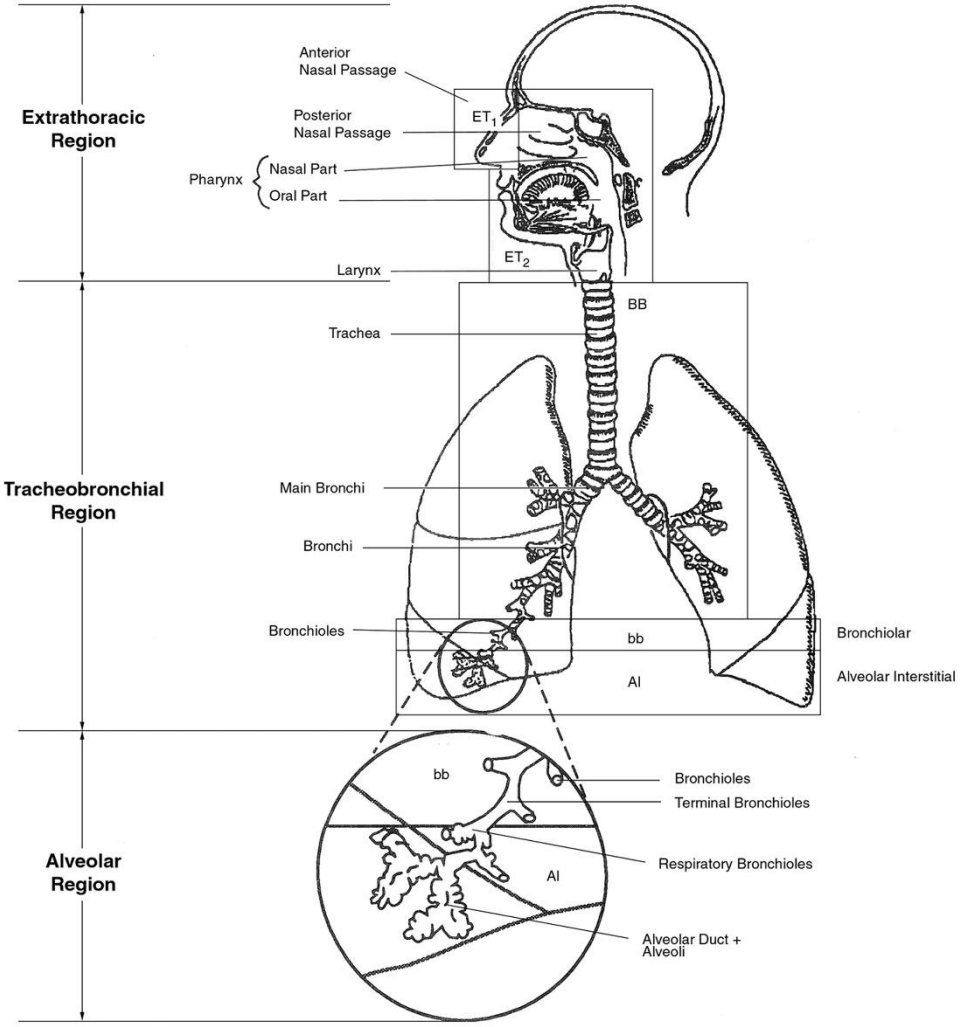


Figure 1.9 Major anatomical regions of a Normal Human Respiratory system. The upper region called extrathoracic region including anterior nasal passages, posterior nasal passages, pharynx, and larynx. The tracheobronchial region includes trachea, bronchi, bronchioles and terminal bronchioles. Alveolar region consists of respiratory bronchioles and alveolar ducts and sacs surrounded by alveoli. Adapted from [93].

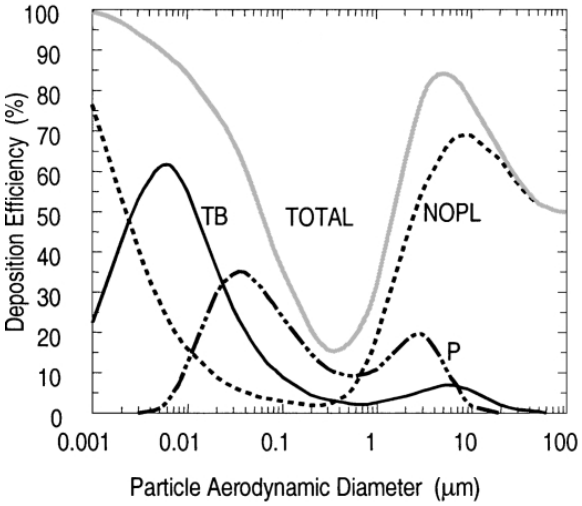


Figure 1.10 Particle deposition in the major regions of the human respiratory tract during normal respiration corrected for the size-dependent inhale ability NOPL: Naso-oropharyngolaryngeal region, TB: Tracheobronchial region, P: Pulmonary region (Adapted from [95].

1.5.2 Carbon monoxide (CO)

Carbon monoxide (CO) is a colourless, odourless, non-irritant and tasteless toxic gas which is mainly produced by the incomplete combustion of carbonaceous fuels like wood, dung, coal, natural gas, petrol and kerosene [99]. The atmospheric CO emissions are from various anthropogenic activities, contributing about two-thirds of the total emissions, whereas natural sources contribute another one third [100]. Incorrectly installed and poorly ventilated cooking and heating appliances that burn fossil fuel are the potential sources of indoor CO in developed countries, whereas combustion of biomass fuel for cooking in a low efficient stove and smoking are the main sources in the homes of developing countries. Various studies in developing countries have reported an increased level of CO in indoor kitchens using biomass fuels

for cooking and heating [20, 101-103]. Individuals present in such an environment are exposed to CO through inhalation during the normal respiratory process. Inhalation is the only route for CO exposure in humans. Exposure to CO is hazardous and may lead to various respiratory, cardiovascular and neurological disorders [100, 104]. However, the effect of CO exposure on human body depends on the degree of exposure [105].

CO is a gas which is slightly soluble in water, blood stream and plasma. CO inhaled into the human lung is first dissolved in the blood and quickly binds with haemoglobin (Hb) to form carboxyhaemoglobin (COHb) [106]. Unlike oxygen binding to Hb, which is quickly and easily dissociated, CO remains bound with Hb for a much longer time. Hence, COHb accumulates in the blood upon continued exposure which results in less available haemoglobin for oxygen transport [107]. This leads to arterial hypoxaemia and tissue hypoxia [108]. The higher percentage of COHb in the blood also enhances the binding strength of oxygen to Hb which results in more difficulty in releasing oxygen into the tissue [109].

1.5.3 Household air pollution Exposure

A rising number of studies around the world have shown high levels of HAP in households using biomass fuel for cooking and heating. The number of HAP studies is not surprisingly disproportionately higher in LMICs compared to developed countries. The emission monitoring studies performed in the past were mainly focused on indoor levels of pollutants

over a fixed period. However, monitoring personal exposure levels of inhabitants to indoor pollutants during cooking has gained more attention in recent years. The majority of these studies where actual exposure from biomass combustion have been measured examine PM of different size factors and CO as a surrogate measure of all HAP.

In a previous study performed in Guatemala, 24-h indoor concentrations of PM_{10} in households using biomass fuel combusted in open fires were 717 $\mu g/m^3$ [110]. High levels of PM_{10}have also been measured in kitchens with biomass burning from other countries such as 1390 $\mu g/m^3$ in rural Mexico [38], 1518 $\mu g/m^3$ in Pakistan [111], 1177 $\mu g/m^3$ in Bangladesh [112] and 3233 $\mu g/m^3$ in Nepal [113]. In more recent studies $PM_{2.5}$ has been used as a PM metric. All those studies from different regions reported a high concentration of indoor $PM_{2.5}$ in the kitchen using biomass fuel. These levels of $PM_{2.5}$ were several folds higher than the WHO recommended safe limit. The levels of 24-hour indoor $PM_{2.5}$ in the study from different countries were 1920 $\mu g/m^3$ in Bangladesh [114], 635 $\mu g/m^3$ in Guatemala [110], 490 $\mu g/m^3$ in Mexico [38] and 430 $\mu g/m^3$ in Sri Lanka [115]. The indoor concentration of $PM_{2.5}$ measured in previous studies performed in various regions of Nepal showed a similar pattern. The highest concentration of $PM_{2.5}$ (3370 $\mu g/m^3$) was recorded in households of Dolakha using biomass fuel for cooking [116]. In the same study, the reported indoor $PM_{2.5}$ concentrations in the other two districts, Dang and Ilam was 2650 $\mu g/m^3$ and 890 $\mu g/m^3$ respectively [116]. The 48

h mean indoor $PM_{2.5}$ concentration was reported to be the lowest (417 $\mu g/m^3$) in households in Janakpur, but the mean cooking period emissions were measured 966 $\mu g/m^3$ [20]. The household using biomass fuel for cooking from other regions of the country also showed a significantly high concentration of indoor $PM_{2.5}$ [19, 117-120]. Taken together, these data show that high levels of personal exposure to indoor $PM_{2.5}$ are likely to be very common in rural households cooking with biomass.

Table 1.2 Pollutant concentrations of $PM_{2.5}$ and CO reported in households using biomass fuel for cooking in different regions of Nepal.

Exposure type/Period	Stove Type	$PM_{2.5}$ ($\mu g/m^3$)	CO (ppm)	Location	Reference
Indoor/48 h	TCS	417.6±686.4	5.4±4.3	Janakpur	[20]
Indoor/Cooking		966±1384	8.3±5.2		
Indoor/24 h	TCS	1336±952.8	36.03±19.1	Palpa	[117]
	ICS	825.4±730.9	27.11±14.2		
Indoor/24 h	TCS	650±959	8.9±8	Sarlahi	[118]
Indoor/24 h	TCS	1376	10.9	Sarlahi	[19]
Indoor/24 h	TCS	630±908	N/A	Bhaktapur	[119]
Indoor/Cooking	TCS	1550±1050	N/A	Dhanusha	[120]
Indoor/24 h	TCS	3370±1650	38.7±13.7	Dolakha	[116]
	ICS	1430±810	17.2±7.7		
	TCS	2650±1950	26.3±23.2	Dang	
	ICS	750±510	8.32±5.53		
	TCS	890±720	8.66±4.74	Ilam	
	ICS	310±190	3.34±1.95		

1.6 **Inflammation**

Inflammation is the body's normal response to pathogens, pollutants and other harmful stimuli to protect tissues from those injurious agents. It can be broadly defined as a non-specific protective reaction of living vascularised tissues to any injurious agent [121]. Inflammation is usually protective and beneficial but also has the potential to cause tissue damage. The classical features of an inflammatory response involve an increase in blood flow, an increase in vascular permeability, an infiltration of cells and proteins into the site of injury and a release of materials at the site of inflammation [121]. In general, the inflammatory mechanism involves the recognition of the injurious agent, recruitment of the inflammatory cells into the site of inflammation, removal of injurious agent from the tissue and repair and resolution of inflamed tissue [122]. The first phase of the normal inflammation process starts within minutes or hours after pathogen challenge and is called acute inflammation. If the resolution process of acute inflammation is incomplete, the process leads to chronic inflammation. The inflammatory cells mainly involved in acute inflammation are polymorphonuclear leukocytes i.e. neutrophils, whereas, in chronic inflammation lymphocytes, monocytes and macrophages are the active inflammatory cells.

Exposure to harmful environmental toxins, pollution and particles from various sources is a major cause of airway inflammation. Airway inflammation is initiated by the recognition of the injurious agent by

particle recognition receptors (PRRs). Toll-like receptors (TLRs) are membrane surface receptors which recognize the molecular patterns expressed by invading pathogens, known as pathogen-associated molecular patterns (PAMPs). In addition, receptors that reside inside the cytoplasm known as Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) may also play a role. The inhaled particles or the molecular patterns expressed by the pathogens that are first recognized by TLRs activate in turn dendritic cells, alveolar macrophages and epithelial cells. The activation of these cells increases levels of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and leads to the production of growth factors, chemokines and pro-inflammatory cytokines such as IL-8 and TNF-α. IL-8 is a chemotactic agent for neutrophils and TNF-α enhances expression of endothelial cell adhesion molecules. These increased expressions of inflammatory mediators activate and recruit the inflammatory cells into the tissue. The pathogen or any foreign agents are then taken up by alveolar macrophages by phagocytosis. After successful host defence repair and resolution of tissue injury occurs. An overview of general inflammatory signalling pathways is shown in Figure 1.11 (taken from [123]).

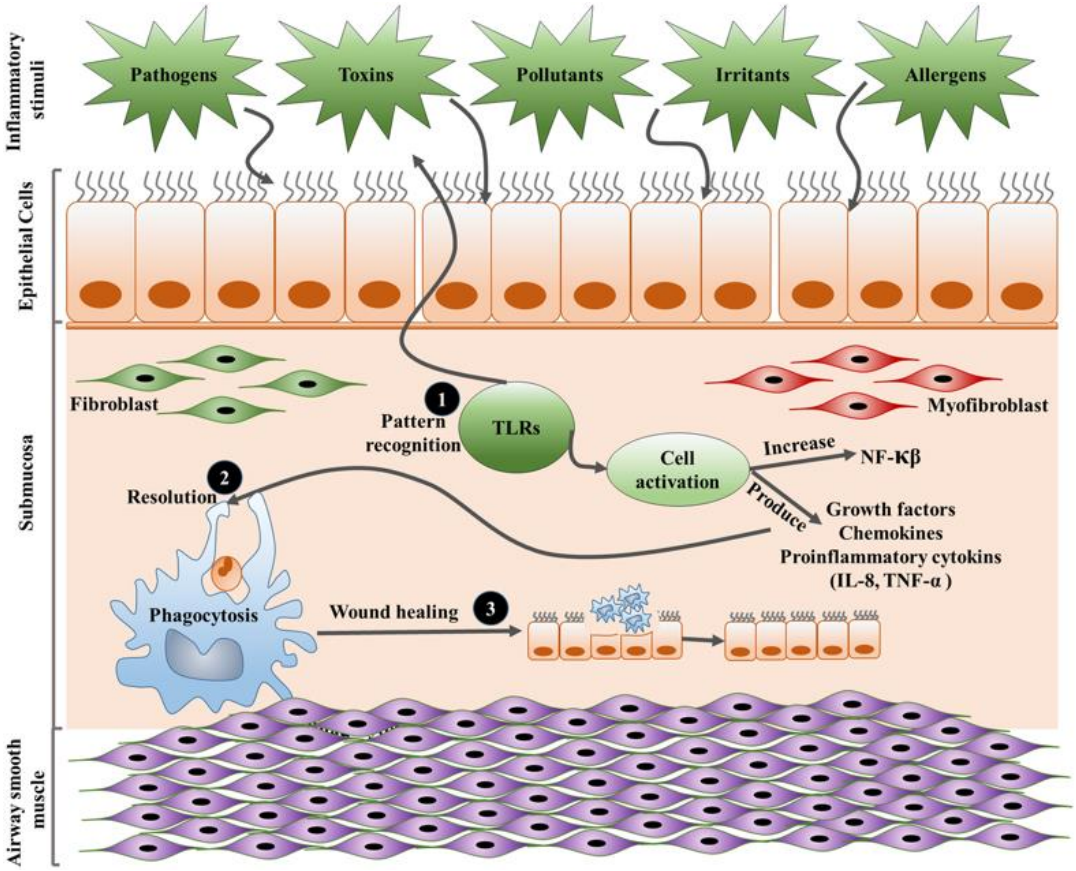


Figure 1.11 General inflammatory signalling pathways in airways disease. Foreign inflammatory stimuli like pathogen, toxins, pollutants, allergens interact with airway epithelial cells and activate it. The surface receptors like TLRs recognize the molecular pattern produced by the pathogens and activate inflammatory cells like NF-kB and produce growth factors, chemokines, pro-inflammatory cytokines like IL-8, TNF-α. The activated cells start killing the pathogen by phagocytosis, followed by wound healing to repair damaged epithelial cells. TLRs: Toll-Like-receptors, NF-kB: nuclear factor kappa-light-chain-enhancer of activated B-cells, IL-8: Interleukin 8, TNF-α: tumor necrosis factor-alpha. (Adapted from the review article [123]).

1.6.1 Role of Inflammation in COPD

Chronic inflammation plays an essential role in an inflammatory disease like COPD resulting in both small airway remodelling and narrowing and

emphysema. Small airway fibrosis, alveolar wall destruction and mucus hypersecretion in COPD are accompanied by increased numbers of alveolar macrophages, neutrophils and CD8+ T-lymphocytes [124]. The initial site of contact with any inhaled antigens in the pulmonary system is the airway epithelial cells which protect the respiratory tract from pathogen attack by secreting a variety of substances like mucins, defensins, lysozyme, lactoferrin and nitric oxide [125]. Both epithelial cells and macrophages become activated in response to inhaled irritants such as cigarette smoke and biomass smoke, and other foreign antigens present in the airways. These activated cells produce several inflammatory mediators, cytokines, reactive oxygen radicals and platelet-activating factor to recruit inflammatory cells into the site of injury [122]. These inflammatory mediators attract CD8+ T-lymphocytes, neutrophils and monocytes into the affected areas. T-lymphocytes and neutrophils are responsible for activation of airway fibroblasts which in turn results in small airway fibrosis. In addition, macrophages and neutrophils release a large number of proteases like Cathepsin G, Neutrophil Elastase and matrix metalloproteinases-8 and -9 (MMP-8 and -9) which are also responsible for mucus hypersecretion and alveolar wall destruction. An overview of inflammatory response in COPD is shown in Figure 1.12 (taken from [126]).

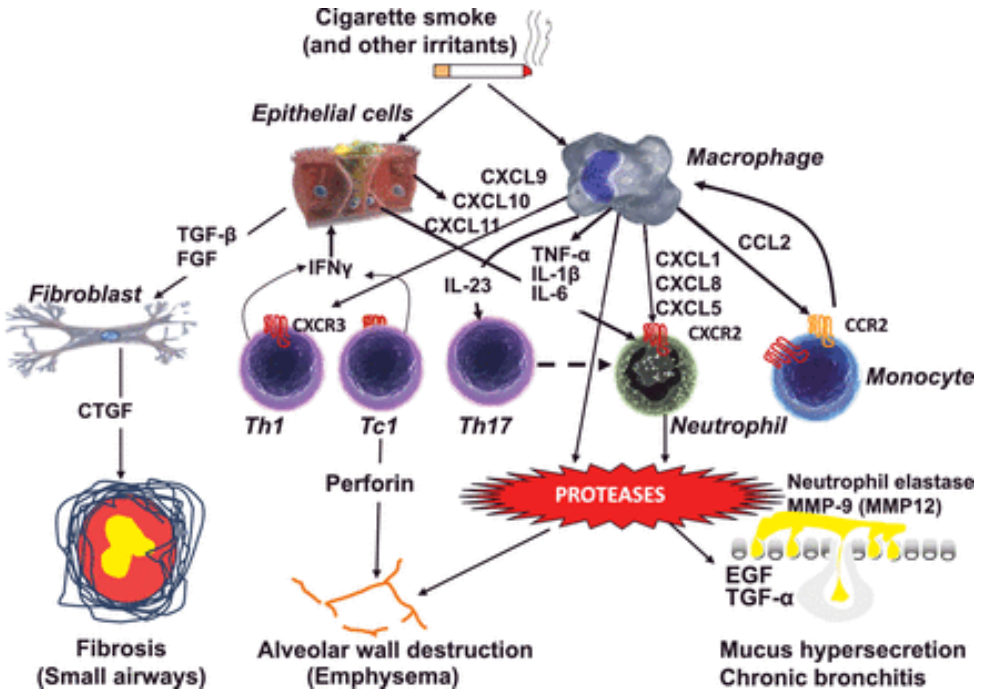


Figure 1.12 Inflammatory response in COPD. An inhaled irritant such as cigarette and biomass smoke and other noxious particles activate epithelial cells and macrophages. The activated macrophages release chemotactic factors including CCL2 and CXCL1/CXCL8, which act on CCR2 and CXCR2 to attract monocytes and neutrophils in the airways. Similarly, activated epithelial cells secrete CXCL9, CXCL10 and CXCL11 which act on CXCR3 to recruit T-lymphocytes (Th1 and Tc1 cells). Macrophages also secrete IL-23 to attract Th17 cells which enhances neutrophilic inflammation. The activation of these inflammatory cells release proteases like MMP-9, MMP-12, which causes alveolar wall destruction and mucus hypersecretion. The secretion of TGF-β by epithelial cells and macrophages causes fibroblast proliferation which results in small airways fibrosis. Adapted from [126].

1.6.2 Inflammatory Cells

In COPD, both inflammatory cells recruited from the blood into the lungs and structural cells in the lungs actively participate in the inflammatory response [126]. Both innate immunity cells like eosinophils, neutrophils,

macrophages, mast cells, natural killer cells, innate lymphoid cells and DC, and adaptive immunity cells like T and B lymphocytes are present in the lungs during the process. The structural cells involve in the process are epithelial cells, endothelial cells and fibroblasts.

Dendritic cells: Dendritic cells are antigen presenting cells, whose primary function is to recognize antigen, process and present it to resident T cells [122]. DCs originate in the bone marrow and reach tissue through blood circulation. The DCs are found residing in and below the airway epithelium, the alveolar septa, pulmonary capillaries and airway spaces in the lung [127]. DCs show a key role in the immune system acting as messengers between the innate and the adaptive immune system and activating a variety of inflammatory and immune cells including macrophages, B and T-lymphocytes and neutrophils [127, 128]. An increase number of activated DC are present in the lungs of patients with COPD [129].

Epithelial cells: Airway epithelial cells in the lung are the first barrier against a wide range of inhaled environmental contaminants [130]. The airway epithelial cells are actively involved in both innate and adaptive immune responses to foreign particles present in the airways [131]. Increasing scientific evidence suggests that the innate immune response is critical for the development of chronic respiratory diseases like COPD and airway epithelial cells play an important role in the pathogenesis of these diseases [132]. An activated airway epithelial cells secrete various

inflammatory mediators, including TNF-α, TGF-β, IL-1β, IL-6, IL-8 and GM-CSF in response to inhaled pollutants, such as biomass fuel smoke [133]. It also protects the respiratory tract from the injurious foreign agent by producing antimicrobial substances like defensins, lactoferrin and cationic proteins [134].

Macrophages: Macrophages, a family of mononuclear leukocytes, play a vital role in the immune and inflammatory responses in patients with COPD [135]. Alveolar Macrophages (AM) are the predominant defence cell types in the normal lung and during chronic inflammation in diseases like asthma and COPD [136] and are found in the surface of alveoli [137]. The number of AM is elevated in airways, lung parenchyma, bronchoalveolar lavage (BAL) fluid and sputum of patients with COPD [138]. In smokers, a significantly increased number of macrophages and CD8+ T-cells are found in bronchial biopsies and lung parenchyma [79, 139]. The increased number of macrophages in the airways correlates with the severity of COPD [140]. The activated macrophages secrete inflammatory mediators, including IL-8, IL-6, IL-1, TNF-a, monocytes chemotactic peptide (MCP)-1, leukotriene (LTB4), and reactive oxygen species (ROS) [126, 141]. These mediators recruit and activate neutrophils from the blood circulation into the airway lumen through chemotaxis [141]. The neutrophilic inflammatory response in COPD may be caused by the activation of AM in response to inhaled smoke or particulates [136, 141]. Elastolysis occurs at the surface of alveoli due to the release of elastolytic enzymes, like MMP-2, MMP-9, MMP-12, Cathepsins K, L and S and

Neutrophil Elastase (NE) from AM [142, 143]. AMs from COPD patients were found to secrete more inflammatory proteins which eventually increases the elastolytic activities, and this is found further to increase with exposure to cigarette smoke [144] (Figure 1.13).

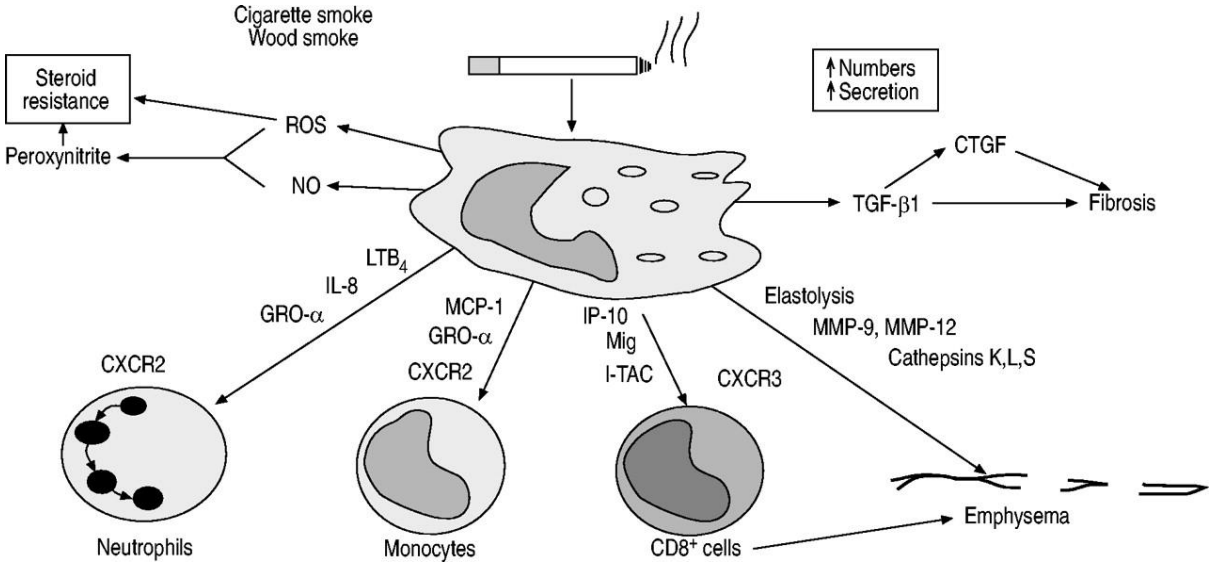


Figure 1.13 Macrophages in COPD: Macrophages activated by the noxious particle secrete inflammatory mediators like IL-8, LTB_4, GRO-α, MMP-9, MMP-12, cathepsins K, L and which in turn brings many other inflammatory cells like neutrophils, monocytes into play. Neutrophils activated by IL-8 and LTB_4 may be responsible for emphysema as they release proteases which damage the elastic fibres. Elastolysis may occur due to the release of elastolytic enzymes like MMPs and cathepsins. The process releases TGF-α and TGF-β1. Mucus hypersecretion and fibrosis may occur due to activated EGFR and CTGF. Macrophages also produce ROS and NO, results in steroid resistance. Adapted from [124].

Neutrophils: Neutrophils, polymorphonuclear leukocytes, are derived from stem cells [145] in the bone marrow and have a role in the innate immune response against invading pathogens [146]. In inflammation, neutrophils are attracted from blood vessels to the site of inflammation by chemical signals including IL-8, TNF-α, IFN-γ and LTB_4. Elevated levels of

activated neutrophils are present in the sputum and BAL fluid of patients with COPD [147-149]. Activated neutrophils secrete serine proteases, mainly Neutrophil Elastase (NE) which is responsible for damaging elastic fibres [144]. In COPD, the pathological changes in airways and lung parenchyma are in part due to these neutrophil proteinases, particularly NE secreted by the activated neutrophils [150]. Damaged elastic fibres and basement membrane have been observed in the alveolar interstitium of patients with emphysema, and these patients also have elevated levels of NE [151]. NE in patients with emphysema was found to be in higher quantities in the granules of neutrophils and around the elastic fibres of the alveolus and basement membrane of the epithelium and endothelium [152]. An imbalance between proteases and anti-proteases has been reported to result in an increased risk of COPD [153], which also correlated with the rate of decline in FEV_1 [154].

Lymphocytes: Lymphocytes are present in airways and lung parenchyma, and are inflammatory cells which normally mediate adaptive immune responses to inhaled pathogens and pollutants. Thymus-dependent T cells and bone marrow-dependent B cells are the two major populations of lymphocytes. CD4+ and CD8+ are two subsets of T lymphocytes. CD4+ T lymphocytes are further divided into helper T cells Th1 and Th2 which release specific patterns of cytokines. T lymphocytes play an essential role in coordinating the inflammatory response in COPD and elevated levels of T cells, mainly CD8+ cells are found in lung parenchyma and airways of COPD patients [144]. The amount of alveolar

destruction and airflow obstruction in COPD also depends on the number of T-cells present. The increased expression of chemokines CXCL9, CXCL10 and CXCL11 by epithelial cells and macrophages activate CXCR3 on CD4+ and CD8+ T cells and contribute to the accumulation of T cells. The activated T cells then release IFN-γ, which in turn causes further release of these chemokines. CD8+ T cells are cytotoxic and produce perforin and granzyme B, which contribute to alveolar cell apoptosis and the development of emphysema [155] (Figure 1.14).

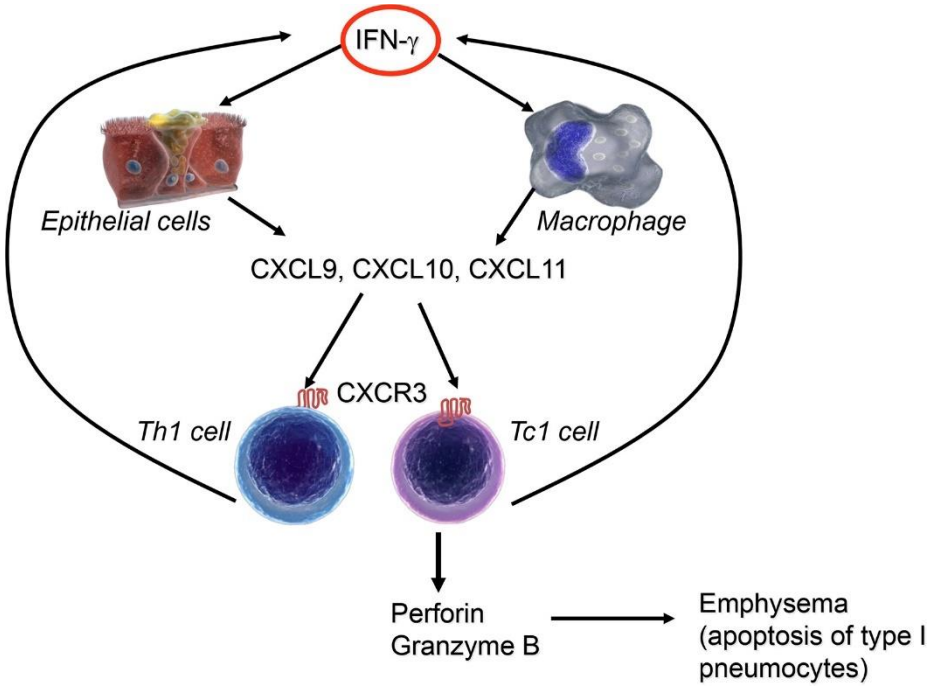


Figure 1.14 Lymphocytes in patients with COPD.IFN-γ secreted from Th1 and Tc1 cells acts on epithelial cells and macrophages, which in turn release the chemokines CXCL9, CXCL10, and CXCL11.These chemokines activate CXCR3 on Th1 and Tc1 cells. Tc1 cells produce perforin and granzyme B, which contribute alveolar cell apoptosis and the development of emphysema. Adapted from [155].

1.6.3 Inflammatory mediators

Inflammatory mediators are chemical messengers secreted from various inflammatory and structural cells that interact with other cells to promote inflammation. Inflammatory mediators play a significant role in mediating the inflammatory response in inflammatory diseases like COPD and asthma. A large number of different inflammatory mediators including lipids, free radicals, cytokines, chemokines and growth factors are present in the site of inflammation.

Pro-inflammatory cytokines are a group of inflammatory mediators that are secreted from immune cells like helper T cell (Th) and macrophages and other structural cells like epithelial cells. The main functions of inflammatory cytokines are to initiate the inflammatory response and regulate the host defence against pathogens. The signalling molecules TNF-α, IL-1β, IL-8, IL-6, IFNγ and GM-CSF are amongst the important pro-inflammatory cytokines involved in airway inflammation [156].

TNF-α is a pro-inflammatory cytokine produced by various cells, including macrophages, T-cells, mast cells, and epithelial cells. TNF-α plays an important role in orchestrating the cytokine cascade in many inflammatory diseases including COPD. TNF-α exerts cellular effects through interacting with its transmembrane receptors, including TNFR1 and TNFR2 [157]. These interactions with the receptors initiate intracellular signal transduction which leads to many cellular events, including cell death, survival, cell proliferation, differentiation, and

migration [158]. Other cytokines including IL-1, GM-CSF and IFN-γ, enhance the production of TNF-α from macrophages. TNF-α is a potent activator of NF-kB, which in turn increases the secretion of IL-8 from epithelial cells and neutrophils [159].

Interleukin-1β is a pleiotropic and immune regulatory cytokine which belongs to the IL-1 cytokine family. It interacts with the transmembrane receptor IL-1R, which regulates a range of cellular activities including cell proliferation, differentiation and apoptosis. It is a crucial cytokine which attracts neutrophils from the bone marrow and stimulates the secretion of many other cytokines, including IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF, IFN-γ from a range of different cells. IL-1β plays an important role in neutrophilic airway inflammation in COPD [160]. IL-1β is produced by various cells including monocytes/macrophages, fibroblasts, T cells, neutrophils and bronchial and alveolar epithelial cells [161]. It also increases the production of neutrophil attractant chemokines such as CXCL1 and CXCL2, and MMP-9 and MMP-12 [162].

IL-8 is a chemoattractant cytokine, produced by monocytes/macrophages and other cells like epithelial cells, airway smooth muscle cells and endothelial cells. IL-8 serves as a chemotactic factor primarily for neutrophils and lymphocytes [163]. IL-8 acts on CXCR1 and CXCR2 receptors to promote pathogen elimination, and also contributes to processes like tissue injury and fibrosis [164].

IL-6 is a cytokine produced by various inflammatory cells including monocytes/macrophages, T-lymphocytes, fibroblasts and airway epithelial cells. IL-6 plays a key role in many aspects of immunity and stimulates target cells through membrane bound interleukin-6 receptors [165]. It exerts stimulatory effects on several immune cells, including B-lymphocytes and T-lymphocytes to increase cytokine production [166].

Over 50 different cytokines have been found to be elevated in the airways in COPD, however, the role of these cytokines in the pathogenesis of the disease progression is yet to be fully identified [167]. TNF-α and IL-8 are potentially the most important markers in COPD patients as high concentrations of these cytokines are present in the sputum of COPD patients [147] which increases further during exacerbations [155]. Also, an increased concentration of IL-8 is found in BAL fluid of patients with COPD [168]. The primary pro-inflammatory markers present in the airway inflammation caused by PM exposures are TNF-α, IL-8, IL-6, IL-1β and GM-CSF. An increased level of these pro-inflammatory mediators has been measured in the lung epithelium and blood after exposure to PM [169, 170]. Experiments using different cell lines have reported increased levels of pro-inflammatory cytokines following *in vitro* exposure to PM. For example, Kocbach et al. reported a significant increase in TNF-α, IL-6 and IL-8 from monocytes/pneumocyte co-cultures stimulated with various concentrations of PM [170]. Similarly, human epithelial cells exposed to PM have been found to secrete dose dependent concentration of IL-6

[169]. Hence the growing scientific evidence shows that exposure to PM induces an inflammatory response in the pulmonary system.

1.6.4 Pathogenic Mechanisms of COPD due to Biomass Smoke exposure

Although epidemiological studies show an association between biomass exposure and the development of COPD, little is known about the cellular and molecular changes which occur in people at risk of COPD caused by exposure to noxious particles from wood smoke. It might be expected that there would be similar clinical characteristics in biomass-induced COPD and tobacco smoke-induced COPD [171]. However, several studies have suggested some significant differences in the histopathology of COPD from biomass and COPD from tobacco. Camp et.al. mentioned that airway-predominant COPD is more associated with biomass smoke, whereas tobacco smoke is more responsible for emphysema-predominant COPD [9]. On the other hand, pro-inflammatory mediator release and activation of T helper cells have been found in patients with COPD caused by both smoking and biomass smoke exposure. A study conducted on women by Villavicencio et al. found significantly higher levels of TH17 cells activated in patients with tobacco smoking-COPD than in patients with biomass smoke-COPD [172]. In the same study, TH2 cell number and IL-4 serum concentration were found to be at higher levels in patients with biomass smoke-induced COPD than in patients with tobacco smoke-induced COPD [172]. Furthermore, a recent study performed by

Krimmer et al. examining the extracellular matrix (ECM) protein from primary human lung fibroblasts *in vitro* showed an increased production of fibronectin following exposure to both cigarette smoke extract and biomass smoke extract [173].

It is clear from the literature that biomass smoke exposure causes lung inflammation and that higher levels of inflammatory cells are found in COPD patients exposed to biomass smoke. This is accompanied by higher levels of expression of specific genes: a recent study conducted by Guarnire et al. described the direct association of biomass smoke exposure with higher gene expression of some pro-inflammatory mediators including IL-6, IL-8, TNF-α, MMP-9 and MMP-12 [174].

One interesting possible inflammatory mechanism proposed to mediate in part the effects of biomass smoke exposure is via activation of Transient Potential Receptor (TRP) ion channels in various lung cells [175]. The activated TRP channel mediates the flow of cations across the plasma membrane to the cytoplasm causing cell transcription, transduction, contraction and migration [176]. It has been shown that TRPA1, V1, V4 and M8 can stimulate inflammation following acute exposure of wood smoke PM [175]. Activation of TLRs on airway macrophages and epithelial cells is considered as another possible pro-inflammatory mechanism for biomass smoke exposure driven effects. Miyata et al. suggested that smoke PMs induce an inflammatory response through the endotoxin TLR-4 pathway and reactive oxygen species generation [177]. This suggests

that TLR-4 and TLR-2 antagonists or blocking antibodies could reduce the production of inflammatory mediators IL-6 and IL-8 in AM and epithelial cells exposed to biomass particulate matters [178].

1.6.5 Experimental studies

Some toxicological experiments support epidemiological evidence showing an association of solid fuel smoke exposure with the development of COPD in an individual. Very few controlled human exposure studies have investigated the effects of wood smoke exposure on human health. In one study, controlled exposure to wood smoke fine particles in healthy humans showed association with respiratory symptoms. For example, 13 healthy humans were exposed to 240-280 $\mu g/m^3$ concentration of wood smoke in a controlled environment for two 4-hours sessions, 1 week apart in an experimental protocol by Barregard et al.[179]. Changes in levels of inflammatory mediators and coagulation factors in healthy human exposed to short term wood smoke particulates were identified. In addition to this, an increase in inflammatory responses and signs of increased oxidative stress in the lower respiratory tract was reported [180] in a separate study with the same exposure level and conditions. Conversely, no effect on systemic inflammation, oxidative stress, DNA damage, cell adhesion, cytokines and microvascular function in atopic subjects was recorded by Forchhammer et al.[181]. 20 non-smoking atopic volunteers were exposed to three different concentrations of particulate matter generated from a wood stove for 3 hours in a

controlled environment in this study. In addition to this, Riddervold et al. [182] found only mild inflammatory responses with short term exposure in non-smoking atopic volunteers and no statistically significant effect on lung function. Moreover, Muala et al. concluded in a recent study that short term exposure from incomplete combustion does not effect on acute neutrophilic inflammation [183]. However, in the bronchial wash and bronchoalveolar lavage of healthy volunteers exposed to wood smoke, significant reductions in macrophages, neutrophils and lymphocytes numbers were observed [182].

1.6.6 Interventional studies

The risk of COPD and other respiratory diseases can be minimized by reducing HAP through a wide range of interventions. Despite many efforts, solid fuels use for cooking in developing countries has not significantly reduced, and in fact, remained unchanged over the last three decades affects around 2.7 to 2.8 billion people worldwide [32]. Alternative clean fuel, improved efficient technologies (e.g. better designed cooking stoves), improved and proper ventilation of the cooking and living area and changes in user behaviour are some of the promising interventions to reduce smoke exposure from the kitchen environment [184]. However, limited interventional studies have been done to date to observe the effect of such interventions on human health.

Interventions to reduce HAP include schemes to promote the use of cooking stoves and the dissemination program has already distributed millions of improved cookstoves in various parts of the developing world. However, as mentioned above, the problem of HAP and its health consequences has not reduced [185]. As an example, several times higher HAP levels than national and WHO standards were measured in China even after dissemination of 130 million improved cook stoves [186]. Though the majority of studies report a positive reduction of HAP after the installation of an improved cook stove, the emission is still significantly higher than WHO air quality recommendations. For instance, in one study $PM_{2.5}$ concentration was found to be decreased by 48% in homes using ICS compared in homes using TCS [187]. However, the concentration was still six times greater than the WHO recommended targets [188]. The lack of awareness and a lack of affordable stoves and clean fuels might be the possible cause of the limited success of emission reduction interventions [189]. Another possible cause why the improved cooking stoves have not been able to reduce the emissions to the desired level could be due to the lack of adequate exposure-response studies [190].

1.7 **Cooking Stoves**

A simple structured biomass combustion stove made from clay without a chimney or open fire having three stones or metal tripods is referred to as a traditional cook stove (TCS). Different forms of TCS being used in various regions of Nepal is shown in Figure 1.15. These types of stoves are easy to construct with locally available material with no cost and hence common in developing countries throughout the world. These stoves are very inefficient resulting incomplete combustion of the fuel, consumption of a lot of fuels and likelihood of producing high concentrations of air pollutants. Throughout this thesis, TCS refers to stoves of these kinds.

Photo © Binaya KC, UoN

Figure 1.15. Different types of traditional cook stove in use in various regions of Nepal.

Various models of efficient biomass combustion cook stoves have been developed throughout the world. The primary purpose of these cook

stoves is to increase the thermal efficiency and hence reduce the emissions of harmful pollutants from the combustion of biomass. The two-pot or three pot mud cook stove with chimney and metallic cook stove with chimney are the most common types of improved cook stove used in Nepal. In this thesis, the stove of these kinds is referred to as ICS. The ICS used in this thesis is shown in Figure 1.16.

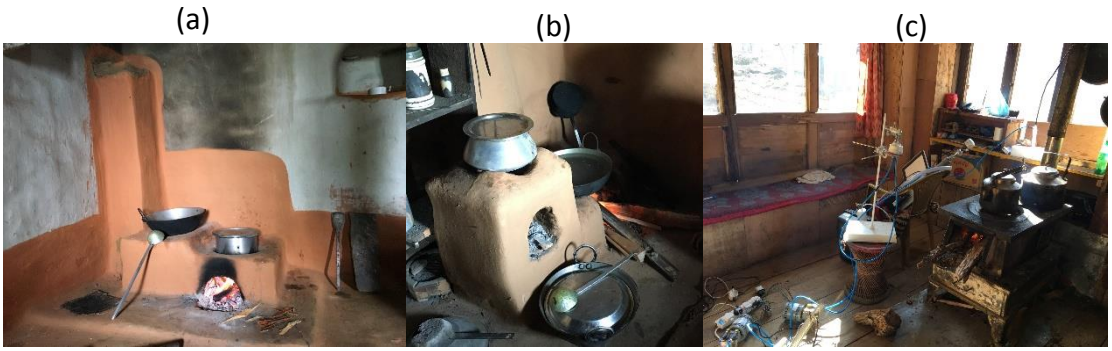


Photo © Binaya KC, UoN

Figure 1.16 Improved cook stove (ICS), commonly used in various regions of Nepal. (a) two pot mud ICS, (b) single pot mud ICS and (c) two pot metallic ICS.

1.8 **Aims of the PHD project**

Copious amount of epidemiological studies have already shown the association of prolonged exposure to the pollutants from biomass combustion for cooking with adverse health outcomes. However the short term exposure and the potential biological mechanisms are yet to be fully elucidated. Although a few studies have shown the effect of PM generated from the biomass combustion in *in vitro* studies using different cell line models, these studies used the PM samples generated in the lab, and hence lack using samples from real-life cooking environment. This is the first study to look into the short term effect of biomass smoke exposure with the samples collected from real-life cooking environment in Nepal using human lung explants model.

My main aims of this PhD work were therefore

- To analyse the emission factor of the most commonly used solid biomass fuels in Nepal for cooking.
- To monitor the range of exposures an individual cooking on biomass combustion stoves experienced in real-life in Nepal.
- To further investigate the potential pro-inflammatory consequences of these exposures using *ex vivo* human lung explants as a model system and *in vitro* human bronchial epithelial cells.
- To investigate the potential effect of exposure reduction using improved cooking stoves on pro-inflammatory responses from human lung tissue and HBEC.

Chapter 2: General Materials and Methods

2 General Materials and Methods

This chapter describes the general materials, methods and techniques used throughout this thesis. The methods specific to each research project contributing to this thesis are explained in the respective chapters.

2.1 Household Air Pollution Exposure Study

This section describes the methods and equipment used in work related to the monitoring of real-time personal exposures to HAP study performed in various regions of Nepal. It mainly includes the description of the study site, the equipment used and technique used for field-based exposure monitoring.

2.1.1 Indoor air pollutants

The real-time personal exposure to particulate matter smaller than 2.5 μm in aerodynamic diameter ($PM_{2.5}$) and carbon monoxide (CO) resulting from the combustion of biomass fuel was measured as a surrogate of indoor air pollutants.

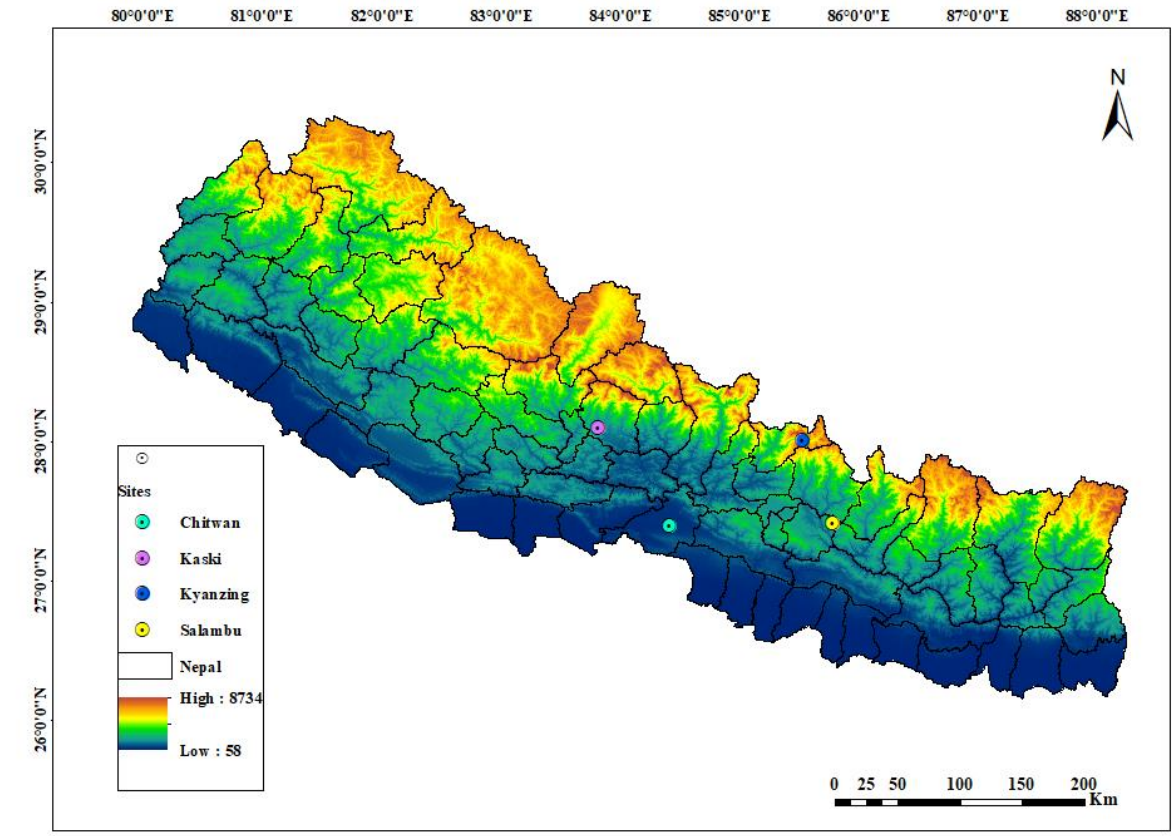
2.1.2 Monitoring Period

All real-time personal exposures were monitored continuously throughout the cooking period. A non-cooking period sampling was also performed before and after cooking. The cooking timing and duration varied among households in each monitoring sites, and mean cooking duration in

households in each monitoring site will be presented in the respective sections.

2.1.3 Study Site

(a)



(b) (c) (d) (e)

Figure 2.1 Study location: (a) map of Nepal with elevation and location of all four monitoring site villages, (b) Kyanzing Kharka, Langtang (4000 m above sea level), (c) Salambu, MajhiFeda (1800 m above sea level), (d) Bhujel Gaun, Pokhara (1200 m above sea level), and (e) Simreni village, Chitwan (200 m above sea level).

The study was conducted in the rural dwellings of four different geographical regions of Nepal; Kyanzing, Salambu, Pokhara and Chitwan (Figure 2.1). The four different regions were selected according to altitude variations ranging from low plain land to high altitude mountainous village. The elevation above the sea levels of each of the monitoring village was 200 meters in Chitwan, 1200 meters in Pokhara, 1800 meters in Salambu and 4000 meters in Kyanzing. All regions are located in the rural part of the country, and biomass fuel combustion was the primary source of energy for cooking and heating in households. However, in a few households in Pokhara and Chitwan clean fuel like Liquefied Petroleum Gas (LPG) and biogas was also used as a substitute source of energy for cooking. The monitoring was performed primarily in the households using biomass fuel in all monitoring sites, although in Pokhara and Chitwan monitoring was also performed in the households using clean fuels for the comparison. The study site description with the total number of households sampled in each site is presented in Table 2.1.

Table 2.1 Study site description with the number of households sampled in each site. N/A: not available, TCS: Traditional cook stove, ICS: Improved cook stove, LPG: Liquefied petroleum gas.

Study Site	Altitude (meters above sea level)	Number of Households sampled	Cooking Fuel and Stove used		
			Biomass (TCS)	Biomass (ICS)	LPG
Kyanzing, Langtang	4000	14	5	9	N/A
Salambu	1800	25	17	8	N/A
Bhujel Gaun, Pokhara	1200	27	24	N/A	3
Simreni village, Chitwan	200	37	30	N/A	7
Total		**103**	**76**	**17**	**10**

2.1.3.1 Kyanzing Kharka, Langtang

The study in Langtang region was conducted in a small village called Kyanzing Kharka (Figure 2.1 (b)), which is the highest settlement located at 4000m in the Langtang Valley. The Langtang valley is situated in Rasuwa district, north of the capital city Kathmandu of the country. It is one of the three central regions of Langtang National Park (LNP), which extends from Syafrubesi at 2000m to Langtang Lirung peak at 7245m.

Langtang valley is a famous mountain trekking destination for the tourists coming to Nepal from different parts of the world. The trekking starts from Syafrubesi, which is about 80 kilometres and a 7-8 hour drive on rough single-lane roads by bus/car from Kathmandu. It usually takes three days to reach Kyanzing from Syafrubesi and trekkers usually spend the first night at Lama hotel (2500 m) and the next night at Langtang (3400m). During our field study, we trekked three days to reach Kyanzing, and porters and mules carried all scientific equipment and goods. An estimated population of 415 individuals live within a total of 152 households in the Langtang valley (before the earthquakes in 2015) [191]. The majority of people in the valley are from the Tamang community. The primary source of income in this region is related to tourism activities. The people run hotels, lodges and restaurants as a source of income generation. At the time of our study, Kyanzing Kharka had a total of 35 households, out of which 95% of households ran hotels and restaurants.

The fieldwork was conducted from 18 October to 26 October 2017. A total of 16 households, including one in Sherpa gaun (2560m) and one in Thangshap (3200 m) during our two days stay at the village before reaching Kyanzing were monitored. Out of 16 sampled household, 15 households were from households operating as hotels. We analysed only 14 households for this thesis, excluding two households which were monitored elsewhere than Kyanzing.

(a) (b)

Figure 2.2 Types of cook stove in Kyanzing (a) Improved cook stove (ICS) and (b) traditional cook stove (TCS).

Biomass fuel combustion was the primary source of energy for cooking and heating in all 14 houses. Most of the houses had metallic improved cook stove (Figure 2.2 (a)) with a chimney in it, but out of total household sampled 36% households were found using traditional cook stove (Figure 2.2 (b)) without having a chimney. The kitchen in a typical household running a hotel and restaurant was located in a separate house. The cooking timing and period depended on the flow of tourists and the frequency of orders for the food. Typically, the stove remained on for 16-17 hours a day once it was started in the early morning. People used the stove to cook food whenever they received an order, and for rest of the time, they used it for heating purposes. The exposure monitoring was done in Kyanzing in households using both ICS and TCS, and the cooking period was fixed to 120 minutes.

2.1.3.2 Salambu, Kavrepalanchowk

The fieldwork was conducted from 18 December to 23 December 2017 in Salambu. Salambu is a small village in the MajhiFeda VDC of Kavrepalanchowk District, Nepal (Figure 2.1 (c)). The district lies in the mid hilly region of the country which is a subtropical climate zone with an elevation range of 280m to 3018 m. The study site is about 80 km east from Kathmandu and takes typically 6-7 hours on a rough gravelled road by bus/car. During the monsoon season access becomes challenging, and it is sometimes impossible to reach the village due to the poor road condition. The study site had a total of 88 households at the time of our study, which is scattered in the hilly region at an elevation ranging from 1600m to 1800 m. The typical home had a kitchen generally located on the ground floor of the home with a separate bedroom on the upper floor. Where households had been damaged by the earthquake, the inhabitants had shifted to a small house having a single shared room for cooking, eating, sitting and sleeping. Most houses were constructed from locally available materials including mud, stone, wood, dung, thatch and straw. The newly built houses after the earthquake were mostly built of processed materials including bricks, cement, iron and concrete.

A total of 25 houses from the village were selected for the study. The only source of household energy for cooking in all 25 houses was the combustion of locally available biomass fuels. The practise of using biomass fuel for cooking was relatively similar and fuelwood along with

some agricultural residue, mainly used to ignite the fire, has been a common source of biomass fuel in households. In all households, the cooking was done inside the house. People customarily cooked three times a day and the cooking timing and period varied between the households. The average cooking duration was 78.12±15.9 minutes. The early morning cooking usually started from 5 to 6 am and finished around 7 to 8 am. The second cooking in the morning started at around 10 to 11 am after returning from the farm work. The cooking in the evening usually began after 5 pm and finished at around 8:30 pm.

The households were noted to have two different types of cooking stove in use; improved cook stove (Figure 2.3 (a)) and traditional cook stove (Figure 2.3 (b)). Personal exposure monitoring was conducted during cooking periods using both traditional cook stoves and improved cook stoves.

(a) (b)

Figure 2.3 Biomass cooking stove in Salambu. (a) Improved cook stove (ICS) and (b) Traditional cook stove (TCS).

2.1.3.3 Kaskikot, Pokhara

The fieldwork was conducted from 4 January to 11 January 2018 in a small village called Bhujel gaun (Figure 2.1 (d)). Bhujel gaun is a small cluster of around 40 households (at the time of the study) in Kaskikot, Gandaki district located at a distance of 12 km west of Pokhara. The energy requirement for cooking was usually fulfilled by the combustion of biomass fuel, whereas few households also used clean fuel like LPG as an alternative energy source. The cooking activities were done indoors in a simple single pot mud or clay stove (Figure 2.4) with no chimney or any other possible source of ventilation. None of the households had an improved type of cooking stove installed at the time of our study. The kitchen was usually in a separate room on the ground floor but usually connected to the other rooms of the house. The typical house was constructed with the combination of locally available materials like mud, stone and wood. However, commercially available materials like brick, cement and iron were used in recently built households.

The exposure monitoring was performed in households using biomass fuel as well as in household using LPG. Mostly two meals a day were prepared in every household. The cooking start time and duration were relatively similar among households. The cooking starts typically at 7-8 am and 5-6 pm for morning and evening cooking. The average cooking duration in this village was 79.3±24.4 minutes. The monitoring was performed in two households at a time for both morning and evening cooking.

Figure 2.4 Traditional mud cook stove in Bhujel Gaun, Kaskikot.

2.1.3.4 Chitwan, Chitwan National park

The fieldwork in Chitwan was carried out from 20 March to 25 April 2017 in a semi-rural village called Simreni located around the Chitwan National Park (CNP). CNP spans an area of ~952 km^2 in the subtropical low lands of Nepal. It is well known for its rich biodiversity and is surrounded by residential communities belonging to different tribes in its peripheral regions. Hence, it is a renowned destination for tourists, conservationists and researchers interested in a range of different subjects. It is surrounded by rural and semi-rural areas with small clusters of villages and townships. The winter season in this region is often affected by winter fogs while summers are dry, dusty and often affected by forest fires. Care was taken to choose sites so that no major roadway passed through the study sites to make sure that vehicular emissions would not directly affect the sampling frame. The households in this area have some livestock, so livestock-related emissions were a possibility, and the households were

surrounded by paddy fields and forest areas, which are potential sources of agricultural emissions. It was observed that in this region, the people had options for cooking with LPG, biogas, and biomass, the latter in a traditional 3 stone stoves (Figure 2.5). As biomass fuels are readily available from the nearby forest with no cost, households usually cook their daily meal using biomass fuel despite ownership of LPG and biogas stoves. The exposure monitoring in this village was performed in households using biomass fuel as well as in households using LPG.

Figure 2.5 Traditional mud cook stove in Simreni Village, Chitwan.

2.1.4 Monitoring Equipment

This section describes the list of equipment used for monitoring indoor and ambient air pollutants including $PM_{2.5}$ and CO. Other equipment used for the measurement of physiological responses like pulse, oxygen saturation level (SpO2) and exhaled breathe CO concentration in individual using biomass fuel are also presented. List of all equipment used throughout this thesis is presented in Table 2.2.

Table 2.2 List of equipment

Equipment Name	Manufacturer	Measurement Unit	Used for	Reference
E-sampler	Met One Instrument, Incs., USA	Particulate matter (PM)	Indoor and ambient air $PM_{2.5}$ monitoring	[192, 193]
Aerocet 831	Met one Instrument, Inc., USA	Particulate matter (PM)	Indoor and personal exposure to $PM_{2.5}$	[194, 195]
Indoor air pollution meter	IAP meter 5000 series, Aprovecho Research Centre, USA	Particulate matter (PM) Carbon monoxide (CO)	Personal exposure to $PM_{2.5}$ and CO	[196]
Indoor air quality meter	GrayWolf® sensing solutions, USA	Carbon Monoxide (CO) Temperature (T) Relative Humidity (RH)	Indoor concentration of CO Indoor temperature and RH	
GRIMM-EDM-180D	Grimm Aerosol Technik GmbH & Co. Kg. Dorfstrasse-9, Germany	Particulate matter of different size	$PM_{2.5}$ data correction measured in field using above low cost sensor equipment	[197]
Pulse Oximeter	Microlife® AG, Switzerland	Oxygen saturation (SpO2) Heart rate (Pulse)	To monitor SpO2 and Pulse of individual involved in cooking	[198]
Micro CO meter	CareFusion, UK	Exhaled Breathe CO concentration % of carboxyhaemoglobin (% COHb)	Cooking and non-cooking period CO concentration in exhaled Breathe	[199, 200]

2.1.4.1 E-sampler

E-sampler was used to monitor indoor and ambient $PM_{2.5}$ concentrations. E-sampler is a type of nephelometer which uses the principle of forward laser light scatter to measure and record a real-time airborne particulate matter of different aerodynamic diameter including PM_{10} (less than 10 μm) and $PM_{2.5}$ (less than 2.5 μm) or total suspended particles (TSP). The equipment has a sensitivity of 1 $\mu g/m^3$ with a flow rate of 2 L/min. It has an additional built-in 47 mm filter sampling unit that can be used optionally to collect the particulate in a filter membrane for subsequent gravimetric mass analysis. An internal diaphragm pump draws a sample air into the E-sampler. The flow rate is controlled through sharp-cut cyclones to determine the sampled volume accurately.

An internal visible laser diode beam is directed through the sample air stream drawn through the laser optical module. The particulate matter presents in the sample air stream scatters the laser light which is collected onto a photodiode detector at a near-forward angle. The resulting electronic signal is then processed to determine a continuous, real-time measurement of airborne particulate mass.

2.1.4.2 Aerocet

Aerocet is a small, lightweight, battery-operated, handheld aerosol mass monitor which simultaneously monitors levels of particulate matters with various sizes including PM_1, $PM_{2.5}$, PM_4 and PM_{10}. The aerocet monitors the

level of particles every 1 minute with a fixed flow rate of 2.83 L/min. Aerocet uses the particle count to mass conversion principle and runs in a continuous mode with a resolution of 0.1 μg/m^3. The aerocet counts and sizes particles in five different size ranges then used a proprietary algorithm to convert count to mass concentrations [201]. It calculates a volume for each detected particle then assigns a standard density for the conversion.

2.1.4.3 IAP meter

The IAP meter is a simple, portable, reliable and easy to use aerosol mass monitor for logging $PM_{2.5}$ and CO concentration. It consists of two sensors, one each for $PM_{2.5}$ and CO concentration measurements. The CO sensor is an electrochemical cell. The range of CO is 0-1000 ppm with a resolution of 1 ppm. The PM sensor is a red laser light scattering photometer. The range of $PM_{2.5}$ is 0-60000 μg/m^3 with the resolution of 25 μg/m^3. The IAP meter can be used in three different sampling speed (a) fast mode: one sample every 9 seconds, (b) Medium mode: one sample in every 51 seconds, and (c) Slow mode: one sample in every 9.7 minutes.

The principle underlying the IAP is that the conductivity of the electrochemical cell changes in proportion to the concentration of CO present in the sample air. The PM sensor consists of a laser and a light receiver. When the sample air enters into the sensing chamber, the light of the laser bounces off the particles present in the sample air into the

receiver. More light reaching the receiver indicates more particles in the chamber. This level of light has been calibrated against a laboratory-standard nephelometer to relate the amount of reflected light to the concentration of the particles.

2.1.4.4 IAQ meter

Indoor air quality (IAQ) meter was used to measure indoor CO concentration. IAQ meter can simultaneous measures VOCs, CO_2, CO, %RH and temperature. The CO sensor works in the principle of electrochemical cells. Diffusion of CO gas in the meter activates electrochemical CO sensor which produce an electrical signal. The amount of current flow between two electrode is proportional to the amount of CO enters into the meter. The range of CO concentration of a meter is 0-500 rpm with an accuracy of ± 2ppm.

2.1.4.5 GRIMM

Grimm (Grimm Technologies Model EDM 180-D) is an optical mass monitor used for the continuous measurement of particulate matter concentration in the air [197]. It works on light scattering principle and can simultaneously measure real-time multiple PM values (PM_{10}, $PM_{2.5}$, PM_1, total counts and particle number distribution). It is a standard stand-alone monitor which is fully approved and certified by US-EPA, MCERTS, CMA and EN.

2.1.4.6 Micro CO meter

Exhaled breath carbon monoxide concentration was monitored using MicroCO Meter (CareFusion) [199, 200]. It is a handheld battery-operated portable device which gives the concentration of CO in the breath and also calculates the percentage of carboxyhaemoglobin (%COHb) in the blood. The meter consists of an electrochemical fuel cell sensor. The change in conductivity of the cells through the reaction of CO generates an electrical current proportional to the concentration of CO present in the sample air. The sensor output is monitored by the microprocessor, which detects peak expired concentrations of alveolar gas and converted to %COHb. The range of the meter is 0-100 ppm with a resolution of 1 ppm. The meter was calibrated before using in the field as per the manufacturer's instruction.

2.1.4.7 Pulse Oximeter

Pulse oximetry is a non-invasive technique to monitor a person's peripheral oxygen saturation (SpO_2) and heart rate [202, 203]. A fingertip pulse oximeter was used to monitor heart rate and oxygen saturation level of individuals involved in cooking using biomass fuel. The meter consists of a sensor and a pair of light-emitting diodes (LED) with different wave lengths (660 nm for red lights and 940 nm near-infrared light). The device passes these two wave lengths of light through the fingertip to photodiodes. The amount of light transmitted is measured,

and the ratio of red light measurement to the infrared light measurement is calculated and converted to SpO2 by the processor.

2.2 **Equipment calibration and Data Correction**

All portable, low-cost sensor exposure monitoring devices used in this work were either sent to the manufacturer for factory calibration or calibrated in the lab following the protocol using calibration accessories provided by the manufacturer. PM monitoring sensors E-sampler and Aerocet were sent to Met One instrument Inc, USA for the calibration before the commencement of the fieldwork. Similarly, factory calibrated IAP meter was purchased from Aprovecho research centre, USA only for this fieldwork. The CO gas sensors were calibrated following the protocol in a lab at 0 ppm using a zero-air gas (Specialty Gases Ltd, UK), and at 81 ppm using CO calibration gas mixture (Alchemic Gases and Chemical Pvt. Ltd, India). Micro CO meter was calibrated according to the instruction provided in a Micro CO meter operation manual using 20 ppm CO calibration gas provided by CareFusion (Cat. No 36-MCG020).

Though all particle monitoring devices were calibrated, these low-cost sensors might overestimate or underestimate particle mass due to differences in particle density at different locations. Hence, the data measured from these devices were compared with the data obtained using more reliable instruments at the reference site (at the laboratory) in order to correct the measured concentrations. E- Sampler and aerocets were validated with GRIMM (Grimm Technologies Model EDM 180-D),

factory calibrated stand-alone instrument for $PM_{2.5}$. Two sets of E-sampler and four sets of aerocets was used during the field campaign. Pre and post field correction runs were conducted for each set of field studies. The validation techniques described elsewhere [204, 205] were used in this study. Briefly, E-sampler and aerocets were run in parallel with the GRIMM for the simultaneous measurements of $PM_{2.5}$ concentration for over 18 hours. Based on this concentration data, the linear scale factor A (slope) and offset B (intercepts) between the two curves were calculated for each pollutant. This correction factors of linear equation $X = A.x + B$ was used as coefficients for the raw readings x to calculate the final corrected concentration X.

Correction for $PM_{2.5}$

Separate correction factors were derived for each of two E-sampler set and four aerocet sets for $PM_{2.5}$ with GRIMM data. A strong linear correlation between the GRIMM and exposure monitoring equipment with $R^2 > 0.77$ in all cases for all aerocet sets and all E-sampler sets was noted. The correction details of instruments used for each fieldwork will be described in the respective result sections. A representative plot of linear relationships between two sets of aerocet and GRIMM is presented in Figure 2.6. The overall correction factor of each set used in all field site is presented in Table 2.3. Though we used the methods previously adapted by others to validate and for data correction [204, 205], it is of note that some other statistical methods can be used for the comparison of

measurements made using the two different forms of equipment. One possible method would be a difference plot (Bland-Altman plot) which gives the differences in measurements between the data from the two techniques which would allow the study of any relationship between the differences and true values.

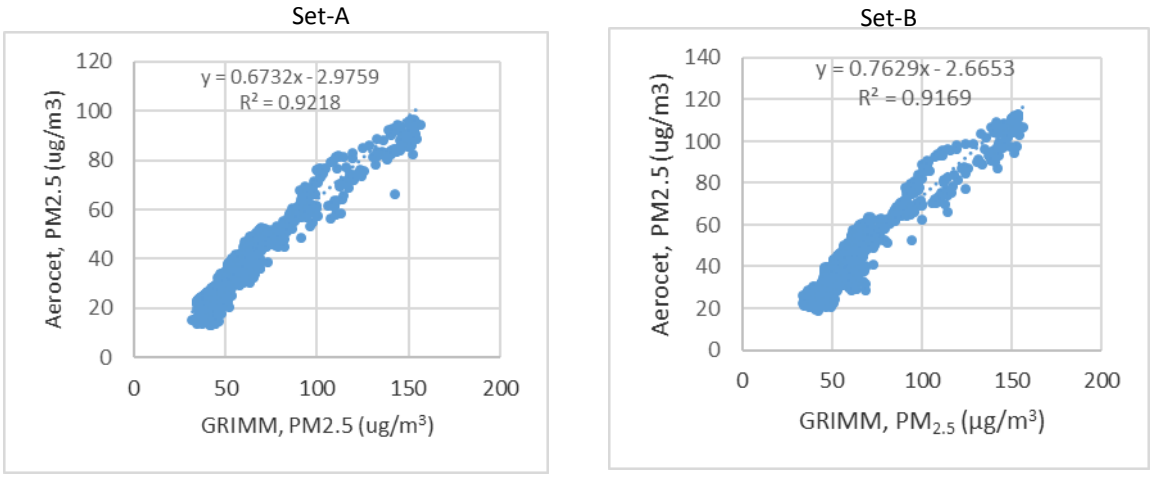


Figure 2.6 Representative plot of correlation between $PM_{2.5}$ concentration ($\mu g/m^3$) measured by aerocet and GRIMM.

Table 2.3 Correction factor derived for all sets of equipment in all monitoring sites for $PM_{2.5}$

Study Site	Instrument set	Correction factor	Root mean square value	P value
Langtang	Aerocet-Set 1	Slope=0.7412 Intercept = -1.1734	0.927	<0.0001
	Aerocet-Set 2	Slope=0.7856 Intercept = -1.0348	0.9384	<0.0001
Salambu	Aerocet-Set 1	Slope=0.4587 Intercept = 3.574	0.9537	<0.0001
	Aerocet-Set 2	Slope=0.4585 Intercept = 9.057	0.9474	<0.0001
Pokhara	Aerocet-Set 1	Slope=0.6732 Intercept =-2.976	0.9218	<0.0001
	Aerocet-Set 2	Slope=0.7614 Intercept = 1.774	0.9174	<0.0001
Chitwan	Aerocet Set 1	Slope=0.6299 Intercept=2.3028	0.893	<0.0001
	Aerocet Set 2	Slope=0.5608 Intercept=3.3675	0.7742	<0.0001

2.3 **Total Emission from Various Species of Biomass Fuel**

The experiments to monitor total emission from the combustion of commonly used biomass fuels for cooking in Nepal were carried out in a controlled laboratory setting using standard equipment following a standard protocol for water boiling test (WBT). The experiment was performed primarily to measure the emission pattern of various biomass fuels commonly used in the rural households of Nepal for cooking and

heating. Low and high power emission matrix was chosen as it gives a comparison between stove types and fuel types.

2.3.1 Laboratory Emission Monitoring System

The laboratory emissions monitoring system (LEMS), designed and manufactured by Aprovecho Research Centre, USA, measures the performance of stoves regarding total emissions from stove combustion including CO, CO_2 and PM [206]. With a solid sheet metal hood and variable speed blower, LEMS is capable of accurately measuring the emissions of simple household stoves and large institutional stoves. LEMS measures the total emissions produced by the stove during combustion with sensors for each pollutant. The CO sensor is an electrochemical cell. The conductivity between two electrodes in the cell changes when exposed to CO in proportion to the concentration of CO present. The CO_2 sensor uses non-dispersive infrared (NDIR) to measure CO_2 concentration and output voltage. The scattering photometer sensor estimates the smoke particle concentrations, whereas the gravimetric system within LEMS provides a total PM concentration using filter-based sampling. The scattering photometer sensor consists of a laser and a light receiver. The smoke particles in the sensing chamber scatter the laser light into the receiver. More light reaching the receiver indicates more smoke in the chamber. The level of light is calibrated with a laboratory-standard nephelometer to relate the amount of reflected light to the concentration of smoke particles. The gravimetric system consists of a vacuum pump

which pulls a sample through the sample line and the critical orifice. The flow through the sample line is maintained at a steady 16.7 l/min with the help of a critical orifice. A cyclone particle separator helps to collect all PM on a glass fibre filter during the combustion period. The weight difference of filter calculates the total smoke particulate mass before and after the test. The water temperature of the pot is recorded using a temperature sensor thermocouple (TC). An experimental set-up for testing using LEMS is presented in Figure 2.7.

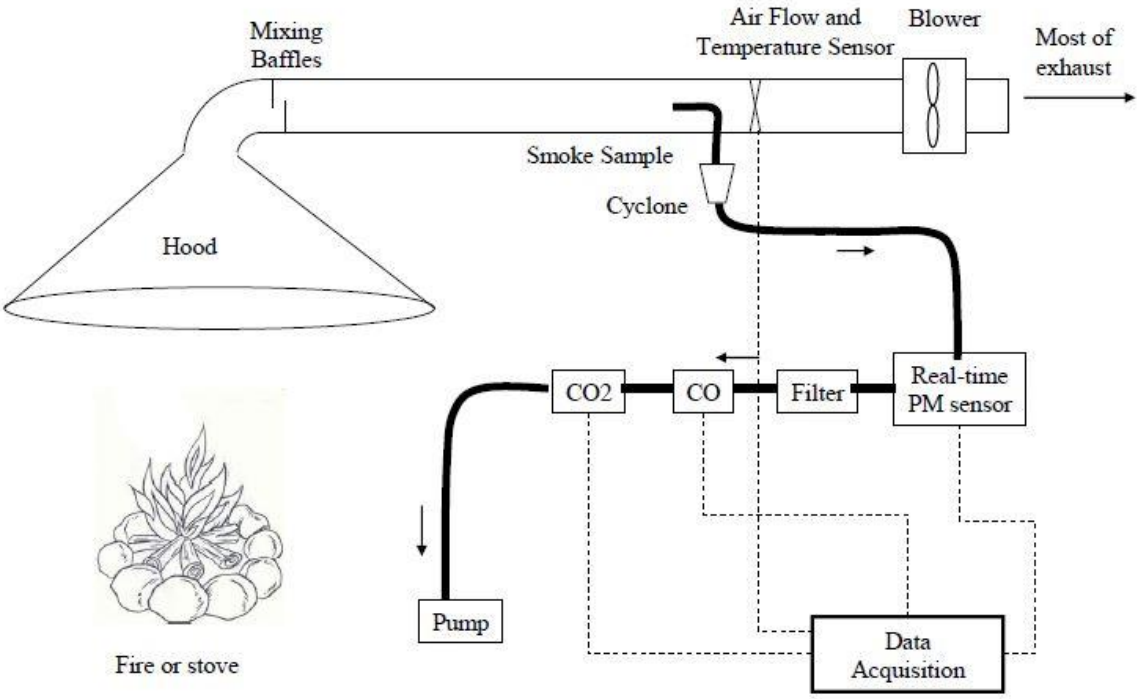


Figure 2.7 Experimental set up for testing emissions. Thick solid lines show smoke collection; arrows indicate the direction of airflow; dashed lines are electrical connections [207].

2.3.2 Water Boiling Test

The water boiling test (WBT) is the simplest way of measuring the efficiency of a stove using particular types of fuel to heat water in a cooking pot and the emissions produced whilst cooking [208, 209]. It is a simplified simulation of the cooking process, normally adopted to assess stove performance in a controlled environment [207]. The WBT provides initial or laboratory assessments of stove performance in a controlled setting and normally used to compare the effectiveness of different designs at performing similar tasks. However, it is an approximation of the cooking process generally conducted in controlled conditions by trained technicians, and the results of the test might differ from the real

cooking tests performed in the field even if efficiency and emissions are measured in the same way for both tests.

The complete WBT consists of three different phases that need to be carried out immediately one after another. A full stove test should always include all three test phases. Temperature variations in all three phases of WBT are presented in Figure 2.8.

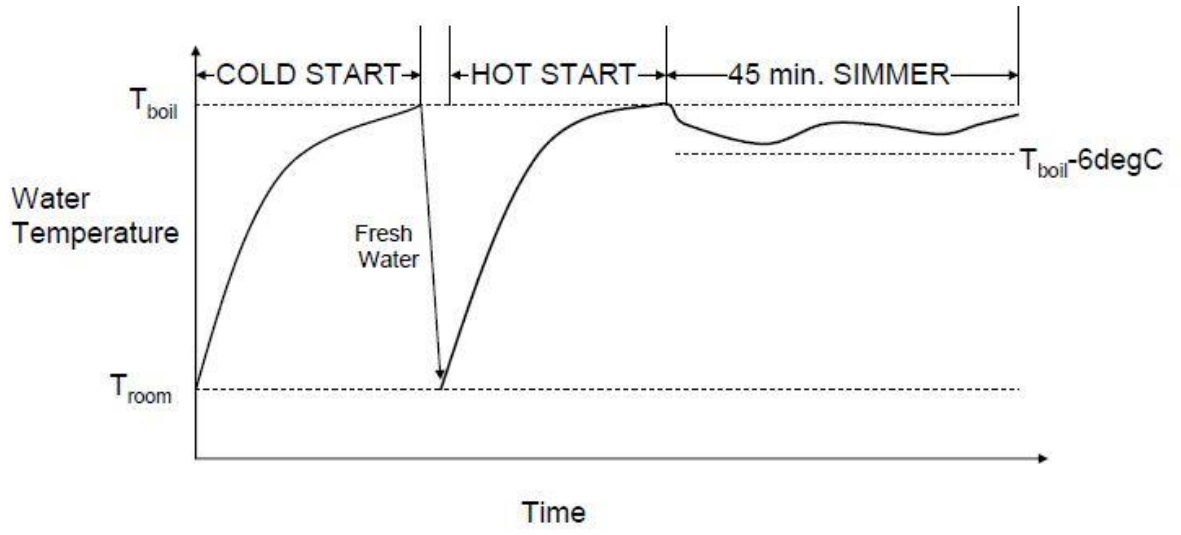


Figure 2.8. Temperature during the three phases of the water boiling test (Figure credit: Nordica MacCarty, [207]).

a) First Phase: Cold-start High Power

The WBT test begins with a cold start high power phase in which a measured quantity of water in a standard pot is boiled from a pre-weighted bundle of fuel. This phase uses the stove at room temperature. After the water reaches its boiling temperature, the fire is completely stopped, and ambient-temperature water in a fresh pot is used to perform the second phase.

b) **Second Phase: Hot-start High Power**

The cold start phase is followed by a hot start high power phase which is conducted while the stove is still hot. Again the same quantity of fresh water in the same pot is boiled from a pre-weighted bundle of fuel. This step helps to quantify the differences in stove performance when it is hot and when it is cold.

c) Third Phase: Simmer Phase

The simmer phase in the test is carried out to calculate the fuel required to simmer a known amount of water at just below boiling point for 45 minutes. The step helps in stimulating longer cooking periods.

2.3.3 Emission monitoring Metrics

The WBT is usually carried out to measure fuel efficiency. However, it can also be used to measure the emissions from the stove. The measured pollutant concentration in the WBT provides an assessment of the actual emissions from the stove, irrespective of the indoor air concentration or personal exposure concentration. In this study, The WBT was performed to measure total emissions from the combustion of various biomass fuels. The IWA metric for high power total emissions for PM and CO, which gives the emissions per Mega Joule of energy supplied to the pot was used for the data analysis. This metric is preferably used for high power phases (cold start and hot start) as it enables comparisons between stoves and fuel types in terms of total emissions.

2.3.4 Fuel Used

Various types of biomass fuelwood, agricultural residues and animal dung were collected from various parts of Nepal. The details of all fuel used for this experiment are presented in the respective section.

2.4 Biomass Smoke Sample Collection

2.4.1 Filter Based PM sample

Particulate matter ($PM_{2.5}$) resulting from the combustion of biomass fuel during cooking in a real cooking environment was collected in a membrane filter (PALL Corporation, 47 mm 2 μm pore size). The combustion by-product was passed through the filters placed in the filter holder of a collection unit. A locally made collection unit was used which consists of a pump (operated at 5 L/min) connected with the assembly of filter holder (URG-2000-30RAF-1, 47 mm filter pack) and cyclone (URG-2000-30E-5-2.5-S, 5 L/min, 2.5 μm) as shown in Figure 2.9. The total collection time in each household was throughout the total cooking period. A cyclone with a cut-off point at 2.5 μm helps to collect particles less than 2.5 μm in diameter present in the sample air on a filter during the full combustion period. The total amount of $PM_{2.5}$ collected in each filter was noted before the filters were sealed airtight and transferred to the University of Nottingham, UK. The filter, kept in a desiccator overnight to dry, was weighed before it was used. After the experiment,

the filter was again kept in a desiccator overnight to dry before taking its resulting weight. Amount of PM in each filter was calculated by taking filter weight difference after and before the experiment.

Figure 2.9 Cyclone and filter holder assembly for the collection of $PM_{2.5}$ from the combustion of biomass fuel.

Particulate matter collected from controlled burning laboratory studies was also collected. Briefly, dried firewood was combusted in a closed chamber of LEMS in the stove testing laboratory at the Centre for Rural Technology, Nepal (CRT/N). The combustion by-product was passed through the filters attached to the system collection unit in which respirable sized fine particles (less than 2.5 μm in diameter) were collected. The collection unit in LEMS primarily works on a gravimetric system, consisting of a vacuum pump for pulling the smoke through the sample line and critical orifice for maintaining the flow at a steady level of 16.7 L/min. A cyclone particle separator in the unit helps to collect all PM samples on a glass fibre filter during the full combustion period. The total test duration, the amount of wood used and the total amount of PM

collected in each filter were noted before the filters were sealed airtight and transferred to the University of Nottingham, UK. The filters were kept in a desiccator overnight to dry and were weighed before use. After the experiment, the filter was again kept in a desiccator overnight to dry before taking its resulting weight. Amount of PM in each filter was calculated by taking filter weight difference after and before the experiment.

2.4.2 Media Based Smoke sample

Figure 2.10. Media based smoke sample collection unit.

Biomass smoke resulting from the combustion of biomass fuel for cooking was also collected in cell and tissue culture media Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich). The smoke produced from the combustion of biomass fuel for cooking was channelled through 10 ml of media. The pump (flow rate of 3 L/min) and impinger set up was prepared, as shown in Figure 2.10. The smoke was collected throughout the cooking period in each household.

2.5 **Mammalian Cell Culture Methods**

2.5.1 Normal Human Bronchial Epithelial Cells (Commercial)

Normal human bronchial epithelial cells (NHBECs), were obtained from Lonza®, Castleford, UK (Product code CC-2540) and recovered from frozen vials and used for subsequent studies. Information on the donors used in this thesis is presented in Table 2.4.

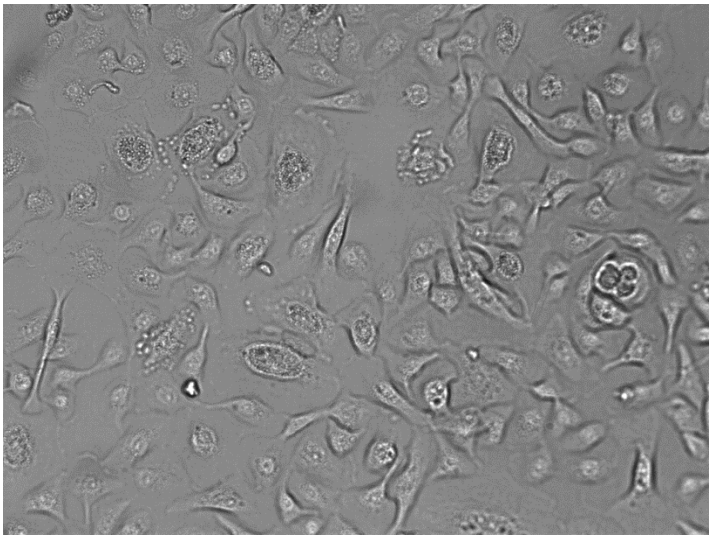


Figure 2.11 Morphology of a human bronchial epithelial cell (HBECs) in submerged in culture media. Commercial normal HBEC (Lonza®Clonetics™) donor 6 (x 20 objective).

Table 2.4 Details of commercially sourced HBEC used in this thesis. The details were obtained from Lonza® (Castleford, Uk)

Donor ID	Donor number	Age	Gender	Viability	Doubling time	Seeding efficiency	Alcohol	Smoker
355687	5	38	Male	75%	23 hrs	68%	No	No
369144	6	43	Male	87%	18hrs	41%	No	No

2.5.2 Culturing Cell Lines

Normal human bronchial epithelial cells (NHBECs) were cultured in bronchial epithelial basal media (BEBM^{TM}Lonza), which is defined as bronchial epithelial growth media (BEGM TM Lonza) after supplementation with growth factors and additives (2 ml Bovine Pituitary Extract, 0.5 ml Insulin, 0.5 ml Hydrocortisone, 0.5 ml retinoic Acid, 0.5 ml Transferrin, 0.5 ml Trilodothyronine, 0.5 ml Epinephrine and 0.5 ml Epidermal Growth factor in 500 ml BEBM)[210]. HBECs were taken out from liquid nitrogen, thawed and seeded at a density of 250,00 cells per T75 flask containing 15 ml of pre-warmed (37°C) fresh BEGM. After every 48 hours, the spent media was changed with fresh pre-warmed media until the cells were ready for seeding further into the culture vessels. Monolayers of cultured cells in T75 flasks at 80-90% confluence were detached and seeded into 24 well plates at a density of 20,000 cells per well.

Cells were cultured following a pre-established protocol. Briefly, the cells were detached from the culture by adding 5 ml of pre-warmed (37 °C) trypsin-EDTA solution (Sigma) after the spent media was aspirated off from the flask. After 5 minutes of incubation at 37°C and ensuring all the cells were detached from the flask, 5 ml of pre-warmed trypsin inhibitor (Sigma) was added to the flask to deactivate the effect of trypsin on cells. The solution was then transferred to the centrifuge tube. The flask was then washed with 5ml of media and transferred into the same centrifuged tube which was then centrifuged at 200 x g for 5 minutes. The cell pellet was then suspended in 1 ml of media, counted using a haemocytometer and seeded at the recommended density by Clonetics [211].

2.5.3 Cryopreservation of cultured cells

Cells for long-term use need to be stored under conditions to maintain cell viability. For this, the cultured cells were frozen at a temperature of -180°C in liquid nitrogen. The cells were first suspended in a freezing medium (10% v/v solution of DMSO (dimethyl sulfoxide, Sigma Aldrich) in BEGM) and transferred into the liquid nitrogen for long-term storage. NHBECs cells were frozen in freezing media at a cell concentration of 2.5 x 10^6 cells/ml in each vial.

2.5.4 Cell counting

The cell density in each cultured flask was determined by counting using a neubauer haemocytometer. The cells were detached from the culture and pelleted as described in section 2.7.2 and re-suspended in 1 ml of fresh media. 10 µl of cell suspension was added under the cover slip of the assembled haemocytometer, and the average number of cells was counted in the four corner squares. The cells were counted using an inverted light microscope, and the average number of cells was determined. The total number of cells per ml was calculated by multiplying the average value by the volume of the total square, which is equal to $1X10^4$.

2.6 Tissue Culture

2.6.1 Human parenchymal lung tissue

The human parenchymal lung tissue was obtained with informed written consent from the patients undergoing lung resection surgery at University Hospitals, UK. The tissue was sourced from Nottingham Health Science Biobank (NHBS, Study number 56, ethics Ref. 10/H1008/72), from Papworth Hospital Tissue Bank (PHRTB, Study number 61, ethics Ref. 08/H03/56+5) and Arden Tissue Bank (ATB, Study number 62, ethics Ref. 12/SC/0526). The patient demographics of all tissue used in this thesis are presented in the appendix II. A representative tissue sample is shown in Figure 2.12.

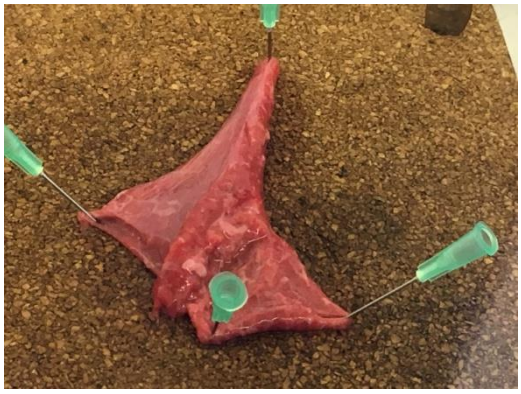


Figure 2.12. A representative sample of lung tissue used for the experiment.

2.6.2 Tissue processing

The tissue was stored at 4°C in DMEM + 1 X PSF after surgery and transferred to the lab for further processing in the same solution maintaining the temperature [15]. The tissue was usually transferred on the same day or the next day after surgery. The tissue was first washed in Tyrode's buffer solution, and whole tissue weight was taken. Tissue sample was then dissected into small pieces of 50-100 mg (wet weight) and incubated for 24 hours in 24 well plate with 1 ml of RPMI 1640 media (with 2.05 mM L-glutamine and 25 mM HEPES) (Sigma, 51536C) containing antibiotics and antimycotics (PSF, penicillin, streptomycin and fungizone) (Sigma, A5955). On the following day, the media was replaced, and explants were stimulated with lipopolysccirade (LPS) and samples as per the experimental design. The samples were then incubated for a further period (usually 48 hours), followed by the collection of supernatants for subsequent protein analysis. All experimental conditions for all tissue work in this thesis were performed in duplicate.

2.7 **Stimulus preparation**

2.7.1 Wood smoke extract preparation

Wood smoke particulate matter ($PM_{2.5}$) was collected in a glass fibre filter (Whatman® glass microfiber filters, grade GF/A, 1.6 µm pore size, 90 mm diameter, Sigma-Aldrich) for laboratory-based study and PTFE membrane filter (PALL Corporation, 47 mm 2 µm pore size) from field-based studies as described earlier. The particles from the filters were extracted in methanol by water bath sonication, dried under nitrogen gas and stored at -80°C until further use. Briefly, one half of the filter was immersed in 4 ml of methanol and sonicated for 1h in a water bath sonicator. The extraction was repeated twice with the other half of the filter, and the solution was kept in a single falcon tube. The combined solution from each filter was then purged under a gentle stream of nitrogen gas for 2h. The total weight of the remaining extracts after evaporation of all solvent was taken. The extract was then dissolved in 1ml of Phosphate buffer solution (PBS), and the total concentration of the solution was calculated. The solution was then diluted with the culture media (BEGM and RPMI) to get the required concentration for cell and tissue culture experiments.

2.8 **Protein Analysis**

2.8.1 Enzyme-Linked Immunosorbent Assays (ELISA)

An enzyme-linked immunosorbent assay is used to detect and measure peptides, proteins or antibodies in samples including culture supernatant, blood serum [212]. Measurement of Interleukin-8 (IL-8) and interleukin-6 (IL-6) levels was carried out using sandwich Enzyme-linked Immunosorbent Assays (ELISA) (Human IL-8 DuoSet® ELISA, Catalog number DY208-05, R&D SystemsTM and Human IL-6 DuoSet® ELISA, Catalog number DY206-05, R&D SystemsTM) as per the manufacturer's instructions. Unlike other ELISAs, sandwich ELISAs capture the protein in between two layers of antibodies, the capture antibody and the detection antibody, using two separate epitopes on the protein.

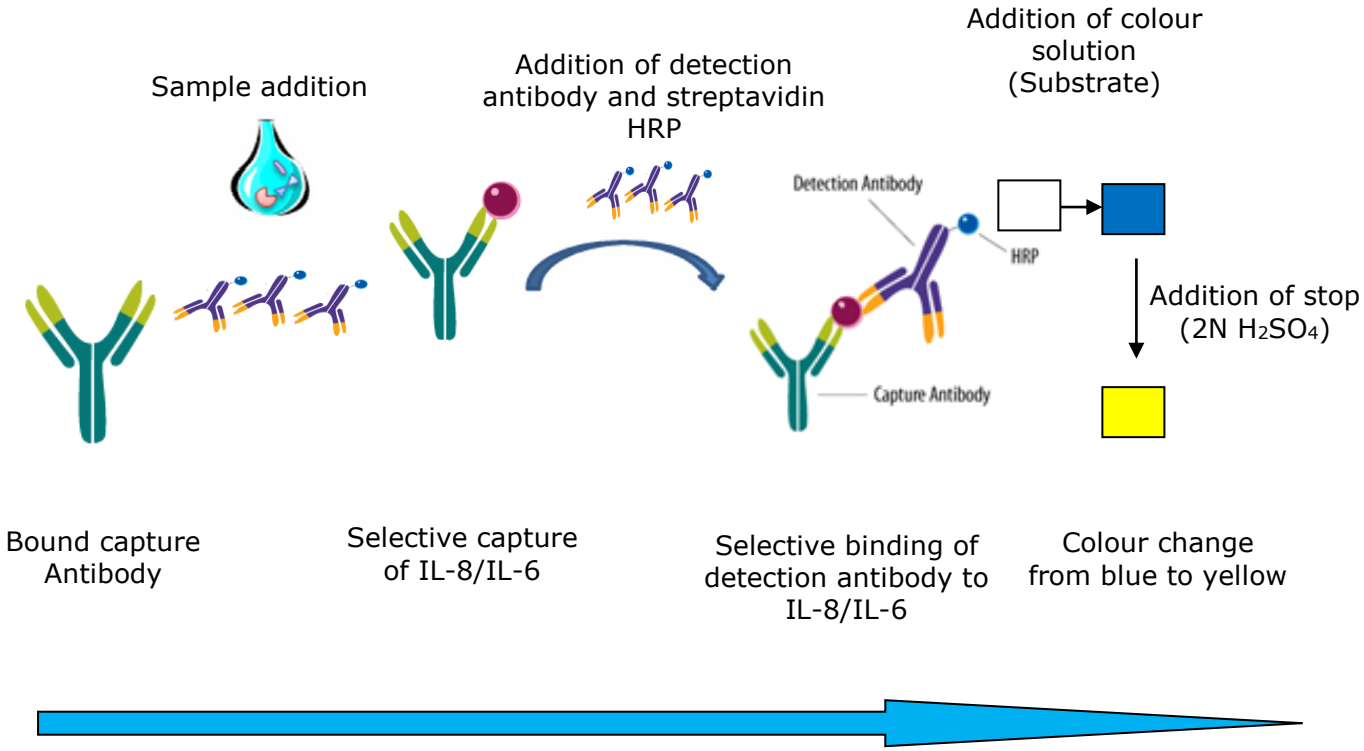


Figure 2.13 Sandwich ELISA procedure. A 96-well plate is coated with capture antibody and left overnight to bind to each well, which is on the following day blocked by adding block buffer (1 % w/v BSA in PBS). The analytes of interest specifically bind to the capture antibody, which in turn captures the detection antibody. Streptavidin HRP binds to the detection body, which allows the reaction with the colour solution, changing from transparent to blue colour. The plate is read using a plate reader to quantify the amount of analytes of interest after adding the stop solution (2N H_2SO_4) which change the colour from blue to yellow.

In summary, a non-sterile 96 well plate was coated with a capture antibody diluted in phosphate buffered saline (PBS) and incubated at room temperature overnight, which allows the capture antibody to bind to the well. The following day, the solution was removed, and the plate was washed three times with wash buffer (0.05 % v/v Tween® 20 in PBS)

(PBS-T) prior to blocking the uncoated plate with block buffer (1% w/v BSA in PBS). After an hour of incubation time at room temperature, the plate was washed again 3 times with wash buffer followed by addition of standard and samples, diluted in reagent diluent (1% w/v BSA in PBS for IL-6 and 0.1% w/v BSA, 0.05% v/v Tween®20 in Tris-buffered saline for IL-8) as required in duplicate for a period of 2 hours at room temperature. This was followed by the addition of a detection antibody diluted in reagent diluent for 2 hours. Following 2 hours of incubation time, streptavidin HRP diluted in reagent diluent was added in each well and incubated for 20 minutes in the dark, preventing direct light. The colour substrate solution (1:1 solution of substrate reagent A and substrate reagent B, R&D SystemsTM, catalogue number DY994) was added for 20 minutes. The plate was washed three times with PBS-T after each incubation and before the addition of each new solution. Following the incubation period, the plate was ready for reading by the addition of stop solution (2N H_2SO_4), which changes the colour from blue to yellow. The plate was then read immediately using a Flexstation 3 microplate reader (reading at 450 nm and corrected at 570 nm). The standard curve obtained from the reading was used to calculate the concentration values.

2.8.2 Luminex Assay

For the simultaneous detection of various human biomarkers along with IL-8 and IL-6, which was previously detected by ELISA, a magnetic Luminex screening Assay (Human Premixed Multi-Analyte kit, Magnetic

Luminex®Screeeing Assay, R&D SystemsTMCatNo#LXSAHM) was used to analyse cell and tissue culture supernatants as per the manufacturer's instruction [213-215]. Unlike other assays, the Luminex assay is used to detect multiple biomarkers of interest in a single sample. Suspended colour coded microparticles coated with antibodies against analytes of interest can be read with the Luminex MAGPIX® CCD Imager or Luminex 100/200TM or Bio-Rad®Bio-Plex®, dual laser, flow-based sorting and detection platforms.

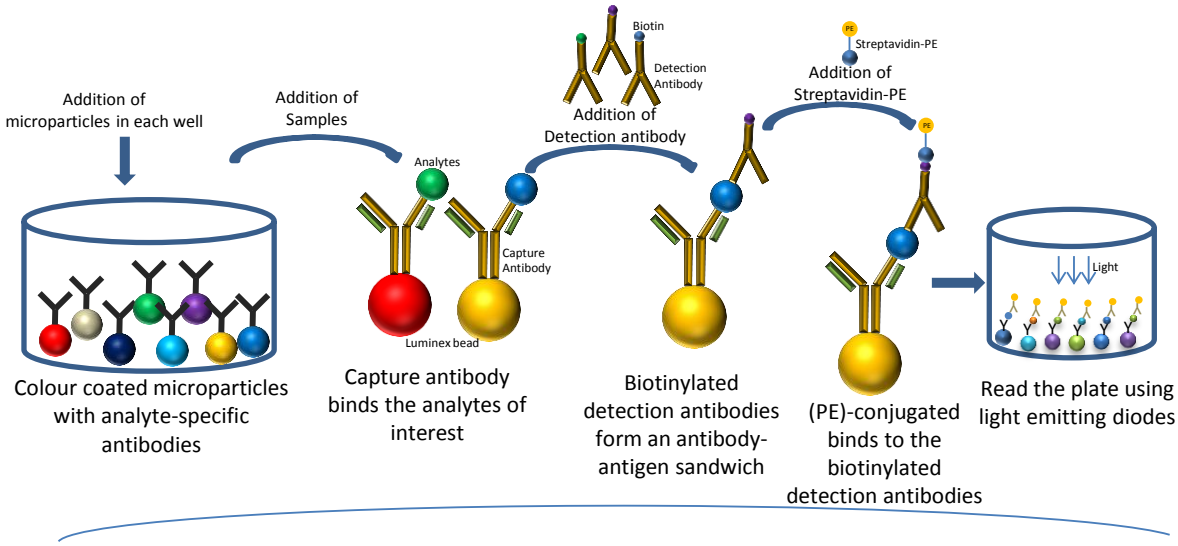


Figure 2.14 Luminex Assay Principle. Samples and standards were added with colour coded Luminex beads which bind the analytes of interest via analyte-specific capture antibody present in the beads. The analytes of interest bind the biotinylated detection antibody, which in turn attached with the streptavidin-PE. The microparticles were then kept in suspension by adding a buffer, and the microparticles were read using a dual laser flow-based system.

Briefly, the 96 well Luminex assay micro-plate was coated with colour-coded magnetic microparticles, pre-coated with analyte-specific antibodies. The standards and samples diluted as required in duplicate

were added straight after pipetting the microparticles in each well and incubating for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12” orbit set at 800 ±50 rpm). During the incubation time, the immobilised antibodies bind the analytes of interest. A biotinylated antibody cocktail specific to the analytes of interest was pipetted into each well after washing away any unbound substances. After a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE) was added to each well which binds to the biotinylated antibody. Any unbound streptavidin-PE was then washed after 30 minutes of incubation followed by re-suspending the microparticles in a buffer. The plate was then read using a dual laser, flow-based system. In summary, a dual laser flow-based system consists of a magnet which captures and holds the super paramagnetic microparticles in a monolayer and two separate, distinct light-emitting diodes illuminate the beads by identifying the analyte and measuring the magnitude of the PE-derived signal. A CCD camera is used to image each well.

2.9 **Endotoxin Quantification**

Endotoxin concentration present in the smoke sample collected from different locations was quantified using a Limulus Amebocyte lysate (LAL) assay (Pierce^{TM}LAL Chromogenic Endotoxin Quantification Kit, ThermoFisher Scientific, US). Quantification of endotoxin level was performed as per the protocol suggested by the manufacturer

(ThermoFisher Scientific, US) under sterile conditions. Dilutions of endotoxin standard (Escherichia coli 011:B4) were prepared using endotoxin-free water, and a four-point standard curve was generated (R^2 = 0.996). All the samples were used in 50 fold dilution prepared using endotoxin-free water, and neat endotoxin-free water was used as a blank. All the samples in the assay plate were run in duplicate. The absorbance was measured at 405 nm in a plate reader. The final concentration of endotoxin for each sample was calculated subtracting mean concentration in the blank from the mean concentration of each sample.

2.10 Statistical Analyses

All statistical analyses were carried out in GraphPad Prism (GraphPad Prism software Inc. Version 7.01). All data generated from tissue culture work were normalised using wet tissue mass before conducting statistical analysis.

2.10.1 Analysis of variance

The variance in two independent groups was checked using either student's t-test (parametric) or Mann-Whitney test (non-parametric) depending on the number of samples analysed. The non-parametric test was carried out when using small dataset ($n<20$). When comparing more than two independent groups, either one-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was carried out. All statistical analysis was performed in a raw data and data presented, mostly for inflammation study, is the fold change of treated samples over the untreated basal condition. For example, fold increase in cytokine response from lung tissue stimulated with biomass smoke is presented over the basal condition.

2.10.2 Correlation Analysis

Correlation analysis was performed to compute a correlation coefficient which estimates the degree of linear association between two variables. The correlation coefficient can be defined as the number of standard deviations that the outcome of one variable changes for a standard

deviation change in another variable. A correlation coefficient is always a number between -1 to +1 where r=-1 indicates two variables have a strong negative correlation, and where r=+1 indicates a strong positive correlation between two variables tested. If two variables do not correlate, the value of r will be close to 0. The dataset within this thesis for correlations analyses are non-normal (non-parametric), and hence the non-parametric version of the statistical tool (Spearman's Correlation) was used.

2.10.3 Simple regression analysis

Simple linear regression was used to develop the relationship between two variables and to generate the best fit straight line using the method of least squares. The equation of a straight line from the regression would best describe how the value of a dependent variable (normally plotted in 'y' axis) differs or changes with the changes in the independent variable (normally plotted in 'x' axis). It generates the equation in the form of y=mx + c, where m is the slope and c is the line intercept, which is then used to compute the data for the dependent variable (y) with the given value of an independent variable (x). This linear regression method was mainly used to develop the correction factor between the exposures measured from two independent equipments and to correct the measured data.

Chapter 3: Characterization of Emission Factors of commonly used biomass fuel in Nepal

3 Characterization of Emission factors of commonly used biomass fuel in Nepal

This chapter presents work related to the emission and personal exposure to indoor air pollutants from the combustion of biomass fuels commonly found and used in Nepal. The work presented here is divided into two sections. First, the emission patterns of commonly used biomass fuels will be presented. Then the real-time personal exposure of indoor pollutants resulting from the combustion of such biomass fuels will be discussed.

3.1 Introduction

3.1.1 Energy Consumption patterns in Nepal

Biomass fuel is an important and primary source of energy for cooking in many rural parts of developing countries throughout the world [216]. According to the World Health Organization (WHO), over 3 billion people worldwide use biomass fuel for daily cooking and heating. In Nepal, more than 60% of the population cook their daily food using polluting biomass fuel in low efficient stoves [217]. The use of biomass fuel for cooking and heating is more prominent (more than 75%) in the rural area of the country where more than 75% of the country's population lives [217]. Biomass fuel provides 35% of primary energy consumption in developing countries and 14% of total energy consumption globally [218-220]. In Nepal according to the economic survey 2014/15 report, an estimated 78% of all energy consumed was derived from biomass fuel including

fuelwood (70%), crop residue (3.4%) and cow dung (3.6%) [221]. The report also highlighted that the residential sector consumed approximately 80% of total energy consumption in national energy demand for domestic use [221]. Biomass resources, like fuelwood along with agricultural residue and animal waste, have been extensively used in the household for cooking and heating, providing 96.5% of all household energy [221].

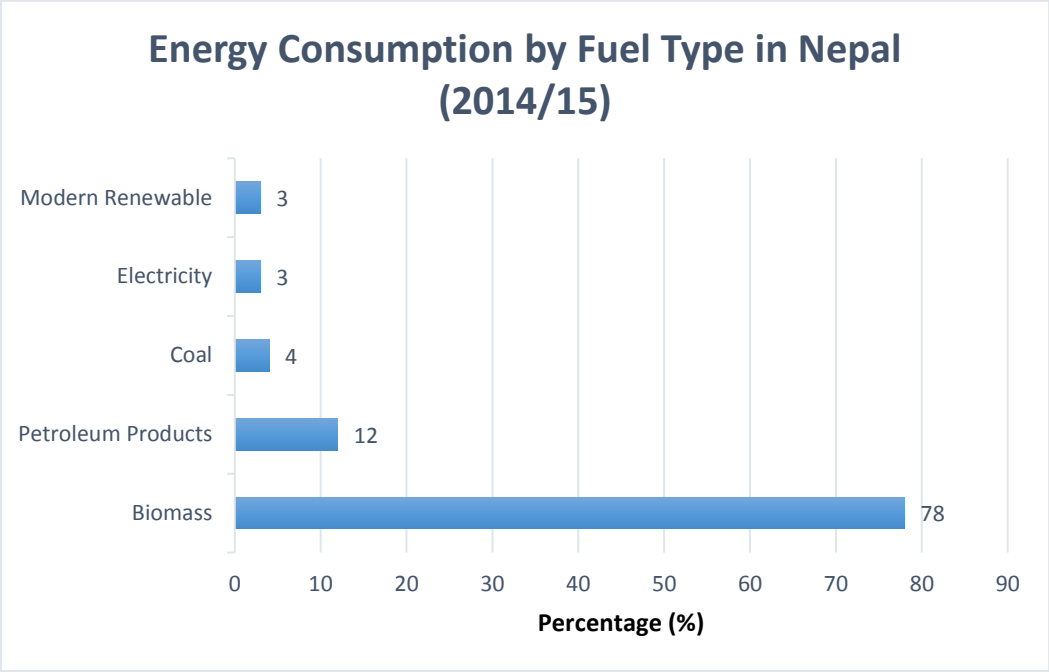


Figure 3.1 Energy consumption by fuel type in Nepal in the year 2014/15. The data published in national economic survey report conducted for the year 2014/15 [221].

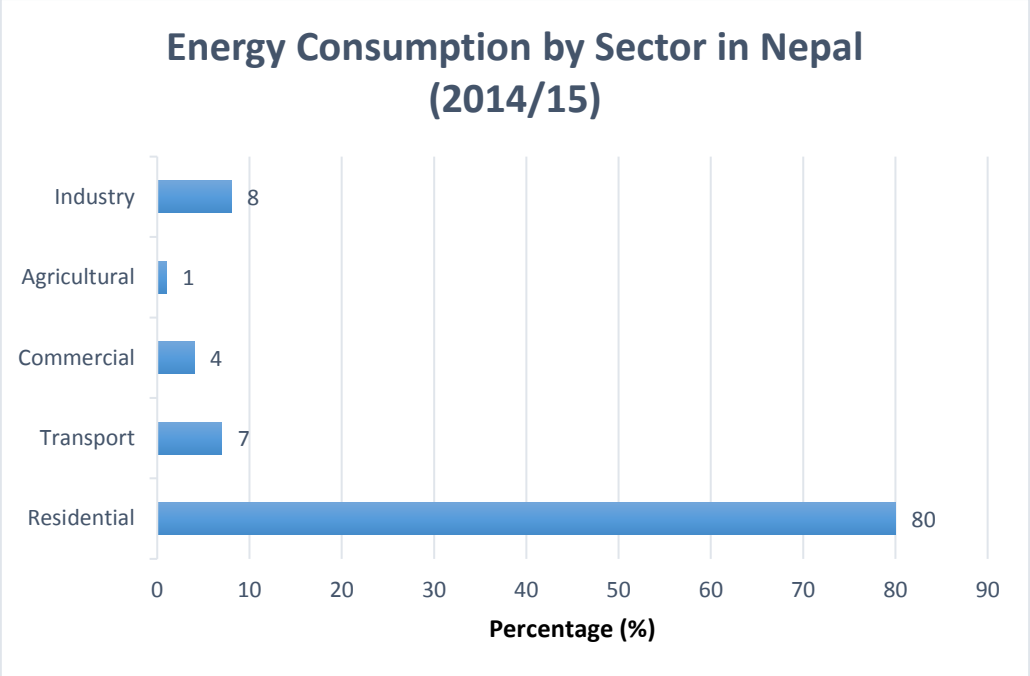


Figure 3.2 Energy consumption by sector in Nepal in the year 2014/15. The data published in national economic survey report conducted for the year 2014/15 [221].

3.1.2 Biomass fuel Sources in Nepal

3.1.2.1 Fuelwood

Nepal is a country well recognised for its rich biodiversity and culture. Within a total area of 147 181 km^2 and a distance of less than 150 km, the country provides a unique opportunity to visit both lowland regions at less than 100 meters above sea level (masl) in the tropical Terai in the South, and the Himalayas which culminate in the highest point in the world, Mount Everest (8848 m), on the edge of the Tibetan plateau. This dramatic difference in altitude provides the country with varying climatic condition ranging from sub-tropical to Arctic-alpine. The diverse climatic conditions provide Nepal with a large number of different plants, including

5400 species of vascular plants, over 254 species of endemic plants and 700 species of medicinal plants [222]. Many of these species of plants and trees are distributed among the forest covering approximately 37% of the total area from tropical forest below 1000 m to alpine scrub forest above 4100 m. The species of *Shorea robusta* are commonly found in a tropical forest while other species like *Acacia catechu*, *Dalbergia sissoo* and *Terminalia* are also found. *Pinus Roxburghii*, *Schima Wallichii*, *Castanopsis indica* and *Alnus nepalensis* are the common species of tree found in sub-tropical forests [223]. The species of a tree commonly found in a sub-alpine forest located at above 3000 m to below 4000 m are *Abies Spectabilis, Betula Utilis* and *Rhododendron [224].* The species of *Shorea Robusta*, *Pinus Roxburghii*, *Schima Wallichii*, *Castanopsis Indica* and *Alnus Nepalensis* account for the major share of forest area in the country [223]. Wood as a source of energy for domestic use, particularly for cooking and heating is the most important product harvested from the forest [225].

3.1.2.2 Agricultural Residue

The temporal, altitudinal and topographical variation has also made the nation rich in numerous crops and cultivated plants [226]. Agriculture supports the livelihood of 65% of the total population contributing a significant share of the country's GDP (36.1%) [227]. The major agricultural products are rice (45%), maize (20%), wheat (18%), millet (5%) and potatoes (3%) [228]. Crop residues are commonly used as a

source of fuel for cooking in many rural areas. Maize is the most important crop in terms of area and production. It is produced in all three regions of the country. Maize is produced mainly in the mid-hill region contributing 70% of total production while the Terai region and high-hill region share 22% and 8% respectively [228]. Therefore, in terms of using agricultural residue as a fuel for cooking, maize stalk and corn cob contributes the major share among the agricultural residues.

3.1.2.3 Animal Waste

Another form of source of energy for cooking in rural areas is dried animal waste. Livestock is one of the major cash income sources of farmers. Nearly 70% of total households raise some type of livestock, including cattle, buffalo, goats, pig, yak [226]. Farm families in the high hill/ mountain regions generally raise Yak or Chauri while in mid-hills and Terai cow, buffalo, sheep and goat are common [226]. The farm families make cash income by selling milk, meat from the livestock while the animal waste, especially yak dung, cow dung and buffalo dung are used as a fuel for cooking and heating. The animal dung is typically used as cooking fuel by making it into a cake shape and drying it in the sun. However, in some places, especially in the Terai region, animal dung has been used to produce biogas which is considered as a cleaner fuel for cooking.

With the tremendous potential of fuelwood from the forest, the majority of biomass fuel for domestic use in Nepal comes from fuelwood. The study

conducted in 1995 showed that 92% of total energy was supplied by biomass fuel out of which fuelwood, agricultural residue and animal waste shared 75%, 16.3% and 8.7% respectively [229]. The use of different biomass fuels has remained the same over the recent period. A study in 2010 showed that out of 87% total energy supplied by biomass, 77% was from fuelwood, and the rest was from other sources like agricultural residue and animal waste [230]. Similarly, fuelwood shared 71.2% of the total energy supplied by biomass in the year 2016, while the rest was from other sources. A study conducted recently in southern Nepal revealed that two-thirds of homes use biomass fuel in which wood and animal dung were the most common, comprising 37.1% and 27.7% respectively [120].

The combustion of such biomass fuels in a low efficient stove, and open hearths result in the production of a variety of health-damaging pollutants. The pollutants from biomass burning include particulate matter (PM), carbon monoxide (CO), methane, polycyclic aromatic hydrocarbons (PAH), and volatile organic compounds (VOC) [17, 231, 232]. The people in the household using biomass fuel are exposed to higher concentrations of indoor pollutants compared to the households using clean fuel [39, 233], which lead to a wide range of adverse health outcomes. Several strategies have been introduced globally to reduce emission and exposure to HAP. Shifting from using polluting biomass fuel to clean fuel like LPG, biogas or electricity could be the best possible way to reduce exposures. However, rural communities have limited, or no access and also the lack

of affordability of clean fuel due to low socioeconomic status leads to continued use of polluting biomass fuel. Improving combustion efficiency of stoves by designing efficient cook stove (ICS) has been a successful intervention to reduce IAP. Several ICS designs have been implemented and tested in different countries [102, 117, 234], and comparative studies on exposure concentration using TCS and ICS suggest significant reductions of exposure levels can be achieved using ICS [117, 234].

HAP from biomass burning for cooking using inefficient combustion devices has been a serious concern in Nepal. Several studies have already been done monitoring HAP from the combustion of biomass fuel for cooking in various region of Nepal [20, 117, 120, 235]. However, most of these studies monitored indoor concentrations of pollutants over a fixed period, considering all source of fuel (fuelwood, agricultural residue or animal dung) as a common biomass fuel. Few studies have looked at the variation in emissions separately while using fuelwood, animal dung or agricultural residue [20]. None of the previous emission monitoring studies performed in Nepal has measured the emission from specific types of fuelwood along with agricultural residue and animal dung. As biomass fuels have been a primary source of domestic energy for cooking in Nepal, it is important to study the variation in emission factor using those species of biomass fuel. As emissions vary significantly depending on the source, types of fuel, stove designs and cooking practices [236], I attempted to monitor the emission factor of traditional biomass fuels being used for cooking in various ecological regions of Nepal.

3.2 **Aim**

The main aim of this study was to provide further insight into the emission factor variation among combustion of various species of biomass fuel for cooking in Nepal.

3.2.1 Specific Aims

- To measure the emission of $PM_{2.5}$, CO and CO_2 resulting from the combustion of different species of fuelwood, agricultural residues and cow dung.
- To monitor personal exposure to $PM_{2.5}$ and CO using various sources of biomass fuel combusted for cooking in rural households of Nepal.
- To monitor the percentage reduction of personal exposure to $PM_{2.5}$ and CO, which can be achieved using a more efficient cookstove.

3.3 **Method:**

3.3.1 Total Emission Factor

3.3.1.1 Biomass samples

In this study, biomass samples were collected from different regions of Nepal and included commonly used fuelwood, agricultural residue and animal waste. The biomass samples used were *Pinus Roxburghii (Salla), Alnus Nepalensis (Utis), Schima Wallichii (Chilauni), Shorea Robusta (Sal), Quercus Leucotricophora (Sajh), Corn Cob (Khoya), Maize Stalk (Dhod)* and *Cow Dung Cake (Guitha).*

Table 3.1 Fuel properties

Fuel Species	Local Name	Moisture Content (%)	Energy content (MJ/kg)	Source
Corn Cob	Khoya	12.9	17.62	[207]
Cow Dung cake	Guitha	13.4	11.8	[237]
Maize stalk	Dhod	12.5	16.1	[237]
Pinus Roxburghii	Khotesalla	11.3	20.79	[238]
Alnus Nepalensis	Utis	10	19.4	[239]
Schima Wallichii	Chilaune	10	20	[238]
Shorea Robusta	Sal	12.2	27.9	[240]
Quercus Leucotricophora	Baajh	11.6	18.9	[239]
Prunus Cerasoides	Payun	13.8	19.1	[207]

3.3.1.2 Experimental procedure

Detailed experimental methods and materials have already been discussed in chapter 2.3. Briefly, all experiments to measure emission factor from biomass combustion was carried out at the cook stove testing laboratory at the Centre for Rural Technology Nepal (CRT/N), Kathmandu-Nepal. The laboratory emission monitoring system (LEMS, Aprovecho Research Centre, Cottage Grove, OR, USA) was used to perform water boiling tests (WBTs) following a set protocol proposed by Global Alliance for Clean Cookstove (GACC) [207]. 5L of water is boiled for three phases (high power-cold start, high power-hot start and low power-simmer) of standardised WBT and emission from fuel combustion and stove efficiency is measured. All experiments were carried out in a controlled laboratory environment. A total of 8 different biomass fuel, including five species of fuelwood, two agricultural residues and one animal dung was used for this study. The full WBT was carried out for n=3 with each fuel except for *maize stalk* (n=1) due to unavailability of enough fuel at the time of the study. The stove used for the entire test was the traditional type of cook stove (Figure 3.3) which represents the stove design commonly used in the majority of rural communities. The results of this study in this section are presented as the average of cold start and hot start, commonly referred as high power phase (refer WBT from section 2.3.2), for all the measured parameters [241]. The data presented for each fuel is the overall mean of three independent full water boiling test except for *maize*

stalk where the data is from the single test. The moisture content of all fuels was between 11-15%.

Figure 3.3 Traditional cook stove used for the emission factor experiment.

3.3.1.3 Performance and efficiency measures

Performance and efficiency measures include the parameters which describe the quality of fuel and stove used regarding time, the quantity of fuel used and thermal efficiency.

Boiling time: The boiling time refers to the total time taken for the water to reach boiling temperature from the starting temperature with each fuel. For the comparison of boiling time between fuels, the temperature corrected time reflecting a temperature rise of 75°C from start to boil was taken for the analysis. This is calculated using the following equation $\Delta t = \left(t_f - t_i\right) \times 75/(T_f - T_i)$ where Δt = temperature corrected time to boil, t_f =final time t_i = initial time, T_f = final water temperature (°C) and T_i= initial water temperature (°C).

Specific Fuel Consumption: Specific fuel consumption is a measure of the amount of fuel required to boil 1L of water. It is usually calculated by the equivalent dry fuel used minus the energy in the remaining charcoal, divided by the litres of water remaining at the end of the test. The fuel consumption for each fuel presented is the temperature corrected specific fuel consumption to reflect temperature rise of 75° C from start to boil. The temperature corrected values were used as it normalizes the differences in initial or final water temperatures and makes it easier or simple to compare different tests with different fuels.

Thermal Efficiency: Thermal efficiency is a measure of the percentage of heat or energy produced by the fuel delivered to the water in the pot. The thermal efficiency indicates the greater ability of the system or fuel to transfer the produced heat to work. The thermal efficiency of each fuel while completing water boiling test was measured and compared.

3.3.1.4 Emission Measures

Among various pollutants, emissions of $PM_{2.5}$, CO and CO_2 were taken into consideration and measured from the combustion of each of the fuel for this study. The emissions per mega joule of energy delivered to the pot matrix (emission factor) were used for the comparison as it gives the emissions in terms of the desired output, cooking energy and enables comparisons between stoves and fuels [242]. The cooking energy delivered to the pot is a combination of the sensible heat that raised the pot water temperature and the latent heat that produced steam. Also, a

total amount of emission, the amount of pollutant emitted per kg of fuel burnt and indoor emission rate were measured.

3.3.2 Personal exposure monitoring

Having demonstrated the emission factor of biomass fuel combustion in a laboratory setting, I then investigated the real-time personal exposure to these emissions using biomass fuels for cooking in an actual real-life cooking environment. The monitoring was performed in the kitchen of rural households while the actual food was being cooked without any interference on regular cooking routines. The exposure monitoring was performed for each fuel in two different stove designs throughout total cooking durations. This section describes the work related to personal exposure monitoring using different biomass fuels for cooking in a rural setting.

3.3.2.1 Biomass fuel

Biomass fuels used in this part of the study were *Pinus Roxburghii (Salla), Alnus Nepalensis (Utis), Schima Wallichii (Chilauni), Prunus Cerasoides (Payun), Corn cob (Khoya), Maize Stalk (Dhod)* and *Cow Dung Cake (Guitha).* These biomass fuels were the conventional fuels being used in the study sites. All the fuels except *Shorea Robusta* and *Quercus Leucotricophora* were the same fuels I used in the previous laboratory experiment. However, I included one more fuelwood *Prunus Cerasoides,*

another common fuelwood in the study sites, which was not included in the previous laboratory-based emission factor study.

3.3.2.2 Study site

The site for this study was selected on the primary basis of the percentage of household using biomass fuel as a primary source of energy for cooking. The study was conducted in households of Pyakurel Gaun, a small cluster of dwellings in Mathurapati Phoolbari VDC of Kavrepalanchowk District. The village is located in the Eastern part of Kathmandu (27°58′ N, 85°60′ E) at an approximate altitude of 1400-1700 meter above sea level. The people in the village are from a mixed ethnic group; the majority includes Tamang, Bhramin, Chhetris and Sunuwars, spreading in a total of about 80 households. According to the CBS 2011, 95.27% of the houses in this VDC are mud bonded stone houses with 84.17% Galvanized iron roof, and 1.01% tile roofs [243]. The village was an ideal site for our study as the majority of the population (about 74%-data obtained from the preliminary survey) relies on biomass fuel as a primary source of energy for cooking. In addition to these various sources of biomass fuel, including different species of fuelwood, agricultural residue and animal dung were common in use for cooking. The most common types of fuel used in this area with its scientific name (local name) are *Pinus Roxburghii (Salla), Alnus Nepalensis (Utis), Schima Wallichii (Chilaune), Prunus Cerasoides (Payun), Corn Cob (Khoya), Cow Dung Cake (Guitha), Maize Stalk (Dhod)*. Another important aspect of our

study was to monitor the potential reduction in emissions, and personal exposures using a more efficient cookstove commonly called improved cook stove (ICS). I found from the initial survey that among biomass users, about 55% of households used a traditional type of cook stove and other 45% of households use an improved type of cook stove. This allowed me to work in a single study site to test the effectiveness of interventions.

3.3.2.3 Study Stove

The potential reduction of exposure resulting from the combustion of biomass fuel using a more efficient cook stove compared with the traditional type of cook stove was monitored. The common type of cook stoves found in households of the study site was a traditional single pot mud stove (Figure 3.4(a)) and improved two pot mud stove (Figure 3.4(b)). The traditional stove generally refers to a simple locally made configuration where a cooking pot is typically adjusted in the centre of a triangular configuration of three stones. This type of stove is commonly used in almost all rural part of the country as it is easiest to build with no cost. However, the combustion of biomass fuel in such a stove generates highest level of indoor pollutants as it does not provide any flue or ventilation. Improved cook stoves on the other hand with a chimney attached is considered to be a more efficient stove than the traditional stove. In recent years, various kinds of improved cookstove have been introduced and disseminated in rural households of the country. Of these,

the two pot improved mud stove is the most popular stove design, especially in the mid hilly region of the country. The stove has a simple design, can be manufactured easily using readily available materials with minimum cost. In this study, the exposure from the combustion of various biomass fuels in such stoves was compared with the traditional stove commonly used in the study site.

Photo © Dhital SK, KU

(a) (b)

Figure 3.4 Common type of cook stove (a) Traditional cook stove (TCS) and (b) Improved cook stove (ICS) being used in the study site.

3.3.2.4 Pollutants monitoring and equipment used

Among various pollutants resulting from the biomass combustion, personal exposure to $PM_{2.5}$ and CO were monitored in this study. The exposure monitoring was performed using an IAP meter 5000 series (Aprovecho Research Center, USA) (refer section 2.1.4.3 for more detail of the equipment). All the real-time personal exposure data were collected at 10 seconds interval over each cooking period from the moment the fire started until the fire was put out.

3.3.2.5 Monitoring strategy

The household for the monitoring was selected based on cooking regular meals using either ICS or TCS. A total of 10 households were selected out of which 5 households used ICS and another 5 households used TCS for their regular cooking. The exposure monitoring with 7 selected fuels was conducted one fuel at a time by randomly selecting one household out of 10 households. For example, to test the first fuel on improved cook stoves, we first randomly selected the household having ICS and asked them to use that particular fuel for that particular cooking session. Similarly, on another day, the monitoring was performed with a second fuel type in another randomly selected household with either ICS or TCS. During the monitoring, we did not interfere with cooking, and we asked villagers to cook whatever they decided to cook on that day. The only thing we requested them to use was the fuel we had selected for that particular cooking session. The monitoring was performed throughout the cooking period in each household. The monitoring was performed for both morning and evening cooking session as usually all households cooked food at least two times a day. A total of 62 sets of personal exposure monitoring data were collected, of which 31 each with TCS and ICS. All seven fuels were used at least for n=3 in each stove design. The fuel was air and sun-dried before use to maintain the moisture content. All fuels used in this study had moisture content in the range of 10-15%, which was measured each time before the test by using a moisture meter.

3.3.3 Monitoring method

Real-time pollutant concentrations were measured at 10 seconds intervals over each cooking period from the moment the fire started till the fire was extinguished. The IAP meter was turned on and left in a clean environment for at least 10 minutes for background adjustment before it was worn by the subject (cook). Throughout the cooking period, the wearer had the meter on her back with the flexible tube attached to the meter box. The tube was adjusted with its inlet positioned over the shoulder on top of the cook shoulder near to the breathing zone. The data stored in the SD card was downloaded to the computer after completion of each test and processed through software provided, which was written for Microsoft Excel.

3.4 **Statistical Analysis**

All data in this section were presented as mean +/- standard deviation (SD). All data were analysed using GraphPad Prism software (Version 7, GraphPad Software Inc.). All statistical significance tests were performed using the t-test for comparison of two variables and using ANOVA for comparison of three or more variables. A p-value of <0.05 was considered significant. Correlation analysis between different variables was done by using Spearman correlation. A description of statistical analyses performed can be found in figure legend of each figure.

3.5 **Results**

3.5.1 Total Emission Factors

3.5.1.1 Boiling Time

The overall boiling time for each fuel from each WBT was computed by averaging the time taken in the cold start and hot start [242]. The overall mean (SD) boiling time from three independent tests for each fuel is presented in Table 3.2. Among all fuels tested, fuelwoods took less time to boil 5 L of water compared to the time taken by *maize stalk* and *cow dung*. Among the fuelwoods, *Schima Wallichii* was the fastest to boil water; however, time taken by other fuelwoods remained relatively similar with the highest time taken by *Quercus Leucotricophora* (44.3 minutes). On the other hand, variation in boiling time was observed between two agricultural residues tested. *Corn cob* (36 minutes) took lesser time to boil compared to *maize stalk* (53 minutes). The time taken to boil 5 L of water using *cow dung* (51 minutes) was similar to that observed with *maize stalk*. Though there were some differences in total time taken by each fuel to boil water, none of the differences was significant (probably because of the relatively small number of observations).

3.5.1.2 Specific fuel consumption and thermal efficiency

The WBT protocol suggests to report both specific fuel consumption, and thermal efficiency for any test as only measure of thermal efficiency may sometimes mislead the indicator of performance. It might be possible for a stove that uses more fuel to produce steam can show higher thermal efficiency. Specific fuel consumption gives how much dry fuel used to boil a specific amount of water (1 L). According to the water boiling test protocol, both specific fuel consumption and thermal efficiency were computed by averaging the cold start and hot start values of high power phase.

Table 3.2 presents the mean (SD) of temperature corrected specific fuel consumption and thermal efficiency of each of the fuel used for the test. The data among the fuels showed that higher amount of *maize stalk* required to boil 1 L of water as compared to other fuels. It is obvious to observe lower thermal efficiency while combusting *maize stalks* as compared to other fuels. On the other hand, a higher thermal efficiency measured while combusting *cow dung* as compared to other fuels, however, the amount of *cow dung* used to produce a unit of output was higher than other fuels except *maize stalk*. *Corn cob* seemed to be a better fuel in terms of performance metrics as higher thermal efficiency obtained with the lower amount of fuel required for a unit output. The overall performance in terms of both specific fuel consumption and thermal efficiency seemed better for fuelwoods than for *maize stalk* and

cow dung. Among the fuelwood, *Shorea Robusta* showed lower thermal efficiency with a higher amount of fuel required, whereas *Alnus Nepalensis* seemed to be a better fuel having higher thermal efficiency and lower specific fuel consumption. Because of a relatively small number of observations, the differences observed were not significant.

Table 3.2 Performance results. The data shown are mean (SD) of n=3 independent test for each fuel except for maize stalk (n=1). Variations were checked using Kruskal-Wallis, followed by Dunn's multiple comparison test (Maize stalk was not included in the comparison test). CD: Cow dung, CC: Corn cob, MS: Maize Stalk, PR: Pinus Roxburghii, AN: Alnus Nepalensis, SW: Schima Wallichii, SR: Shorea Robusta, QL: Quercus Leucotricophora.

Performance Metrics	CD	CC	MS	PR	AN	SW	SR	QL
High power thermal efficiency (%)	34.7 (4.6)	34 (7)	19	26.7 (4)	32.7 (4)	24.7 (2.5)	20.7 (2.1)	30 (4.6)
Specific fuel consumption (g/L)	165.3 (38.3)	109.8 (30.6)	248.1	109 (11)	100.8 (19)	112.9 (19.3)	132.8 (23.4)	104.3 (21.5)
Time to boil 5L of water (min)	51 (15.6)	35.7 (7.6)	53	36.5 (5)	43.1 (12.7)	31.6 (8)	41 (6.9)	44.3 (7.4)
Fuel to boil 5L of water (g)	1450.4 (224)	1242.6 (135)	2036.6	1217.5 (103)	1150.4 (125)	1081.5 (106)	1327.2 (235)	1146.7 (76)

3.5.1.3 Emission Metrics

The emission metrics used to measure total emissions and emission factors of $PM_{2.5}$, CO and CO_2 were computed by averaging values from high power cold start and hot start of WBT [207, 241].

3.5.1.4 Total Emissions

From the results shown in Table 3.3 combustion of *maize stalk* emitted a higher amount of CO (140.7 g), $PM_{2.5}$ (16695 g) and CO_2 (2668 g) than any other fuels. The total amount of $PM_{2.5}$ and CO emitted from *cow dung* was 13127 g and 90.5 g respectively, which was higher than any other fuel tested except *maize stalk*. However, CO_2 emission from *cow dung* was slightly lower as compared to the emission from *Shorea Robusta* and *Schima Wallichii.* Although *corn cob* showed better thermal performance, its total CO emissions (77.7 g) were higher than emissions from any other fuelwoods tested, however, its total CO_2 emissions (1599 g) were lower as compared to the fuelwoods.

The total amount of $PM_{2.5}$ emitted from *corn cob* was 5101 g which is slightly lower than the emissions from *Pinus Roxburghii* and *Schima Wallichii*. Among the fuelwoods, the mean total $PM_{2.5}$ emissions were 4943 g (SD 768, range 3986 g – 6063 g) with the highest emissions from *Pinus Roxburghii* and lowest emissions from *Shorea Robusta*. However, the total CO emissions from *Shorea Robusta* showed the highest (62.8 g) among the fuelwoods, where the mean total CO emissions from all

fuelwoods were 55.18 g (SD 5.78). The amount of CO_2 emission showed a similar pattern with the highest emissions from *Shorea Robusta*.

The emission test results (Table 3.3) showed that $PM_{2.5}$ emission rate of 169.9 mg/min for *maize stalk* and the emission rate of 123.3 mg/min for *cow dung* were higher than the emission rate of any other fuel tested. The emissions rate of about 49 mg/min for *Shorea Robusta* and *Quercus Leucotricophora* were the lower emission rate among all fuelwoods whereas *Pinus Roxburghii* and *Schima Wallichii* showed the highest emission rate at about 83 mg/min. The emission rate for *corn cob* was about 67 mg/min. Because of a relatively small number of observations, the differences observed were not significant for all parameters measured.

Table 3.3 Emissions performance and emission factor results. The data shown are mean (SD) of n=3 independent test for each fuel except for maize stalk (n=1). Variations were checked using Kruskal-Wallis, followed by Dunn's multiple comparison test (Maize stalk was not included in the comparison test). CD: Cow dung, CC: Corn cob, MS: Maize Stalk, PR: Pinus Roxburghii, AN: Alnus Nepalensis, SW: Schima Wallichii, SR: Shorea Robusta, QL: Quercus Leucotricophora

Emissions Metrics	CD	CC	MS	PR	AN	SW	SR	QL
CO to boil 5L of water (g)	90.5 (27.1)	77.7 (14.8)	140.7	54.9 (8.4)	47.1 (6.3)	57.8 (15)	62.8 (7.9)	53.3 (12.9)
$PM_{2.5}$ to boil 5L of water (mg)	13127 (4973)	5010 (725.8)	16695.4	6063 (489.5)	4641.3 (1304.1)	5228.3 (1702.2)	3986.3 (844.8)	4800.8 (2258.9)
CO_2 to boil 5L of water (g)	1837.6 (700.6)	1599.6 (344.5)	2668.6	1606.6 (352.8)	1646.7 (408.1)	2049.4 (548.5)	2319.2 (34.2)	1721.4 (294.8)
Indoor CO emissions (g/min)	0.8 (0.1)	0.9 (0.2)	1.2	0.7 (0.1)	0.6 (0.1)	0.9 (0.3)	0.7 (0.1)	0.5 (0.1)
Indoor $PM_{2.5}$ emissions (mg/min)	123.3 (31.2)	66.9 (5.2)	169.9	83.5 (23.6)	58.9 (15.9)	83.1 (27.3)	48.4 (6.6)	49.5 (24.7)

3.5.1.5 Emission Factor

Emissions from the combustion of various biomass fuel normalized either by the amount of energy delivered to the pot or the amount of fuel used, referred as Emission Factor (EF), was also analysed for emission metrics. The mass EF quantifies the amount of emissions per unit of dry fuel consumed or burned (g pollutant/kg fuel), whereas energy EF provides the pollutant emissions per unit of fuel energy produced during combustion [244]. EFs for $PM_{2.5}$, CO and CO_2 from all fuel sources are presented in Table 3.4. EFs for all pollutants follow the similar pattern to total emissions with the highest EFs observed for *maize stalk* at 3007.3 mg/MJ_d, 21.6 g/MJ_d and 411.9 g/MJ_d for $PM_{2.5,}$ CO and CO_2 respectively. This was followed by EFs for *cow dung* where EFs for $PM_{2.5}$ (2191.3mg/MJ_d) and CO (13.4 g/MJ_d) were higher than EFs for those pollutants for other fuelwoods. The EFs for $PM_{2.5}$ among fuelwoods remained similar with the highest EFs for *Pinus Roxburghii* which was ~3 fold and ~2 fold less than EFs for *maize stalk* and *cow dung* respectively. The $PM_{2.5}$ EFs for corn cob (915.8 mg/MJ_d) was just above the EFs for *Alnus Nepalensis*, *Shorea Robusta* and *Quercus Leucotricophora* whereas EFs for CO (12 g/MJ_d) was higher than EFs for all other fuelwoods. The EFs for CO among fuelwoods remained similar with the range from 7.7 g/MJ_d to 10.9 g/MJ_d. The measured mass EFs for all pollutants for all fuels followed a similar pattern to energy EFs (Table 3.4). Because of relatively small number of observations, the differences observed were not significant

Table 3.4 Emissions factors. The data shown are mean (SD) of n=3 independent test for each fuel except for maize stalk (n=1). Variations were checked using Kruskal-Wallis, followed by Dunn's multiple comparison test (Maize stalk was not included in the comparison test). CD: Cow dung, CC: Corn cob, MS: Maize Stalk, PR: Pinus Roxburghii, AN: Alnus Nepalensis, SW: Schima Wallichii, SR: Shorea Robusta, QL: Quercus Leucotricophora.

Emission Factor	CD	CC	MS	PR	AN	SW	SR	QL
$PM_{2.5}$ emission factor (mg/MJ_d)	2191.3 (800)	915.8 (41.3)	3007.3	1155.6 (211)	794 (85.1)	1074.2 (363.1)	746 (147.8)	693.2 (170.6)
CO emission factor (g/MJ_d)	13.4 (3.9)	12 (3.5)	21.6	9.8 (0.3)	7.5 (0.8)	10.8 (2)	10.9 (1.6)	7.7 (1.0)
CO_2 emission factor (g/MJ_d)	318.3 (95.5)	262.4 (71.2)	411.9	275.3 (56.3)	324 (87.9)	368.3 (73.3)	403.2 (10.7)	296.6 (70.5)
$PM_{2.5}$ emission factor (g/kg)	8.9 (3.9)	4.4 (0.7)	8.4	5.3 (0.7)	4.1 (0.8)	4.6 (1.3)	3.3 (0.4)	3.6 (0.7)
CO Emission factor (g/kg)	52.9 (15.6)	56.3 (11.1)	59	45.3 (4.8)	38.7 (5.5)	47.5 (8.8)	47.8 (4.7)	40.3 (7.6)
CO_2 Emission Factor (g/kg)	1270.6 (475.4)	1265.8 (445.4)	1111.8	1307.1 (451.3)	1687.6 (584.3)	1600.5 (188.7)	1790.6 (189.3)	1542.8 (258.1)

3.5.2 Correlation Analysis

Having demonstrated the performance results and emission profile of different biomass fuel, I then investigated whether or not a relationship existed between these two metrics. The relationship among thermal efficiency, specific fuel consumption, time to boil and emission factor of $PM_{2.5}$, CO and CO_2 were established using data from an independent test for each fuel. A level of interaction between these variables was checked using the Spearman correlation coefficient with a p-value <0.05 considered as a significant correlation. A correlation map showing Spearman r value and the corresponding p-value between each variable tested is shown in Figure 3.5. Fuel consumption showed a significant positive correlation with time to boil, $PM_{2.5}$ emission factor and CO emission factor (r=0.64, p=0.001; r=0.82, p =3.1e-5; r=0.85, p=4.2e-7 respectively). There was a positive correlation with CO_2 also, but not significant. Thermal efficiency showed no correlation with $PM_{2.5}$ emission factor, very weak negative correlation with CO and CO_2 and a weak negative but not significant correlation with specific fuel consumption. Time to boil showed a moderate positive correlation with $PM_{2.5}$ emission factor (r=0.53, p=0.01), whereas the correlation with CO emission factor (r=0.4, p=0.06) and CO_2(r=0.46, p=0.02) was weak.

In terms of emissions, a significant positive correlation was observed between total emissions and emission factor for respective pollutants. For example, the total emission of $PM_{2.5}$ showed a significant positive

correlation with $PM_{2.5}$ emission factors (r=0.97, p<0.00001). Similarly, the total emission of CO and CO_2 showed a positive correlation with CO emission factor and CO_2 emission factor respectively (r=0.93, p<0.00001, and r=0.91, P<0.00001). The relationship between co-pollutants was also investigated. $PM_{2.5}$ emission factor showed a strong positive correlation with CO emission factor (r=0.80, p=5.9e-6). However, CO_2 emission factor showed a weak positive correlation with both $PM_{2.5}$ emission factor and CO emission factor. A linear relationship among specific fuel consumption and $PM_{2.5}$ and CO emission factor and a linear relationship between $PM_{2.5}$ emission factor and CO emission factor is also presented in a scatter plot (Figure 3.6).

	TE (%)	*SFC (g/L)*	*TB(min)*	*PM2.5*	*CO*	*CO2*	*EF (PM2.5)*	*EF (CO)*	*EF (CO2)*
TE (%)	1.000								
SFC (g/L)	-0.348	1.000							
TB(min)	0.119	0.640	1.000						
PM2.5	0.098	0.766	0.540	1.000					
CO	-0.050	0.817	0.444	0.848	1.000				
CO2	-0.265	0.441	0.362	0.383	0.469	1.000			
EFPM(2.5)	0.014	0.822	0.535	0.973	0.837	0.415	1.000		
EF (CO)	-0.196	0.854	0.406	0.750	0.936	0.499	0.806	1.000	
EF (CO2)	-0.260	0.391	0.469	0.225	0.266	0.915	0.284	0.355	1.000

Figure 3.5 Correlation of performance measures and emissions measures from WBT using various biomass fuels. Figure shows the correlation coefficient value, r, The correlation between the variables was checked using the Spearman correlation coefficient.

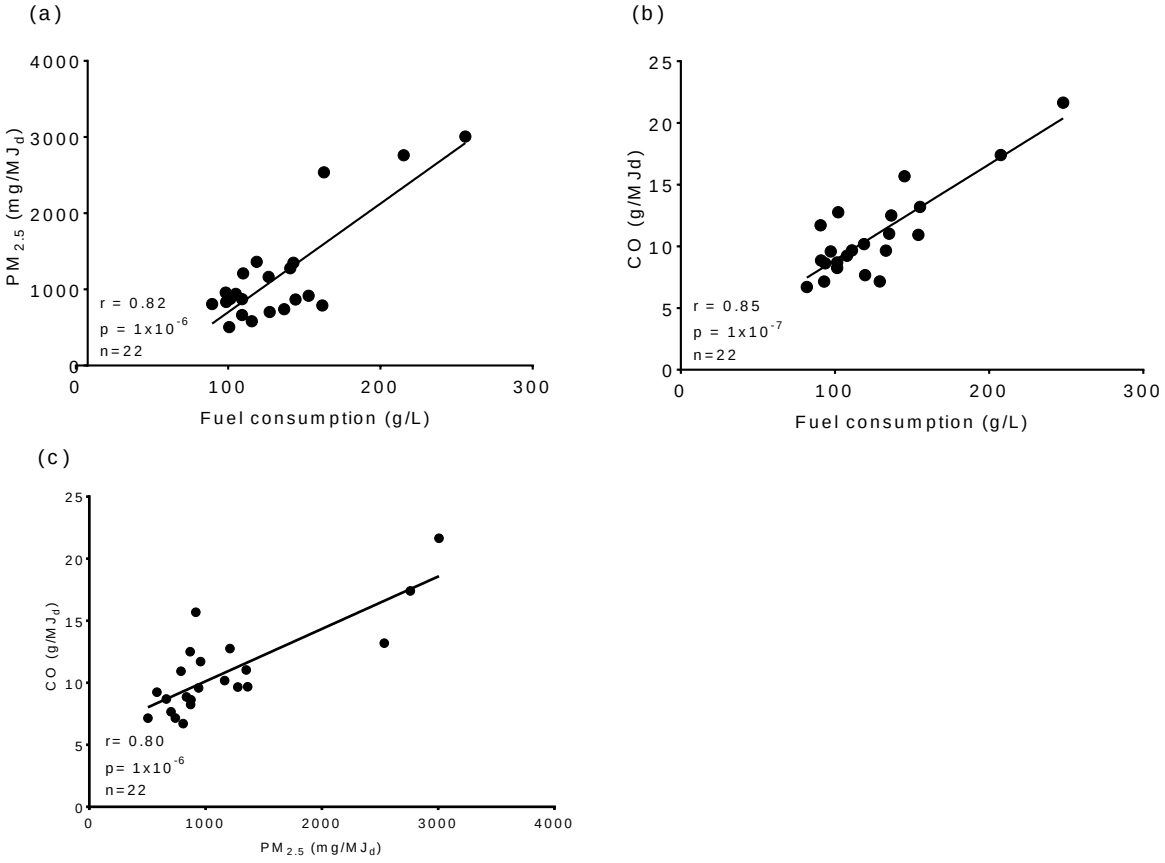
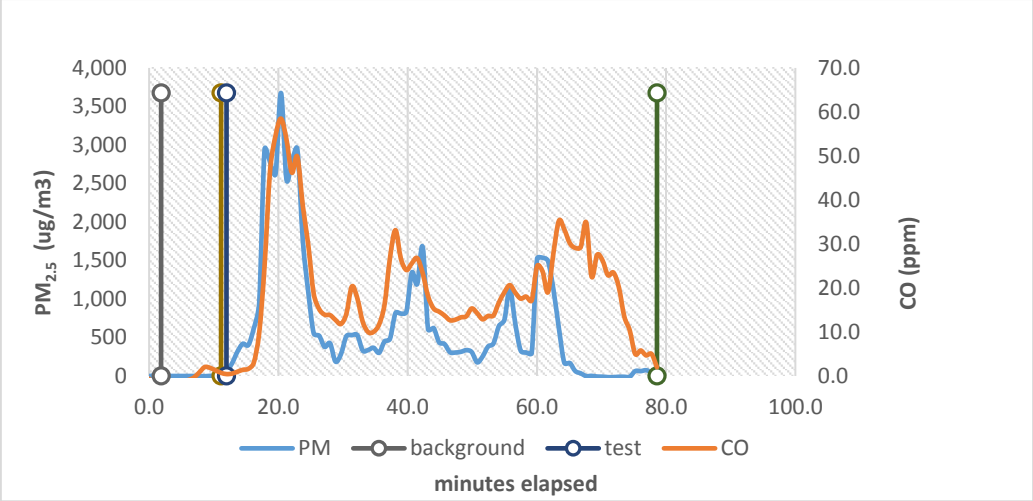


Figure 3.6 Scatter plot (a) $PM_{2.5}$ emission factor and specific fuel consumption, (b) CO emission factor and specific fuel consumption, and (c) $PM_{2.5}$ emission factor and CO emission factor. The data point represents an individual test data for each fuel repeated for n=3 independent tests for all fuels except n=1 for maize stalk.

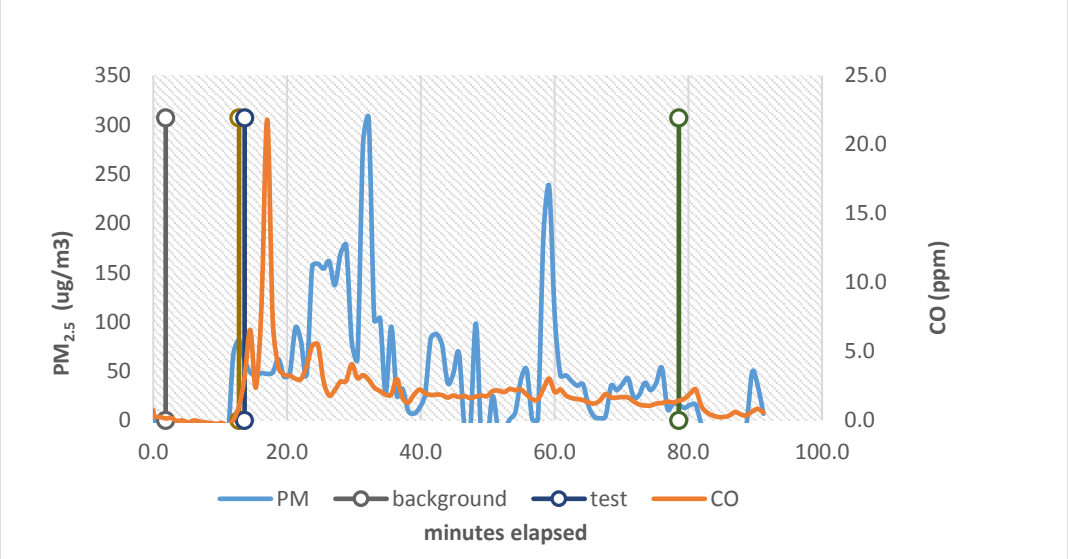
3.5.3 Personal Exposure monitoring

3.5.3.1 Pattern of pollutant exposure

A representative temporal variation in real-life personal exposure to $PM_{2.5}$ and CO concentrations when biomass fuel combusted for cooking in TCS and ICS is shown in Figure 3.7 (a) and (b) respectively. A similar pattern of exposure profiles was observed for all fuels in both stove designs. The concentrations for both $PM_{2.5}$ and CO fluctuated markedly throughout the cooking period. The temporal variation of $PM_{2.5}$ and CO showed the trend that both $PM_{2.5}$ and CO emissions reach peak levels on more than one instance throughout the monitoring period. The instances at which peak concentration observed were different for different tests; however, a peak concentration was notably observed at the start and the end of cooking. The fluctuation and peak concentration variation among different tests depended mainly on cooking activities followed by the cook at the time of a particular cooking session. Though the patterns remained the same for all tests, the range of pollutant concentrations, peak concentration and average concentration was different for the two stove designs. The overall peak $PM_{2.5}$ concentration observed with TCS reached up to 10,721 $\mu g/m^3$ amongst all biomass fuel, whereas it reached up to 8497 $\mu g/m^3$ when combusted in ICS. CO exhibits the same peak concentration trend for both stove design as $PM_{2.5}$. The peak CO levels reached up to 280 ppm with TCS, whereas it was up to 95.4 ppm with ICS.



(a)



(b)

Figure 3.7 Pattern of real-time personal exposure to $PM_{2.5}$ and CO concentration. The graph represents a time series emission exposure profile with (a) TCS and (b) ICS resulting from the combustion of biomass fuel. This is an example of single traces from a single cooking period; similar exposure profiles were obtained for all tests performed. The background marker indicates the start of the period for assessing background levels and Test indicates the total test period throughout the cooking activities.

3.5.3.2 Variation in personal exposure between TCS and ICS

From the results, I first investigated the overall $PM_{2.5}$ and CO personal exposure in households using TCS and ICS without being considered the types of fuel used. For this mean levels of $PM_{2.5}$ and CO exposure from each households using either TCS or ICS was computed. Then the overall mean from households only using TCS and households only using ICS was calculated, and the variation in overall mean for each stove design was compared. Table 3.5 shows minimum mean exposure and maximum mean exposure among households using TCS and ICS for $PM_{2.5}$ and CO. The overall mean exposure of all households using respective stove designs is shown in Figure 3.8. For both pollutants, exposure in households using TCS was higher than the exposure in households using ICS. In households using ICS, $PM_{2.5}$ concentration ranged from minimum 83 $\mu g/m^3$ and reached up to 1615 $\mu g/m^3$ whereas $PM_{2.5}$ concentration in households using TCS reached up to 4784 $\mu g/m^3$. Similarly, the maximum CO concentration in households using ICS was 19.9 ppm, whereas CO concentration reached up to 93.8 ppm in households using TCS.

Table 3.5 Improved cook stove and traditional cook stove personal exposure to (a) $PM_{2.5}$ and (b) CO.

(a)

Pollutant Type	$PM_{2.5}$ (μg/m^3)					
	Min	Max	Mean	Median	SD	95% CI
ICS (n=31)	83	1615	429.9	303	373.8	287.7-572.1
TCS (n=31)	118	4784	868.3	381	1019	480.7-1256
Percentage reduction	65%					

(b)

Pollutant Type	CO (ppm)					
	Min	Max	Mean	Median	SD	95% CI
ICS (n=31)	1.9	19.9	7.55	7	4.54	5.8-9.2
TCS (n=31)	1.3	93.8	14.95	9.1	18.05	8.08-21.8
Percentage reduction	50%					

The overall mean (SD) $PM_{2.5}$ personal exposure in households using TCS was 868.3 μg/m^3 (1019 μg/m^3) while the mean exposure was significantly reduced to 429.9 μg/m^3 (p=0.03) in households using ICS. Similarly, there was a significant reduction in mean CO exposure from 14.95 ppm to 7.5 ppm (50% reduction, p=0.03) in households using ICS than in households using TCS (Figure 3.8). Though comparatively lower

exposures were observed in households using ICS, $PM_{2.5}$ personal exposures were still about 17 fold higher than the safe exposure levels recommended by WHO. Hence the overall results suggest that people using biomass fuel are exposed to high concentrations of indoor air pollutants despite of their ownership of ICS.

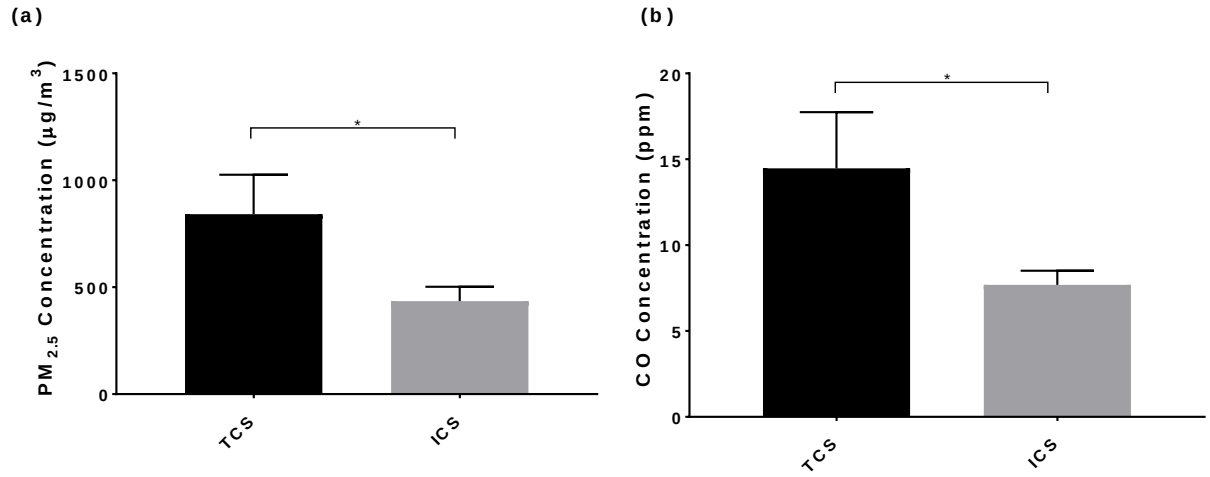


Figure 3.8 Overall mean personal exposure variations between TCS and ICS. The data shown are mean (SD) exposure to (a) $PM_{2.5}$ and (b) CO using different biomass fuel in two stove designs (n=31 for each stove). Variations were checked using the Mann-Whitney test. *=$p<0.05$. TCS: Traditional Cook stove, ICS: Improved Cook stove.

3.5.3.3 Exposure based on fuel type

The overall variations in personal exposures to $PM_{2.5}$ and CO using different fuels were investigated. For this mean personal exposure from the combustion of fuel in both TCS and ICS was computed for each of the seven fuels used. The overall mean of all tests for each fuel was calculated and compared for the variations (Figure 3.9). Without considering the variation in exposures using different cook stove designs, a distinct variation in personal exposures to both pollutants was observed between fuelwood, agricultural residue and *cow dung*. For example, *maize stalk* showed higher $PM_{2.5}$ (1705 $\mu g/m^3$) and CO (28.78 ppm) exposures among all fuels tested. $PM_{2.5}$ exposure resulted from the combustion of fuelwood were relatively less compared to the exposure from *cow dung*. However, $PM_{2.5}$ exposure from *Schima Wallichii* was slightly higher (876 $\mu g/m^3$) than the exposure from *cow dung* (836$\mu g/m^3$). The lowest $PM_{2.5}$ exposure among all fuels tested was from the combustion of fuelwood *Prunus Cerasoides* (271 $\mu g/m^3$). The exposure concentration of $PM_{2.5}$ using other fuels is 375.2 $\mu g/m^3$, 496.5 $\mu g/m^3$ and 552.3 $\mu g/m^3$ respectively for *Alnus Nepalensis*, *corn cob* and *Pinus Roxburghii*. Variations in exposures to both $PM_{2.5}$ and CO among different fuels were not statically significant.

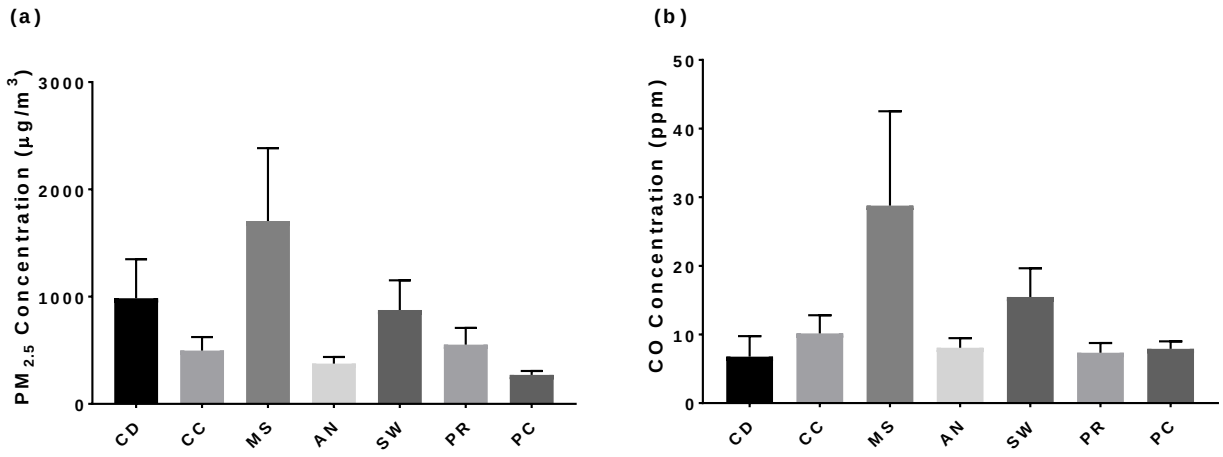


Figure 3.9 Real-time personal exposure variation of (a) $PM_{2.5}$, and (b) CO while using different biomass fuel types for cooking. The data shown are the mean ± SD concentration of each fuel combusted in either ICS or TCS. Variations in exposures among different fuels were checked using the Kruskal-Wallis test, followed by Dunn's multiple comparison test.CD: Cow dung, CC: Corn cob, MS: Maize Stalk, AN: Alnus Nepalensis, SW: Schima Wallichii, PR: Pinus Roxburghii, PC: Prunus Cerasoides

CO exposures among fuelwoods showed a similar pattern with the highest exposure from *Schima Wallichii* (15.4 ppm) which was ~2 fold higher than the exposure from other fuelwoods. *Cow dung*, on the other hand, showed the minimum CO exposure among all fuels despite it showed higher $PM_{2.5}$ exposures. Among the fuelwood, the lowest CO exposure was for *Pinus Roxburghii* 7.3 ppm, whereas the CO concentration using *Prunus Cerasoides*, *Alnus Nepalensis and corn cob* were 7.9 ppm, 8 ppm and 10.15 ppm respectively.

3.5.3.4 Exposure comparison of each fuel between ICS and TCS

Having demonstrated the variation in exposures using different fuel and different stoves, I then investigated exposure variation among each fuel while combusted in either TCS or ICS. The result showed that for each fuel combusted in ICS, the exposure to both $PM_{2.5}$ and CO was comparatively lower than the fuel combusted in TCS. The percentage reduction in exposure concentration when compared for each fuel with ICS and TCS followed the similar pattern of overall percentage reduction between two stoves. The reduction in exposure concentration between TCS and ICS for $PM_{2.5}$ was > 50% for all fuels with the highest reduction of 87%. Similarly, the reduction in exposure concentration between TCS and ICS for CO was > 28% for all fuels with the highest reduction of 85%. The result indicates that ICS users irrespective of variation in fuel types are exposed to comparatively lower indoor air pollutants than the TCS users. However, the concentration levels are still higher than the WHO permissible values of 25 $\mu g/m^3$ and 7 ppm for $PM_{2.5}$ and CO respectively.

Having demonstrated that using ICS results in the reduction of exposure irrespective of fuel used, I then analysed the exposure variation produced by each fuel when combusted in a single type of stove (Figure 3.10). The results showed as expected that the exposure variation observed using different fuels was influenced by the type of cookstove used. The average concentration of $PM_{2.5}$ for each fuel while using TCS was ranged from

340 μg/m^3 to 2383 μg/m^3 whereas it ranged from 150 μg/m^3 to 350 μg/m^3 while using ICS. Similarly, the average concentration of CO for each fuel was ranged from 7.9 ppm to 39.9 ppm and 4.5 ppm to 8.5 ppm while using TCS and ICS respectively. The results showed that no variation in $PM_{2.5}$ and CO exposure among different fuels when fuels were combusted in ICS. However, a difference in both $PM_{2.5}$ and CO exposure among fuelwoods while combusted in TCS was observed (differences are not significant). These results suggest that indoor air pollutant exposures in households using various biomass fuels depends on types of stove used to combust the fuels.

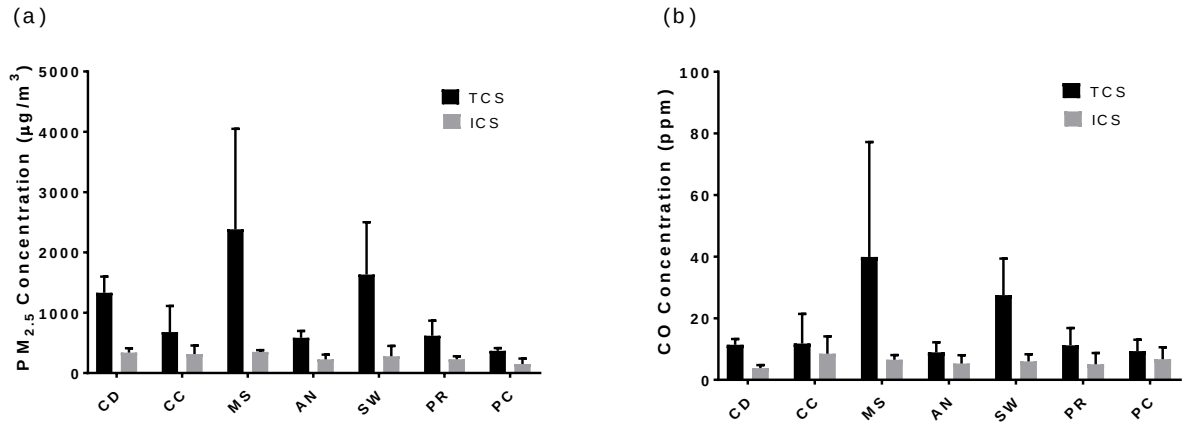


Figure 3.10 Comparison of personal exposure to (a) $PM_{2.5}$ and (b) CO using two different cook stoves with different biomass fuel. Variations in exposures among different fuels were checked using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. CD: Cow dung, CC: Corn cob, MS: Maize Stalk, AN: Alnus Nepalensis, SW: Schima Wallichii, PR: Pinus Roxburghii, PC: Prunus Cerasoides

3.5.3.5 Correlation between exposure to $PM_{2.5}$ and CO

Having established the pattern of personal exposure to indoor pollutants using biomass fuel for cooking in different stove design, I then investigated whether or not a relationship existed between $PM_{2.5}$ and CO exposures. $PM_{2.5}$ and CO exposures in households were monitored simultaneously throughout the cooking period for each cooking session. The correlation analysis was performed between two variables, where mean exposure data from all test (n=62) was used. Mean $PM_{2.5}$ exposures were strongly correlated with mean CO exposures (r=0.9, $p=1e^{-7}$). This correlation further confirms that the emission factor of $PM_{2.5}$ and CO followed a similar pattern.

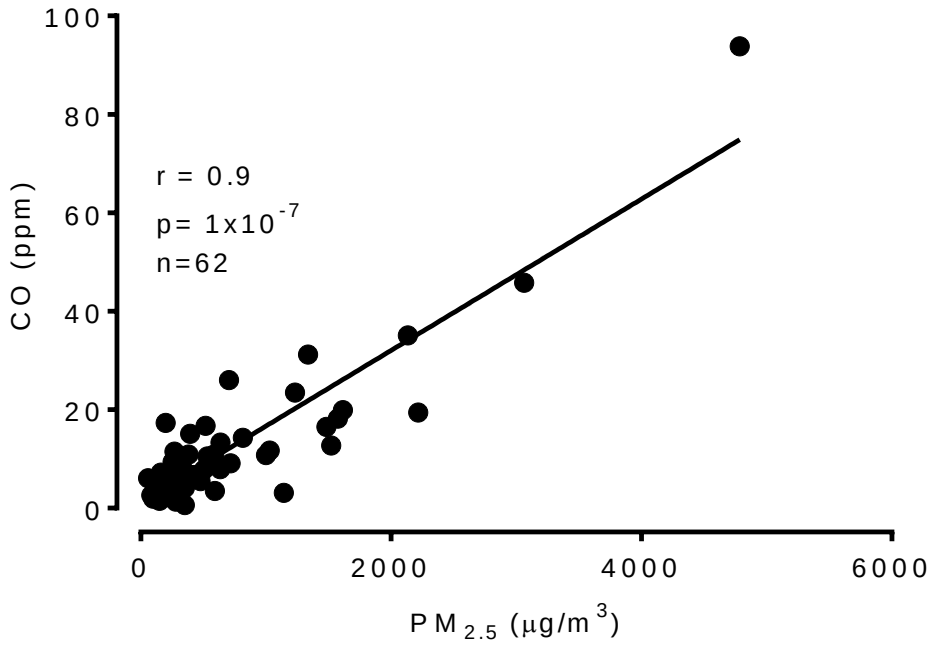


Figure 3.11 Correlation between mean $PM_{2.5}$ and CO exposures in households using different biomass fuels for cooking.

3.6 **Discussion**

The aims of this chapter were to i) determine the emission factors of indoor air pollutants including $PM_{2.5,}$ CO, and CO_2 resulting from the combustion of various biomass fuels; (ii) monitor real time personal exposure to $PM_{2.5}$ and CO resulting from combustion of biomass fuels for cooking in rural households; (iii) identify potential reduction in personal exposures in those households using efficient biomass combustion stoves; (iv) to establish any relationships among those pollutants and other measured variables.

I found that emission factors for $PM_{2.5}$ and CO are highest in agricultural residue burning followed by *cow dung* burning and fuelwood biomass burning. Studies conducted by others comparing emission factors among various biomass fuels have shown similar results [245-247]. Three categories of biomass fuel, including agricultural residues, fuelwood and dung cake, commonly used in the North of India, were compared for emissions in a study conducted by Saud et al.[248]. The results from this study showed that fuelwood had the lowest PM emission compared to the emissions from agricultural residues and dung cake. Unlike our study, this study showed that dung cake had the highest PM emission (15.68 ± 0.70 g/kg) than the emissions from agricultural residue (5.24 ± 0.6 g/kg), which might be because of combusting device or differences in types of agricultural residue used. It has been observed that emissions from combustion of biomass fuel depend on many factors including the type of

combustion devices, types of fuelwood fuel moisture content and specific properties of the fuel [249, 250]. Sen et al. [246] conducted a similar study in Western India which also showed that fuelwood had the lowest PM emissions than Dung cake and agricultural residue. However, this study only considered emissions for particulate matter and not included carbon monoxide emission. Another study conducted by Brassard et al.[247] had measured both PM and CO emissions from the combustion of agricultural residue and fuelwood. The study showed that agricultural residue had the highest PM and CO emissions followed by fuelwood, which is in line with the result observed in our study.

I also found differences in performance measures among these biomass fuels. *Maize stalk* has the lowest thermal efficiency (19%) but has higher specific fuel consumption (248 g/L) and total time to boil (53 min). This is an obvious observation as only a small fraction of heat can reach to the pot and water due to low thermal efficiency of fuel which results in a higher amount of fuel and time required to complete the task. However, I found little different performance measures for *cow dung*. *Cow dung* has the highest thermal efficiency (35%) among the fuels; however, specific fuel consumption (165 g/L) and time to boil (51 min) were also higher. This shows that *cow dung* takes longer time to boil the water and in the process, a large amount of water gets evaporated, which in turn reflect on higher thermal efficiency. Also, higher amount of fuel is required while bringing water to a boil in a slow-boiling process [207].

On the other hand, *corn cob* showed a better performance among all fuels with higher thermal efficiency (34%) with lower specific fuel consumption (110 g/L) and lower time to boil (36 min). Fuelwoods also showed a better performance in terms of thermal efficiency, specific fuel consumption and time to boil. The average thermal efficiency among fuelwood was 27% with 113 g/L specific fuel consumption and 39 min of average time to boil.

Whilst the data presented here show that the emission from the combustion of biomass fuel differs among fuel types, fuelwood being better than agricultural residue and cow dung in terms of emission and performance, there are some potential limitations of this work. First, the relatively small number of repeated tests within fuel types limits the ability to recommend one fuel type over another due to data variation within fuel types. For example, the variation in $PM_{2.5}$ emission factor for *cow dung* between two tests was 1483 mg/MJd. Similarly, the differences in two tests for *Schima Wallichi* were 687 mg/MJd. Hence, though the overall results presented here are in line with the results from previous studies, due to the relatively small number of replicates and the variation seen within fuel types between tests, more studies need to be carried out.

Having established the emission profile and performance measures of various biomass fuels, I then investigated whether or not a relationship existed between these factors. The emission factors of CO and CO_2 were negatively correlated with fuel thermal efficiency. This result is in line with

the results published in a study conducted by Jetter et al. [241]. However, a weak correlation in our study suggests the requirement of more study to establish an actual relationship between these variables. Also, thermal efficiency did not show any correlation with $PM_{2.5}$ emission factors. We found that emission factors of $PM_{2.5}$ and CO are strongly correlated with the specific fuel consumption. This indicates that emissions from the combustion of biomass fuels are directly associated with the amount of fuel burnt to complete the task. The relationships between specific fuel consumption and pollutant emissions have been established in several previous studies [241, 242]. A strong correlation between specific fuel consumption and time to boil was observed, which indicates that fuels with higher consumption rate require a more considerable amount of time to complete the task. Also, the time factor showed some influence on emissions factor as a moderate positive correlation between the amount of time and emission factors of $PM_{2.5}$ and CO was observed.

Having demonstrated the emission factors of various biomass fuels in a control laboratory setting, real time personal exposure to $PM_{2.5}$ and CO resulting from the combustion of these biomass fuels for cooking was monitored. This was performed in households of a rural community of Nepal in a real cooking environment for a total cooking period. The variation in exposures using these biomass fuels while combusted in ICS and TCS was compared. Convincing amount of evidence from the previous studies performed in different parts of the world suggests that

people using biomass fuel are exposed to higher concentration of indoor pollutants [20, 119, 251]. The results from this study also support this evidence as $PM_{2.5}$ personal exposure in households using biomass fuel were found up to 35 fold higher than the safe levels for $PM_{2.5}$ recommended by WHO. I also found that the overall $PM_{2.5}$ and CO exposures were reduced by 65% and 50% respectively in households using ICS to combust biomass than in households using TCS. Both laboratory and field-based studies performed elsewhere have demonstrated the effectiveness of an improved type of cook stove in emissions reduction [102, 116, 252]. A study conducted in rural western Kenya by Yip et al.[102] had found 38.8% and 27.1% reductions in indoor $PM_{2.5}$ and CO concentration in households using ICS. The study had also shown a significant reduction in personal exposure to CO using ICS, however, personal exposure to $PM_{2.5}$ was not conducted. Similarly, a study conducted by Phillips et al.[115] in Sri Lanka had found a reduction in personal exposure to $PM_{2.5}$ from 216 $\mu g/m^3$ for women using TCS to 47 $\mu g/m^3$ for women using ICS. However, none of the study conducted had measured personal exposure using different types of fuelwood in a real kitchen environment. In this study, I monitored the variations in personal exposure to $PM_{2.5}$ and CO using various biomass fuel combusted in two stove designs.

The observed overall percentage reduction between two stove designs was also true when the exposure from each fuel combusted in TCS and

ICS was compared. For both $PM_{2.5}$ and CO, exposure levels were reduced for each fuel while combusted in ICS than combusted in TCS. However, the degree of reduction varied among the fuels. For example, the percentage reduction of *Maize stalk* is 85% and 83% for $PM_{2.5}$ and CO, whereas the reduction for *Pinus Roxburghii* was 63% and 55% respectively.

The overall personal exposure to both $PM_{2.5}$ and CO among various fuels followed the similar pattern observed for emission factors in a laboratory test. *Maize stalk* had the highest personal exposure, followed by *cow dung* and fuelwood. Among fuelwood, *Schima Wallichii* had the highest $PM_{2.5,}$ and CO exposure than any other fuelwood tested.

In conclusion, this chapter has identified that the emission factor and personal exposure to indoor air pollutants resulting from the combustion of biomass fuel depend on the type of fuel and type of combustion device. The knowledge about the emission factor and personal exposure from various biomass fuels could help minimising the emission by selecting less polluting fuel for cooking. It is also an essential factor for the development of strategies for pollution control and helps in a study assessing effects of particle exposure on human health. Also, we have shown that the combustion of biomass fuel in high efficient stove reduced the exposure significantly. However, these improved cook stoves in real cooking practise are not able to reduce the level up to the WHO recommended level. In this study, we have measured the personal

exposure to indoor pollutant in a region using a single type of fuel at a time. However, in a real cooking environment, not a single type of biomass is used for cooking and exposure may also vary between different households in different regions. Hence more exposure study needs to be carried out in a real cooking environment with the combination of available fuel in different regions to have a comprehensive personal exposure data.

Chapter 4: Pattern of personal exposure to household air pollution in four different resource poor settings in Nepal

4 Pattern of personal exposure to household air pollution (HAP) in four different resource poor settings in Nepal

In the previous chapter, I demonstrated that the emission factors and personal exposures from the combustion of biomass fuel are fuel and device dependent. I monitored emission factors from the combustion of specific biomass fuels in a laboratory setting, and variation in personal exposures using specific biomass fuels for cooking in a real-life rural kitchen environment. However, in reality, cooking in rural households is usually performed using a combination of fuelwoods, agricultural residue, animal dung and other fuel sources like bamboo, wood waste from old furniture, but usually not just a single specific biomass fuel. Type and combination of fuel used vary from household to household in each region of the country and depends on the availability and easy accessibility of the fuel. Almost all rural areas in all three geographical regions of the country use some form of biomass fuel for cooking and heating. I attempted to monitor personal exposure to HAP in rural households using biomass fuel for cooking. The monitoring was performed in households of four rural villages of Nepal representing all three geographical regions to see variability in patterns of exposures among households in the various region in a real cooking environment. The variations in exposure patterns using more efficient biomass combustion stoves and using other fuels than biomass were also demonstrated. In addition to the exposure monitoring exhaled breath CO concentration in individuals actively involved in cooking was also monitored. The short term effect of HAP

exposure in individuals pulse and blood oxygen saturation level was monitored in Salambu and Pokhara. Hence, this chapter describes the exposure pattern of HAP in households using biomass fuel and its immediate effect on health in four resource-poor settings in Nepal.

4.1 **Introduction**

Solid biomass fuel combustion for cooking and heating has been a primary source of HAP in homes in low and middle-income countries [253, 254]. Significant proportions of the population around the world are exposed to high concentration of HAP from solid biomass fuel burning indoors for cooking. The State of global air report mentioned that a total of 2.45 billion people were exposed to high concentration of HAP in the year 2016 [26]. Around the world, traditional biomass fuels are mainly combusted indoors in a traditional stove or open fire within a confined space without proper ventilation [255]. The lower combustion efficiency of these device results in incomplete combustion of the fuel, which in turn produces high concentration of particulate matter, carbon monoxide and other health damaging pollutants [256]. The higher concentration of health hazard pollutants remains indoors for an extended period after cooking as there is usually no mechanism to vent the smoke out of the kitchen. The emissions of fine particulate matter in poorly ventilated dwellings using inefficient stoves can reach up to 100 fold higher than the air quality standard set by the World Health Organization [257]. A recent review article reported that the levels of $PM_{2.5}$ in homes using biomass

fuel in Nepal, Pakistan, Bangladesh and India were up to 139, 180, 77 and 40 fold higher than WHO thresholds limit for indoor $PM_{2.5}$ (25 $\mu g/m^3$), respectively [251].

A convincing amount of individual studies around the world indicate high levels of exposure to HAP in homes using biomass fuel for cooking and suggest its association with health risks. For example, an overall mean 48-hour indoor $PM_{2.5}$ and CO concentration of 586 $\mu g/m^3$ and 6.5 ppm respectively were reported in households using biomass fuel in a study conducted in western Kenya [102]. Similarly, a study conducted in homes using biomass fuel in Brazil reported a mean indoor $PM_{2.5}$ concentration of 230 $\mu g/m^3$ [258]. Similar studies from other countries have also reported high concentrations of $PM_{2.5}$ in kitchens using biomass fuel for cooking. For example, average $PM_{2.5}$ concentration reported in different studies conducted in Sri Lanka, Bangladesh, Pakistan and India were 430, 650, 1790 and 1920 $\mu g/m^3$respectively [101, 114, 115, 259].

Similar studies conducted in various ecological regions of Nepal have also reported high concentration of indoor particulate matter in households using biomass fuel. The studies conducted in Sarlahi and Janakpur districts have found a daily average indoor $PM_{2.5}$ concentration of 1376 $\mu g/m^3$ [19] and a 48-hour average $PM_{2.5}$ concentration of 417.6 $\mu g/m^3$ [20] respectively. Similarly, a mean indoor concentration of 2070 $\mu g/m^3$ of $PM_{2.5}$ and 21.5 ppm of CO has been reported in a study conducted in three low and mid-hill districts in Nepal [116]. A study conducted in

Bhaktapur showed annual mean household $PM_{2.5}$ concentrations of 565 $\mu g/m^3$ in homes using biomass fuel as compared to an annual average of 130 $\mu g/m^3$ in homes using LPG [119]. All these previous studies monitored indoor concentration of air pollutants over a fixed period and mostly centred in low and mid-hill regions of the country. None of the studies performed in Nepal to date has monitored real-life exposure to HAP in high altitude regions like Kyanzing. Parajuli et al. monitored indoor air quality in rural mountainous households of Nepal and reported mean indoor CO and $PM_{2.5}$ concentration of 36.03 ppm and 1336 $\mu g/m^3$ in homes using TCS respectively [117]. However, the actual monitoring site in Palpa in the study lies in the mid-hill region of the country as the altitude of the monitoring site mentioned in the study was 1838 m. It is clear from the previous studies that people using biomass fuel are likely to be exposed to high concentrations of HAP. However, the absence of real-life personal exposure data in these settings advocates the need for more exposure monitoring studies.

A range of strategies and interventions throughout the world have been introduced to mitigate the problem of HAP. The primary aim of these interventions was to reduce HAP in home using biomass fuel to reduce the incidence of disease attributed to HAP exposure. The best possible way suggested to reduce HAP and improve health would be the replacement of biomass fuel with cleaner fuels like liquefied petroleum gas (LPG), electricity or solar energy [11, 185, 260, 261]. However, due to financial

constraints and poor socio-economic conditions in rural households, the transition option is not always feasible in rural areas of LMICs [185, 260].

Hence billions of people from LMICs will continue using solid biomass fuel for cooking for many more years [30]. Therefore, developments have been made to improve cook stove design to increase combustion efficiency and reduce emissions. Use of more efficient stoves over the traditional stove is an effective solution to reduce HAP. The *plancha mejorada* cook stove in Guatemala [262], the *pastari stove* in Mexico [263], the *Justa* stove in Honduras [264], *three pot metallic cook stove* and *2 pot mud cook stove* in Nepal [265] are some of the developments made around the world in ICS to try and reduce emissions. In Nepal, the first ICS program was started back in 1950, where Indian models, the *Hyderabad* and *Magan chulas* were introduced [266]. Since then, government companies, NGOs, institutions and other private sectors have been continuously working on promoting ICS in Nepal.

By 2014, around 935,824 mud improved cook stove had been disseminated throughout all ecological regions of the country along with 16,314 metallic improved cook stoves in the high hill region of the country [267]. Significant reductions in PM concentrations have been recorded with ICS in comparison with TCS in several studies conducted worldwide. In Mexico, for example, use of the *pastari* stove had reduced an average PM concentration from 693 $\mu g/m^3$ to 246 $\mu g/m^3$ [263]. Also, an average reduction of 50% on a daily average personal $PM_{2.5}$ exposure

was noted after the installation of the improved stove [263]. Similarly, a reduction in mean $PM_{3.5}$ of 83% and mean $PM_{2.5}$ of 73% has been measured with ICS in comparison with a traditional stove in Guatemala [262] and Honduras [264] respectively. Few studies, however, in Nepal have been performed to date on monitoring the performance of ICSs in terms of reduction in HAP. A study by Ojo et al. conducted in a home-like setting in Nepal has reported 60% and 50% reduction in mean $PM_{2.5}$ with AMBS-I stove and Environfit G-series stove as compared to traditional stove respectively [234].

However, most of the work performed to date has measured mean exposures at a fixed point in a room, rather than measuring an individual's real exposure using personal monitoring system. Because people will be close to the cooking area but also move around during the cooking proves measurements at a single fixed point may well not represent true exposures accurately. Personal exposure assessment is likely therefore, to be an important basis for the proper assessment of the health effects of air pollution on individuals [268]. The studies mentioned above performed in Nepal to monitor indoor pollutant concentration from biomass combustion did not measure patterns of personal exposure using personal monitoring systems.

In this study, I attempted to measure the patterns of personal exposure to HAP resulting from biomass combustion for cooking in households of four resource-poor settings in Nepal. I also examined the effectiveness of

enhanced cook stoves and using cleaner fuel like LPG in reducing HAP in those settings. CO concentration in exhaled breath, changes in pulse rate and levels of SpO2 during short term exposure to HAP were also monitored to observe any physiological changes due to the exposure.

4.2 **Method**

4.2.1 Study Site

The study was conducted in four different villages of Nepal, representing all three geographical regions of the country (Figure 2.1). Nepal is a country in South Asia situated between China in the north and India in the south, east and west. The country is located at latitudes between 26 and 30 degrees north and longitudes between 80 and 88 degrees east. Nepal covers just 0.1% of the earth's surface, with a total area of 147,181 sq.km. The country is rich in biodiversity and culture due to its unique geographical position and altitudinal variation. Due to these different elevation ranges from less than 100 meters above sea level to the highest point on the earth, the country is topographically divided into three regions. The Himalayan region (also called 'Parbat' in Nepali) to the north of the country is situated mostly at 4,000 meters or more above sea level. The region represents 16% of total land area and is the least populated region of Nepal due to the Arctic-Alpine climatic conditions. Only 8% of the total population of the country lives in the region with most permanent settlements at upto 4000 m altitudes. The middle hill region (also called 'Pahar' in Nepali) is situated south of the mountain

region and covers the highest part (65%) of the total land area. The area situated between 1000 and 4000 meters in altitude falls under this region and provides habitat for around 45% of the population of the country. Due to physiographic and climatic difficulties in the higher altitudes (above 2500 meters) in the region, the population distribution is not the same throughout the region. The lower hills and valley within the regions are densely populated while the higher elevation areas are sparsely populated. The Terai region is a lowland tropical and subtropical belt of flat land and covers the southern part of the country which makes up the border with India. The region covers 17% of the total land area with an altitude variation from 70 m to 1000 m above sea level. The region is well known for excellent farming land and provides a home for around 48% of the total population.

Table 4.1 Topographical regions of Nepal

Region	Altitude range (masl)	Total area covered (%)	% of total population
Himalayan	4000 or above	16	8
Hill	1000 to 4000	65	45
Terai	60-1000	17	47

As already mentioned, the study sites were selected according to altitude variation and to cover all three ecological regions of the country. For this, I selected four rural to semi-rural settings located at different elevations. The four sites were Simreni village in Chitwan (~ 200 masl), Bhujel Gaun in Pokhara (~1200 masl), Salambu in MajhiFeda (~1800 masl), and

Kyanzing Kharka in Langtang (~ 4000 masl). The detailed description of each site has already presented in the section 2.1.3. The study site summary detail, along with the number of households monitored with different types of fuel and stove designs, is presented in Table 2.1.

The study in Langtang was conducted in a small village called Kyanzing Kharka, which is the highest permanent settlement located at an altitude of about 4000 m in the Langtang valley. Personal exposure to indoor air pollutant was monitored in a total of 14 households. Out of 14 households, 5 households were sampled using the two pot traditional type of cook stove and 9 households with an improved cook stove.

Salambu is a small village in the MajhiFeda VDC of Kavrepalanchowk district located in the hill region of the country. The study site in Salambu had a total of 88 households at the time of the study, out of which 25 households were sampled for the exposure monitoring. ICSs were distributed among all households in this study site, but over time, most of the households had tended to come back to the traditional mode of cooking on a three stone stove. A few households still cooked on ICS but also had a TCS in use. The exposure monitoring was performed in 17 households using TCS and 8 households using ICS.

The exposure monitoring in Pokhara was performed in a small semi-rural village called Bhujel Gaun, which is located at a distance of 12 km west of the main Pokhara city. Bhujel Gaun had a total of 40 households at the time of the study, out of which 27 households were selected for the

exposure monitoring. Neither of households had ICS installed at the time of our study. However, few households used LPG as a primary source of cooking. The monitoring was performed in households using biomass fuel in TCS (24 households) and households using LPG (3 households).

In Chitwan, the study was conducted in households of a small semi-rural village called Simreni. A total of 37 households were selected where biomass was used as a primary source of cooking. As in Bhujel Gaun, none of the households had ICS installed at the time of our study; however, LPG and biogas generated locally from livestock waste were the common alternative fuels in most of the households. The exposure monitoring was performed in households using biomass fuel (30 households) and in households using LPG (7 households).

4.2.2 Stove designs

Due to the social and geographical variation among the monitoring sites, cook stove design varied among households. The stove used to combust biomass fuel in each monitoring site is presented in the method section 2.1.3. Though there were some variations in cook stove design in each monitoring site, the stoves having chimney like in Kyanzing and Salambu are considered as an ICS, and stoves without a chimney in all monitoring sites are considered as TCS.

4.2.3 Exposure monitoring

Real-life exposure to HAP including $PM_{2.5}$ and CO were monitored in a total of 103 households from all four monitoring sites: details are presented in Table 2.1. The pollutant exposure in each household was measured for a total cooking period. In order to have a clear picture of cooking period and non-cooking period exposure in each household, exposure during non-cooking period was also measured. This was performed by monitoring start at 20 minutes before cooking commence and continued monitoring for 20 minutes after cooking ends. Mean exposure for the total cooking period was considered as cooking period exposure while mean exposure of before and after cooking was considered as non-cooking period exposure. Real-life personal exposure to $PM_{2.5}$ was measured using Aerosol Mass Monitor (Aerocet831, MetOne, OR). Real-life exposure to CO was measured with Indoor air Quality Meter (IAQ meter) (GrayWolf® sensing solutions). Details of these equipment have already been described in the section 2.1.4. All these instruments were factory calibrated prior to the field campaign. Pre-and post-field campaign comparison was also done with standalone instruments or with the calibration systems. Full details of the comparison study and correction factor determination for each instrument is described in the section 2.2.

Personal exposure to $PM_{2.5}$ was monitored throughout the cooking period by keeping the equipment with the person involved in cooking. For this,

equipment was kept in a jacket or a bag, and the cook was asked to carry on their back. The adjustable tube attached to the device was placed with the inlet of the tube adjusted near to the subject's breathing zone, as shown in Figure 4.1. IAQ meter for CO exposure was placed near to the stove to capture direct real-life exposure to CO. In this study, as IAQ meter was used to monitor real-life CO levels, carrying this equipment by cook proved difficulties for the cook to do cooking activities. Hence, the equipment was placed near to the stove and captured direct stove exposure. However, as a biomarker of CO exposure, I also monitored personal exhaled breath CO concentration during cooking.

Figure 4.1 Personal exposure monitoring using different devices in different sites. Exposure monitoring devices were kept in a jacket/bag, and the cook was asked to wear it on their back. The adjustable tube from the device was placed to keep near to the breathing zone of the subject.

4.2.4 Exhaled Breath CO monitoring

In addition to exposure monitoring, exhaled breath CO concentration of the individual involved in the cooking activity was also measured. The main aim of this measurement was to estimate changes in exhaled breath CO and % carboxyhaemoglobin (% COHb) levels due to short term exposure to HAP during cooking activities. Exhaled breathe CO and % COHb were measured using factory calibrated exhale CO monitors (Micro CO (CareFusion, UK)). The CO monitor was checked and calibrated as per the manufacturer instruction prior to each field campaign. For a detail description of the equipment and its calibration see the section 2.1.4.6. The measurement was taken by following a protocol provided by the manufacturer. In all three monitoring sites except Kyanzing, the exhaled breath CO measurements were performed at three different time points representing pre-cooking, during cooking and post-cooking phase. The first measurement was taken before cooking started, the second measurement was taken during actual cooking, and the third measurement was taken immediately after cooking. In Kyanzing, it proved difficult to measure exhaled breathe CO at three different time points because subjects were unable to do repeat measurements. Hence, exhaled breath CO level was measured during cooking phase only. For each phase, measurements were taken three times, and the mean of three readings was calculated.

These measurements were performed in a total of 29 individuals actively present throughout the cooking period in the kitchen of 16 households in Kyanzing. Out of these 29, 11 individuals were measured with TCS and the remaining individuals with ICS. In Salambu, Pokhara and Chitwan, the measurements were performed with a group of 25, 19 and 42 individuals actively involved in cooking.

4.2.5 Pulse and SpO_2 monitoring

Acute effect of short term exposure to HAP on individual's pulse and peripheral oxygen saturation (SpO2) were monitored in Salambu and Pokhara. A fingertip pulse oximeter (microlife® Oxy 300, AG) was used to monitor an individual's heart rate and SpO2 level. The measurements were taken at three different time points representing pre-cooking, during cooking and post-cooking phases in both monitoring sites. For each phase, measurements were taken three times, and the mean of three readings was calculated. The pulse and SpO2 data were collected from a total of 45 individuals actively involved in cooking at the time of our study from two monitoring sites.

4.2.6 Equipment calibration and data correction

Where appropriate, devices requiring calibration from the manufacturer were sent for the calibration before the start of field work. The other devices that can be calibrated manually were calibrated following the

manufacturer instructions. All four sets of aerocet were sent to Met One Instrument for calibration and inspection. All sets of IAQ were calibrated and tested according to the manufacturer's instruction.

The measured concentration of each pollutant from the respective devices was validated as described in the general methods section 2.2. For each instrument, pre-field collocation runs were conducted with reference instruments at the reference site in order to calculate the correction factor. The derived correction factor was used for the correction of field measured data. Although the same sets of devices were used in all field measurements, the correction factor for each equipment was derived before each field work. That means for the same equipment different correction factors were used to correct the monitoring data from different locations.

4.3 **Aim**

The main aim of the work described in this chapter was to further investigate the patterns of real-time personal exposure to HAP in resource poor setting in the different geographical regions in Nepal.

4.3.1 Specific Aims

- To measure real-life personal exposure to particulate matter ($PM_{2.5}$), and real-life exposure to carbon monoxide (CO) in households using biomass fuel in four different areas.
- To monitor the effect of enhanced stove design on real-life exposures to indoor air pollutants.
- To monitor the exposure variation resulting from using cleaner fuels like LPG in the same setting.
- To assess physiological effects of short term exposure to HAP by measuring changes in CO concentration in exhaled breath, pulse rate and SpO2 levels during cooking period exposures.

4.4 **Result**

4.4.1 Cooking practices, cooking time and stove characteristics

The normal cooking practices in households in each monitoring site were monitored in terms of the cooking sessions in the day, cooking frequency and average cooking period. Cooking pattern seen in each household of each monitoring site was different, and it was observed that this depended on the number of people in a family, the nature of the work they are involved in and the number of livestock present. However, in general, cooking practices in Salambu, Pokhara and Chitwan were found to be relatively similar in terms of cooking timing. In households, the stove was lit at least for two main cooking sessions daily, once in the morning and in the evening. The people in these sites used the stove for morning cooking from 7 to 10 and for evening cooking from 6 to 9. However, in some houses, people start using their stove from the early morning at around 5 to prepare food for livestock and to boil water and milk. In comparison to these three sites, the cooking practices observed in Kyanzing were completely different. In Kyanzing, stove use commenced in the early morning at around 5, and the stove remained on throughout the day for around 16-17 hours. The stove was used to prepare the food as well as for heating purposes. Hence, the stoves remained in use throughout the day. However, in each household, we observed that the stove was at least used for two main cooking sessions. Although the stove remained in use for the whole day, the cooking period

and exposure monitoring period in Kyanzing was fixed to 120 minutes to allow comparison with the other sites.

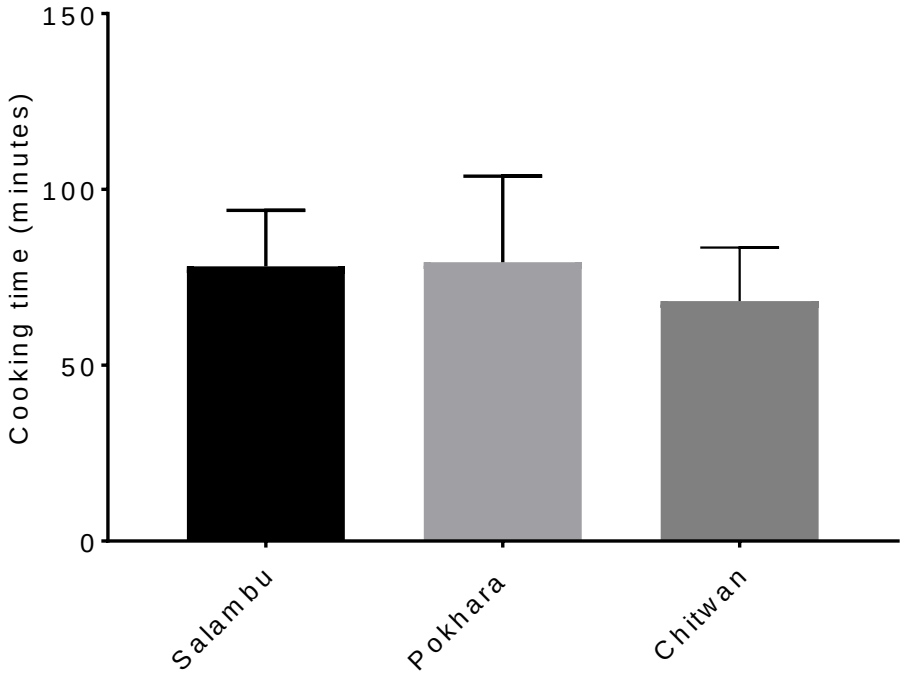


Figure 4.2 Cooking time in households. The data shown are mean (±SD) cooking time of all households in Salambu, Pokhara and Chitwan. The overall mean cooking time in Chitwan was less but not significant than cooking time in Salambu and Pokhara. Kruskal-Wallis test followed by Dunn's multiple comparison test showed non-significant among all variables.

Figure 4.2 presents the mean cooking time of three monitoring sites; Salambu, Pokhara, and Chitwan. Kyanzing cooking time is not included in the figure as it was fixed to 120 minutes as previously mentioned. The mean cooking time in Salambu, Pokhara and Chitwan were 78 minutes, 79 minutes and 68 minutes respectively.

Table 4.2 Cooking time, indoor temperature and relative humidity in all monitoring sites using different cook stoves. The data shown are mean (SD) cooking time of all households in each motoring site using respective stove designs. Each variable among different cook stoves was checked with Mann Whitney t-test. T: indoor temperature in degree Celsius and RH: Relative Humidity in percentage. TCS: Traditional cook stove; ICS: Improved cook stove, LPG: Liquefied petroleum gas

Kyanzing					
	TCS		ICS		p-value
	Mean	SD	Mean	SD	
Cooking time (min)	120		120		
T (˚C)	14.1	2.3	18.1	6.2	0.19
RH (%)	42.1	3.42	34.67	10.8	0.16

Salambu					
	TCS		ICS		p-value
	Mean	SD	Mean	SD	
Cooking time (min)	79	15.8	75	17.08	0.64
T (˚C)	20.65	4.68	17.8	2.3	0.13
RH (%)	42.91	9.92	48.6	7.72	0.23

Pokhara					
	TCS		LPG		p-value
	Mean	SD	Mean	SD	
Cooking time (min)	79.04	24.71	81.66	26.95	0.86
T (˚C)	16.6	3.35	13.8	2.8	0.18
RH (%)	40	9.5	48.7	6.9	0.14

Chitwan					
	TCS		LPG		p-value
	Mean	SD	Mean	SD	
Cooking time (min)	69	16.5	65	6.85	0.61
T (˚C)	27.4	3.87	31.1	3.45	0.22
RH (%)	59.3	11.7	55.06	9.25	0.66

In each of the monitoring site, the cooking activities were typically done indoors in all houses and biomass is the primary cooking fuel. A little variation in biomass combustion stove design was noted in each monitoring site. In Kyanzing most of the houses used an improved metallic cook stove with chimney, but the traditional two pot mud stove was also found in use in some of the houses. Likewise, in Salambu both improved two pot mud stoves and traditional open fire stoves were found in use. However, in Pokhara and Chitwan, none of the households had the improved type of cook stove installed at the time of our study and the cooking was done in a simple single pot mud or clay stove with no chimney. However, in Pokhara and Chitwan few households used LPG as a primary source of cooking fuel. In each monitoring site, the cooking time using different types of stoves was compared. The mean cooking time, indoor temperature and relative humidity in each monitoring site using different stove designs are presented in Table 4.2. The cooking time using different stove design in each monitoring site remained the same. Also, there were no significant changes in temperature and relative humidity in the respective sites. Hence there is unlikely to be an influence of meteorological parameters at the monitoring site on cooking times and exposure between the stove designs.

4.4.2 Temporal variation in personal exposure to HAP

$PM_{2.5}$

Representative personal exposure to $PM_{2.5}$ profiles showing minute to minute variation observed throughout the monitoring periods using biomass fuel in TCS, biomass fuel in ICS and LPG are shown in Figure 4.3. The overall temporal $PM_{2.5}$ exposure followed a similar pattern in all households with considerable variability in exposure levels throughout the cooking period. It is evident from the exposure pattern that the variation in exposure concentration between cooking and the non-cooking period was distinct. $PM_{2.5}$ levels strike peak concentrations more than once during cooking period, with the variation in exposure levels in individual households in each monitoring site being dependent on the cooking practises followed. These exposure variations with few highest peak concentrations were true for households cooking with TCS, ICS and LPG. However, greater variations were observed in households using TCS compared with households using ICS and LPG. Among all households using TCS, peak $PM_{2.5}$ concentrations were reached several folds higher than the instrument detection limit (with the maximum noted concentration of 18,188 $\mu g/m^3$), whereas, in households using ICS and LPG, peak concentrations were up to 2775 $\mu g/m^3$ and 358 $\mu g/m^3$ respectively.

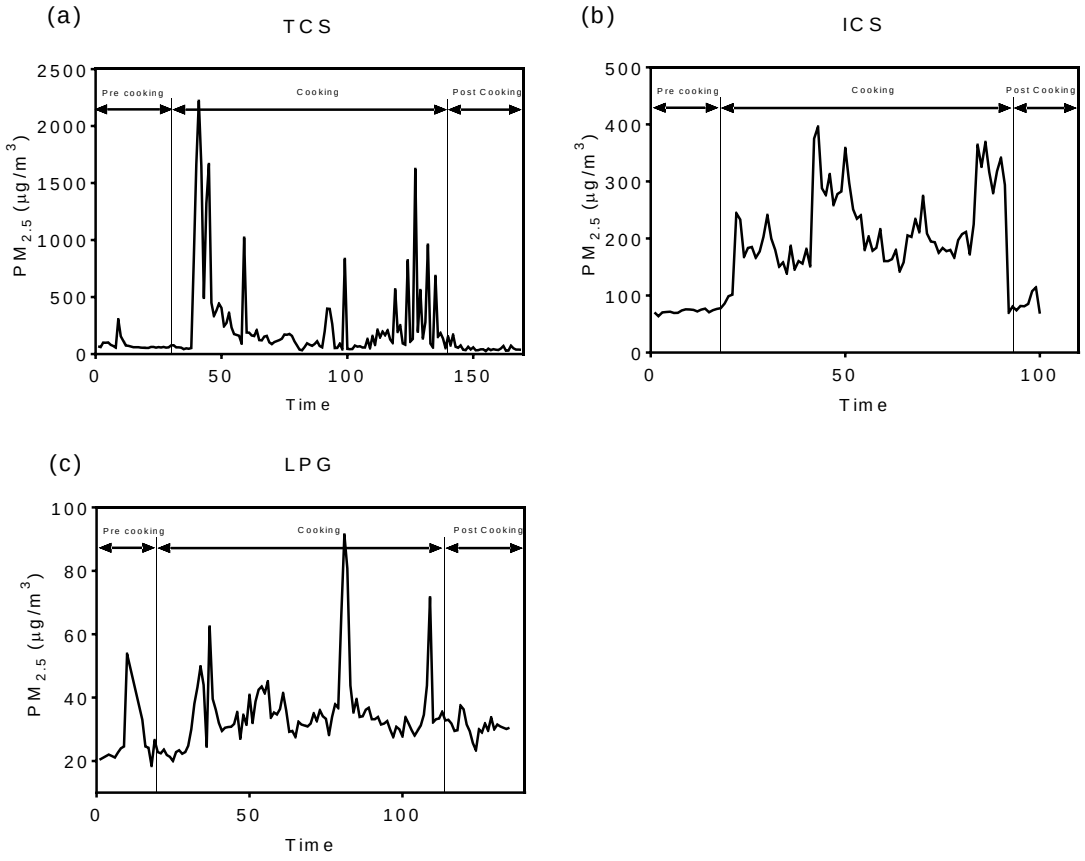


Figure 4.3 Representative temporal variation of $PM_{2.5}$ exposure in households using (a) TCS, (b) ICS and (c) LPG.

Though the overall pattern was similar for all sites, some differences in the minute-to-minute $PM_{2.5}$ variations were observed. The minute to minute data in Kyanzing showed that the $PM_{2.5}$ exposure concentration was under the WHO safe limit (25 $\mu g/m^3$) only for 3.16% of the total monitoring time. In Salambu the $PM_{2.5}$ concentration was above the safe limit all times, whereas only 1.58% and 0.64% of the time the concentrations were under the safe limit in Pokhara and Chitwan respectively. Similarly, the percentage of time the levels of $PM_{2.5}$ reached

above 1000 μg/m^3 were 21.1%, 16.2%, 2.22% and 0.76% in Kyanzing, Salambu, Pokhara and Chitwan respectively (Table 4.3). The minute to minute exposure variation observed among ICS users in Kyanzing and Salambu showed a distinct pattern. In Kyanzing, the exposure level was below the safe limit for about 40% of total monitoring time whereas in Salambu the exposure level was above the safe limit at all of the monitoring houses despite the use of ICS. However, in both places, exposure concentrations above 1000 μg/m^3 were only present for less than 1% of the total time. Similarly, with LPG, the exposure level was from 25 to 100 μg/m^3 for the majority of the time (93% in Pokhara and 84.8% in Chitwan), with none of the data observed being above 1000 μg/m^3.

Table 4.3 Percentage of time $PM_{2.5}$ levels fall under different concentration range. Data shown are derived from minute to minute exposure data from all households in each monitoring site for each stove design.

		$PM_{2.5}$				
Site	Stove Design	Exposure Range (μg/m^3)				
		<25	25-100	100-500	500-1000	>1000
Kyanzing	TCS	3.16	25.14	50.4	0	21.1
	ICS	40.2	43.1	13.51	2.2	0.8
Salambu	TCS	0	13.28	57.58	12.53	16.2
	ICS	0	28.15	67.1	4.1	0.6
Pokhara	TCS	1.58	49.6	39.58	6.9	2.22
	LPG	0.4	93	5.2	1.21	0
Chitwan	TCS	0.64	59.6	37.37	1.58	0.76
	LPG	7.29	84.8	7.9	0	0

CO

Representative exposure to CO profiles showing minute to minute variation observed throughout the cooking periods using biomass fuel in TCS, biomass fuel in ICS and LPG are shown in Figure 4.4. Though there was considerable variability in exposure levels throughout the cooking period, the overall temporal exposure followed a similar pattern in all households. Similar to $PM_{2.5}$ exposure, it is also visible from the minute to minute exposure pattern that CO concentration strikes a peak values more than once throughout the cooking period. Also, like in $PM_{2.5}$ exposure, there was a distinct variation in exposure during non-cooking and cooking period. A greater variation in CO exposure was observed among the households using TCS compared with households using ICS and LPG. Among all households using TCS in all monitoring sites, peak CO concentrations reached up to 290 ppm whereas, in households using ICS and LPG, peak concentrations were reached up to 29.2 ppm and 23.8 ppm respectively.

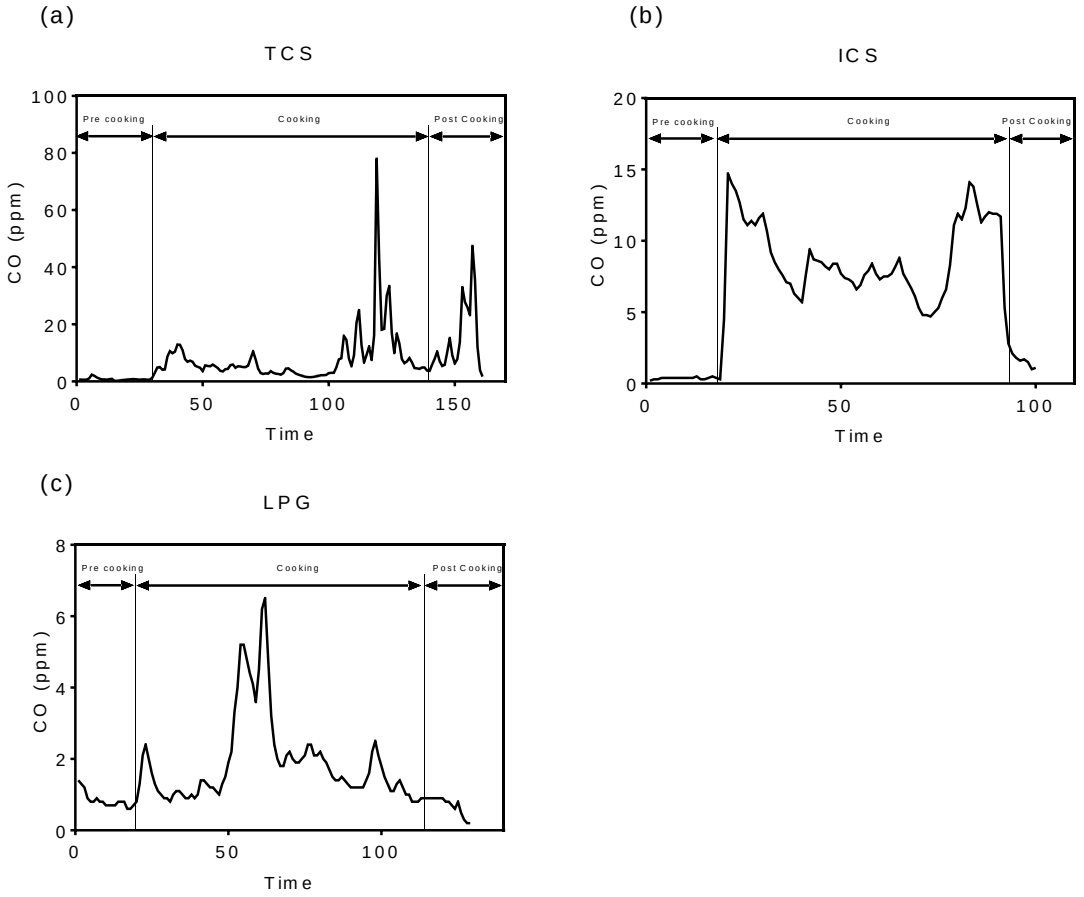


Figure 4.4 Representative temporal variation of CO exposure in households using (a) TCS, (b) ICS and (C) LPG.

Though the overall pattern was similar for all sites, there were some differences in a minute to minute exposure. The minute to minute data showed that CO exposure concentrations were under the safe limit (<7 ppm) for 34.7%, 31.7%, 48.3% and 56.8% of the total monitoring time with TCS in Kyanzing, Salambu, Pokhara and Chitwan respectively. Similarly, the percentage of time the level of CO reached above the concentration of 50 ppm was under 15% in all sites (Table 4.4). The minute to minute exposure variation observed among ICS users in Kyanzing showed that the exposure levels were under the safe limit for

95% of total monitoring time, whereas in Salambu it was just under 55% of total monitoring time. Similarly, the exposure levels in households using LPG was under the safe limit for about 90% of the total monitoring time in Pokhara, whereas it was nearly 99% in Chitwan.

Table 4.4 Percentage of time CO levels fall under different concentration range. Data shown are derived from minute to minute exposure data from all households in each monitoring site.

		CO				
Site	Stove Design	Exposure Range (ppm)				
		<7	8-15	16-35	36-50	>50
Kyanzing	TCS	34.7	29.3	21.18	5.9	8.85
	ICS	95.2	4.45	0.31	0	0
Salambu	TCS	31.7	27.21	23.92	3.78	13.35
	ICS	54.5	38.5	6.91	0	0
Pokhara	TCS	48.31	21.98	16	5.26	8.43
	LPG	89.02	8.13	2.84	0	0
Chitwan	TCS	56.79	28.93	12.67	0.91	0.68
	LPG	98.89	1.1	0	0	0

4.4.3 Personal exposure variation between cooking and non-cooking periods

$PM_{2.5}$

Personal exposure to $PM_{2.5}$ between cooking and non-cooking periods calculated in households from all four monitoring sites using biomass fuel in TCS, biomass fuel in ICS and LPG were compared (Figure 4.5). The mean personal exposure to $PM_{2.5}$ was ~4.6 fold ($p<0.0001$) and ~2.8 fold ($p<0.001$) higher in cooking period than non-cooking period exposures in households using TCS and households using ICS respectively. The overall mean $PM_{2.5}$ exposure in households using LPG was not significantly different between the cooking and non-cooking periods ($p=0.84$).

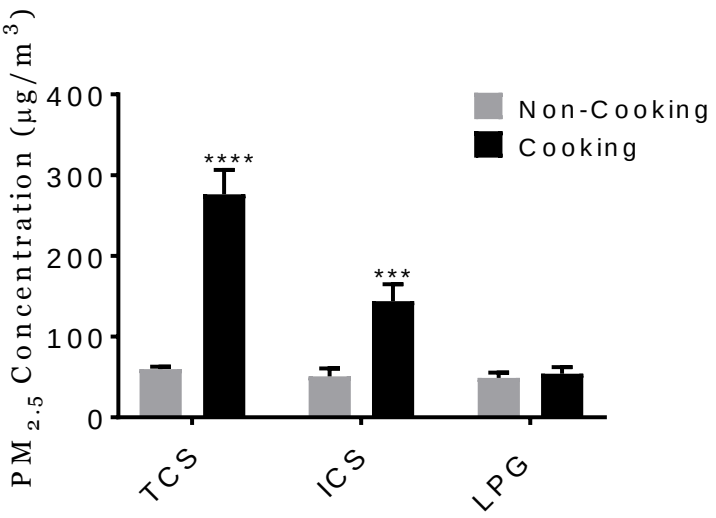


Figure 4.5 Cooking and non-cooking period $PM_{2.5}$ exposure in households using TCS, ICS and LPG. The data shown are mean (±SEM) of all households from all sites with TCS (n=76), ICS (n=17) and LPG (n=10). The variation between two periods for each stove was checked by Wilcoxon t-test, (***=$p<0.001$, and ****=$p<0.0001$).

CO

Cooking and non-cooking period real-life CO exposures in households using biomass fuel in TCS, biomass fuel in ICS and LPG were also compared (Figure 4.6). The overall mean CO exposure was significantly higher in cooking period than in non-cooking period in households using TCS, ICS and LPG stoves. The overall mean cooking period CO exposure was ~8.2 fold ($p<0.0001$), ~3.4 fold ($p<0.0001$), and ~1.5 fold ($p=0.02$) higher than non-cooking period exposure in households using TCS, ICS and LPG stoves respectively.

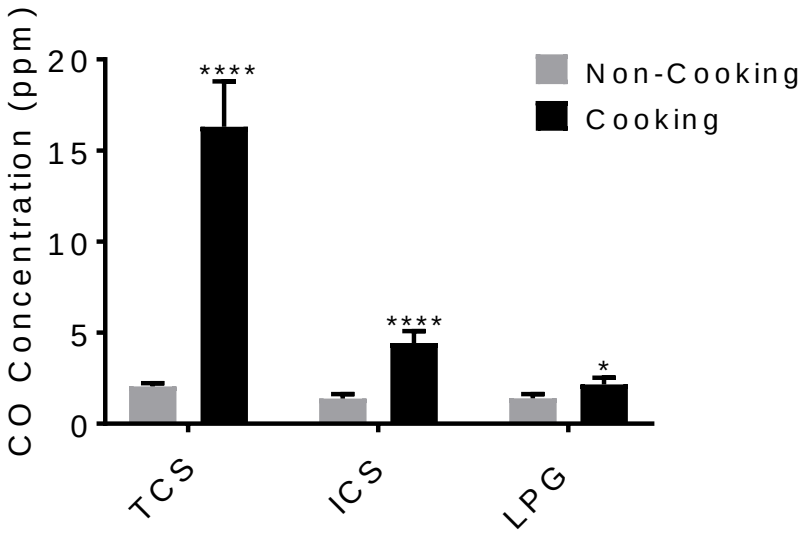


Figure 4.6 Cooking and non-cooking period CO exposure in households using TCS, ICS and LPG. The data shown are mean (±SEM) of all households from all sites with TCS (n=76), ICS (n=17) and LPG (n=10). The variation between two periods for each stove was checked by Wilcoxon t-test, (*=$p<0.05$, and ****=$p<0.0001$).

Cooking and non-cooking period mean exposures to $PM_{2.5}$, and CO using different cook stoves in each monitoring site are presented in Table 4.5. In all monitoring sites, mean $PM_{2.5}$ and CO exposures were significantly higher in cooking period than in non-cooking period exposures in households using TCS. Similarly, households using ICS in both Kyanzing and Salambu the mean exposures to all pollutants were significantly higher in cooking period compared to non-cooking period exposure. In households using LPG on the other hand, mean $PM_{2.5}$ exposure remained the same for both cooking and non-cooking period, whereas mean exposure to CO was significantly higher in cooking period than in non-cooking period. For all these data variations were observed for significance using Mann-Whitney t-test and significance levels for each test is presented in Table 4.5. The temperature and relative humidity remained the same for both cooking and non-cooking period in each monitoring sites for all cook stoves.

Table 4.5 Pollutant exposure concentrations during cooking and non-cooking periods and changes in T: temperature (degree Celsius) and RH: Relative humidity (%) in all four monitoring sites. (a) Kyanzing, (b) Salambu, (c) Pokhara, and (d) Chitwan. Differences were assessed using Man-Whitney tests.

(a) Kyanzing

	TCS			ICS		
	Cooking	Non-Cooking	p-value	Cooking	Non-cooking	P-value
T (˚C)	14.1±2.3	13.75±6.18	0.73	18.01±6.23	20.19±8.2	0.6
RH (%)	42.1±3.42	37.9±11.57	0.55	34.67±10.8	29.6±13.8	0.15
$PM_{2.5}$(µg/m^3)	746±318.9	21.32±16	0.007	91.27±70.1	16.8±15.3	0.001
CO(ppm)	12.56±4.7	1.15±0.69	0.015	2.68±1.47	1.11±0.58	0.001

(b) Salambu

	TCS			ICS		
	Cooking	Non-Cooking	p-value	Cooking	Non-cooking	P-value
T (˚C)	20.65±4.68	18.04±4.09	0.15	17.86±2.3	14.3±3.18	0.4
RH (%)	42.91±9.92	47.1±9.51	0.21	48.6±7.72	58.23±7.9	0.12
$PM_{2.5}$(µg/m^3)	500.6±314.6	104.45±101.15	0.0001	203.25±61.42	84.8±20.1	0.0002
CO(ppm)	25.78±30.4	3.28±3.38	0.0001	7.06±2.48	2.81±3.27	0.01

(c) Pokhara

	TCS			LPG		
	Cooking	Non-Cooking	p-value	Cooking	Non-cooking	P-value
T (˚C)	16.6±3.35	16.3±4.22	0.33	13.8±2.83	11.7±0.14	0.71
RH (%)	40.05±9.51	35.77±8.62	0.20	48.76±6.9	44.55±2.05	0.52
$PM_{2.5}$(µg/m^3)	211.93±133.44	44±15.85	0.0001	52.4±14.5	33.4±0.4	0.1
CO(ppm)	19.08±21.07	1.9±1.78	0.0001	2.96±1.7	0.81±0.16	0.1

(d) Chitwan						
	TCS			LPG		
	Cooking	Non-Cooking	p-value	Cooking	Non-cooking	P-value
T (˚C)	27.4±3.87	27.98±7.52	0.97	31.09±3.45	30.8±3.4	0.81
RH (%)	59.3±11.7	59.39±11.05	0.94	55.06±9.25	56.6±9.1	0.87
$PM_{2.5}$(µg/m^3)	121.9±57.8	79.48±48.9	0.001	54.68±31.02	53.4±20.3	0.71
CO(ppm)	9.08±6.98	5.37±7.91	0.01	1.85±0.94	1.6±0.81	0.24

4.4.4 Overall Exposure variations using different stoves

Having demonstrated that cooking activities in households using biomass fuel enhanced the production of indoor pollutants, I then attempted to observe the overall concentration of indoor pollutants in these settings using different stove designs. The overall mean concentration of $PM_{2.5}$ and CO in households from all monitoring sites using TCS, ICS and LPG stoves were calculated (Figure 4.7). The overall mean $PM_{2.5}$ exposure in households using biomass fuel in TCS was 276.1 µg/m^3, which is about 11 fold higher than the WHO safe recommended concentration for $PM_{2.5}$. The overall mean $PM_{2.5}$ exposure was significantly lower in households using ICS (51% overall reduction, $p=0.04$) and in households using LPG stoves (80% overall reduction, $p<0.0001$). The overall mean $PM_{2.5}$ exposures using ICS and LPG were 143.9 µg/m^3 and 53.9 µg/m^3 respectively. Similarly, high levels of CO exposure were measured in households using TCS as compared to the households using either ICS or LPG stoves. The overall mean CO exposure with TCS was 16.3 ppm, whereas these concentrations were significantly lowered in households using ICS (72%

reduction, p=0.0002) and in households using LPG stove (86% reduction, p<0.0001).

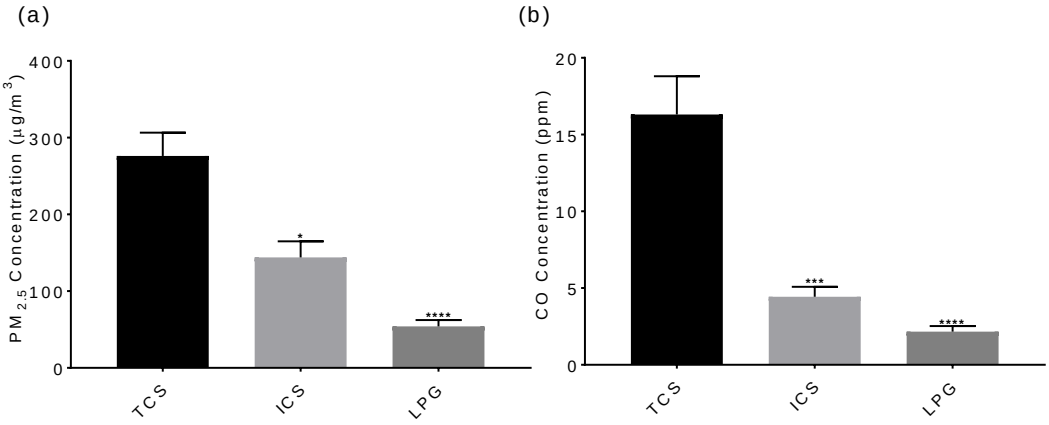


Figure 4.7 Comparison of the overall mean concentration of pollutants in households using TCS, ICS and LPG stoves. (a) $PM_{2.5}$, and (b) CO. The data shown are the mean of all households from all sites using TCS (n=76), using ICS (n=17) and LPG (n=10). Values across the groups were compared using Kruskal-Wallis followed by Dunn's multiple comparisons test (*= p<0.05, **=p<0.01, ***=p<0.001, and ****=p<0.0001).

4.4.5 Variation of exposure among different sites

Having demonstrated the exposures of HAP are significantly higher during cooking period than in non-cooking period and that the exposure depended on the type of stove used for fuel combustion, I then investigated whether or not cooking period exposure to these pollutants varied among different monitoring locations. The mean exposure to $PM_{2.5}$ and CO were computed from all households in each monitoring sites and the variation was assessed using Kruskal-Wallis test followed by Dunn's multiple comparison test (Figure 4.8).

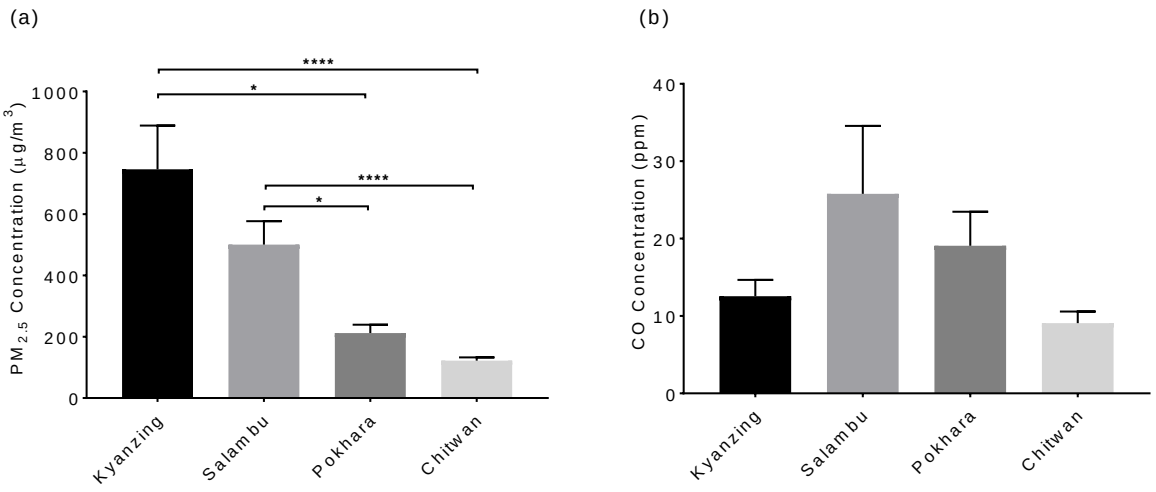


Figure 4.8 Comparison of mean exposure to HAP concentrations between different monitoring sites. The data shown are the mean (±SEM) exposure in households using TCS for (a) $PM_{2.5}$, and (b) CO in Kyanzing (n=5), Salambu (n=17), Pokhara (n=24) and Chitwan (n=30). Values across the groups were compared using Kruskal-Wallis, followed by Dunn's multiple comparisons test. (*= $p<0.05$, **=$p<0.01$, and ****=$p<0.0001$).

The highest mean $PM_{2.5}$ exposure of 746 $\mu g/m^3$ was measured in households at Kyanzing using TCS. The exposure concentration seen at Kyanzing was ~3.5 fold ($p<0.05$) and ~6 fold ($p<0.0001$) higher than the exposure measured in households at Pokhara and in households at Chitwan respectively. The mean $PM_{2.5}$ exposure at Salambu was 500 $\mu g/m^3$, which was ~2.5 fold ($p<0.05$) and ~4 fold ($p<0.0001$) higher than the exposure at Pokhara and Chitwan respectively. Summary data for $PM_{2.5}$ exposure in all four monitoring sites is presented in Table 4.6. The overall exposure data for $PM_{2.5}$ showed that households at higher altitude locations using biomass fuel in TCS generated higher concentration of

$PM_{2.5}$ than in households at lower altitude. The overall mean CO exposure and summary data for CO in all four sites are shown in Figure 4.8 (b) and Table 4.6. The overall mean CO exposure showed a little different pattern. Although there was a trend towards lower exposures of CO at lower altitudes, the highest value was seen in Salambu rather than Kyanzing. However, none of the mean CO exposure variations observed between different locations was statistically significant.

Table 4.6 Exposure summary values from all households in each monitoring site (a) $PM_{2.5}$, and (b) CO

(a)

Pollutant Type			$PM_{2.5}$ (µg/m^3)			
Study Site	n	Min	Max	Mean	Median	SD	95% CI
Kyanzing	5	408.1	1153	746	591.3	319	350-1142
Salambu	17	165.7	1231	500.6	338.4	314.2	339.1-662.1
Pokhara	24	46.1	580.2	211.9	180.8	133.4	155.6-268.3
Chitwan	30	37.02	239.9	122	109.7	57.83	100.4-143.6

(b)

Pollutant Type			CO(ppm)			
Study Site	n	Min	Max	Mean	Median	SD	95% CI
Kyanzing	5	7.3	18.9	12.56	12.1	4.69	6.73-18.39
Salambu	12	2.9	107	25.78	14.25	30.43	6.45-45.12
Pokhara	23	2.5	91.7	19.08	9.8	21.07	9.97-28.2
Chitwan	22	2.7	23.73	9.08	6.9	6.98	5.98-12.18

4.4.6 Exposure concentration variation with stove designs in Kyanzing and Salambu

Having discussed and presented the use of an improved cooking stove and LPG stoves reduced the exposure levels significantly than the exposure levels using the traditional cooking stove, I then investigated whether or not this observation is true for each monitoring sites using different cook stove designs. As discussed above, both improved cook stove and traditional cook stove were in use to combust biomass fuel for cooking in households of two monitoring sites Kyanzing and Salambu. Exposure monitoring in these sites was performed in households using both TCS and ICS and compared for exposure variation between two stove designs. The exposure variations of $PM_{2.5}$ and CO observed between TCS and ICS use in Kyanzing and Salambu is presented in Figure 4.9 and Figure 4.10 respectively. The monitoring data showed that the mean exposure in households using ICS was significantly lower than in households using TCS for both pollutants at both the sites. However, the percentage reduction with ICS in both locations varied. For instance, the reduction in exposure concentration of $PM_{2.5}$ between TCS and ICS was 88% ($p<0.001$) at Kyanzing, whereas the reduction was only 59% at Salambu ($p<0.01$). These results show that ICS reduces exposures; however, the concentration at Kyanzing and Salambu was still 3.6 fold and 8 fold higher than the threshold value of 25 $\mu g/m^3$ recommended by WHO respectively even with the use of ICS. Similarly, the percentage

reduction in CO exposure in households with ICS at Kyanzing and Salambu were 78% ($p<0.001$) and 72% ($p<0.05$) respectively.

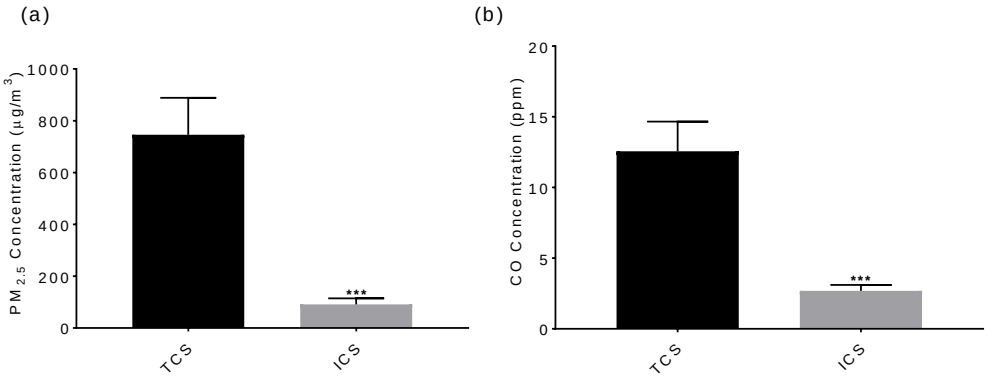


Figure 4.9 Comparison of exposure in households using TCS and ICS at Kyanzing. The data shown are mean (±SEM) exposure to (a) $PM_{2.5}$, and (b) CO in households using TCS (n=5) and ICS (n=9). The variations were assessed using Mann-Whitney t-test (***=$p<0.001$). TCS: Traditional cook stove, ICS: Improved cook stove.

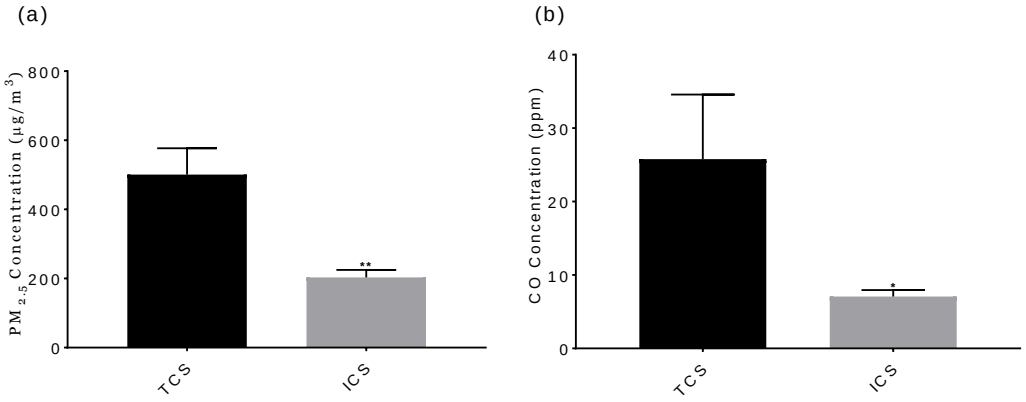


Figure 4.10 Comparison of exposure in households using TCS and ICS at Salambu. The data shown are mean (±SEM) exposure to (a) $PM_{2.5}$, and (b) CO in households using TCS (n=17) and ICS (n=8). The variations were assessed using Mann-Whitney t-test (*=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$) TCS: Traditional cook stove, ICS: Improved cook stove.

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4.4.7 Exposure concentration variation with different fuels

In Pokhara and Chitwan, some households used LPG as a primary source of energy for cooking. The exposure to indoor pollutant in households using biomass fuel in TCS and households using LPG were monitored in these locations. The exposure variations of $PM_{2.5}$ and CO observed between TCS and LPG use in Pokhara and Chitwan are presented in Figure 4.11 and Figure 4.12 respectively. Exposures to all pollutants measured in households using LPG were significantly lower than those observed in households with TCS. The percentage reductions in $PM_{2.5}$ in households with LPG at Pokhara and Chitwan were 75% ($p<0.01$) and 55% ($p<0.01$) then in households with TCS respectively. However, the concentration observed in households with LPG was still 52.4 $\mu g/m^3$ and 54.6 $\mu g/m^3$ at Pokhara and Chitwan respectively, which was still about 2 fold higher than the safe threshold limit suggested by WHO. Similarly, the percentage reduction in CO exposure in households using LPG was 85% ($p<0.05$) and 79% ($p<0.001$) in Pokhara and Chitwan respectively. Hence these data showed that people using LPG are being exposed to a lower concentration of HAP compared to those using biomass fuel in TCS.

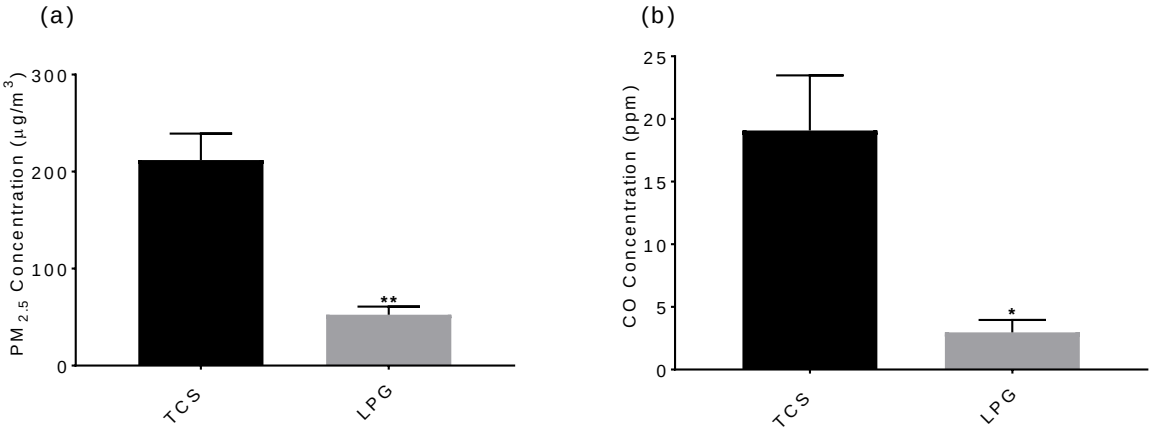

Figure 4.11 Comparison of exposure in households using biomass in TCS and LPG stove at Pokhara. The data shown are mean (±SEM) exposure to (a) $PM_{2.5}$, and (b) CO in households using TCS (n=24) and LPG (n=3). The variations were assessed using Mann-Whitney t-test (*=p<0.05, **=p<0.01) TCS: Traditional cook stove, LPG: Liquefied petroleum gas.

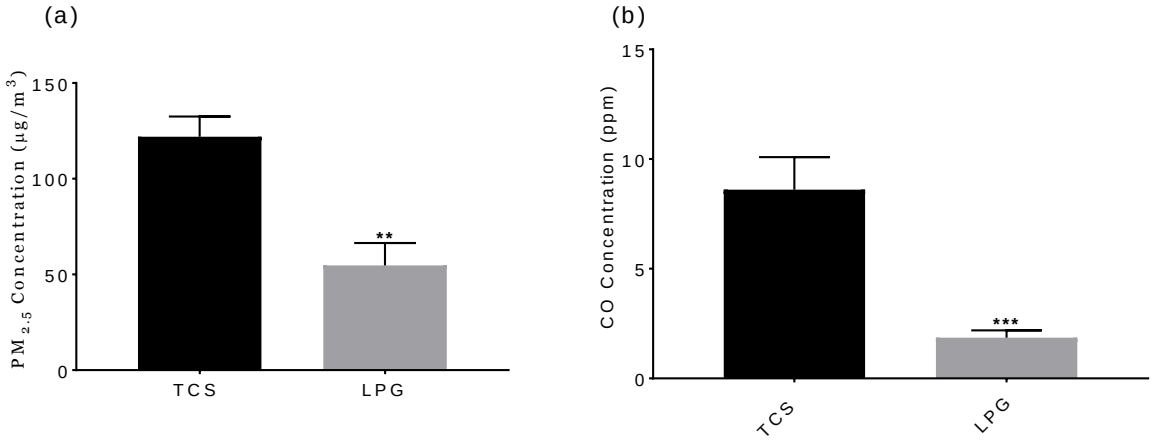

Figure 4.12 Comparison of exposure in households using biomass in TCS and LPG stove at Chitwan. The data shown are mean (±SEM) exposure to (a) $PM_{2.5}$, and (b) CO in households using TCS (n=30) and LPG (n=7). The variations were assessed using Mann-Whitney t-test (**=p<0.01, ***=p<0.001, and ****=p<0.0001)) TCS: Traditional cook stove, LPG: Liquefied petroleum gas.

4.4.8 Ambient $PM_{2.5}$ concentration

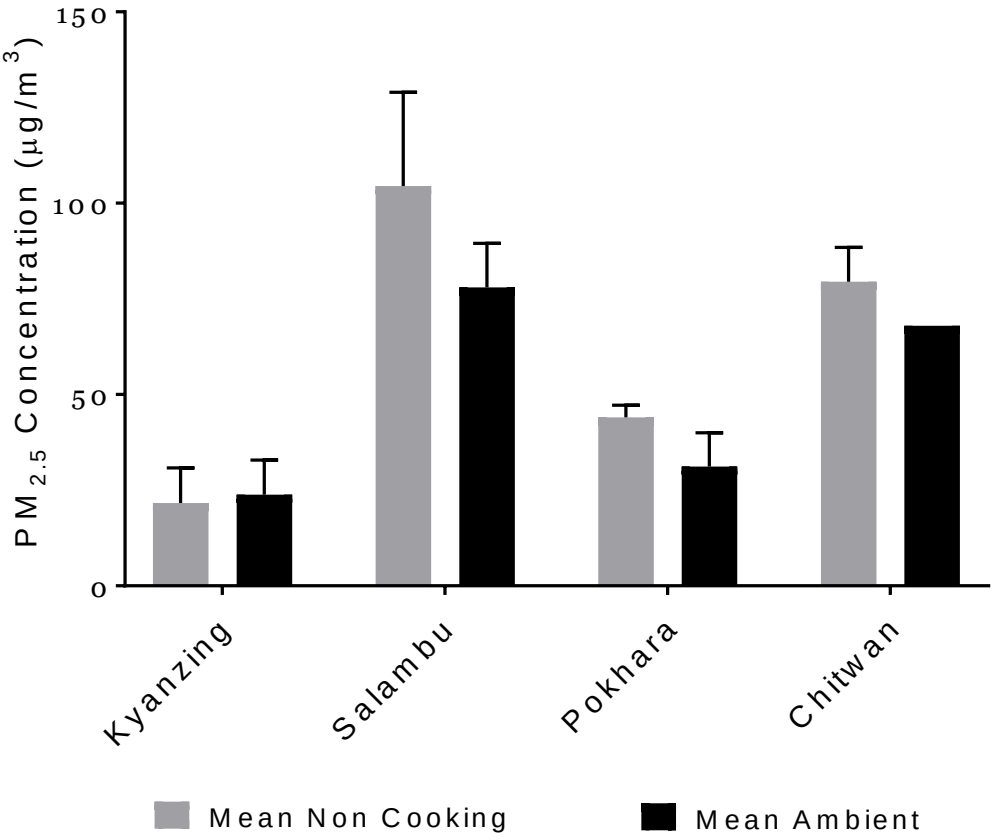

Figure 4.13 Mean ambient $PM_{2.5}$ concentration in each monitoring site. The mean ambient PM concentrations were compared with mean non-cooking period exposure for each monitoring sites. The data shown for ambient $PM_{2.5}$ are mean of n=2 for Kyanzing, Salambu and Pokhara and n=1 for Chitwan.

Ambient $PM_{2.5}$ concentration at the time of this study in each monitoring site was also measured using the same monitoring equipment. Equipment was kept in a central location of the village, and ambient $PM_{2.5}$ concentration was measured for an hour. The number of ambient air samples was relatively low as compared to indoor air sample (2 ambient samples in each location). Hence no statistical analysis was performed for ambient $PM_{2.5}$ samples. However, from the observation, it was found that

indoor $PM_{2.5}$ concentration during non-cooking period in each monitoring site remained similar to the ambient $PM_{2.5}$ concentration in the respective sites. This suggests that the exposures observed in the non-cooking period are could be due to ambient air pollution. It was observed that the ambient $PM_{2.5}$ concentration was comparatively lower at a higher altitude than at a lower altitude.

4.4.9 Correlation with metrology and Co-pollutants

The relationship among the measured co-pollutants, temperature and relative humidity were investigated. The level of interaction between these variables is shown in the correlation map (Figure 4.14). The correlation map shows that there is a significant positive correlation between both co-pollutants. $PM_{2.5}$ and CO concentration showed no correlation with temperature and relative humidity.

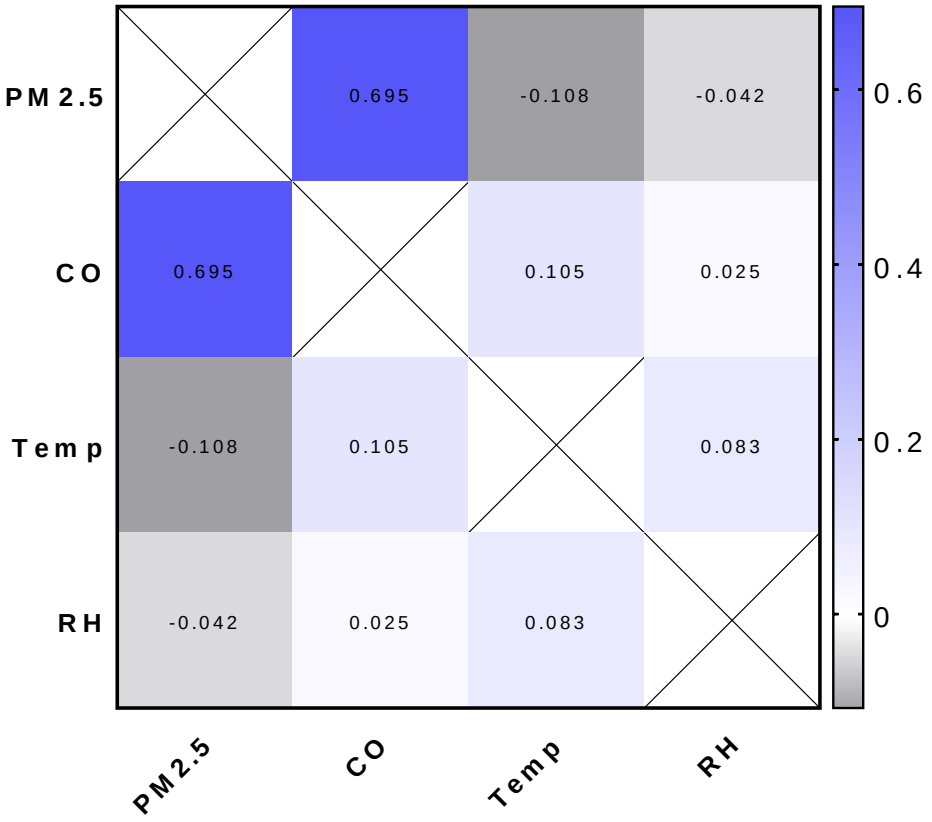

Figure 4.14 Correlation map showing the relationship between the different indoor pollutants and also temperature and relative humidity. The correlations were checked with mean data from all households using biomass fuel either in TCS or ICS (n=93). Correlation coefficient and respective p values were checked using Spearmen's coefficient

Having demonstrated the presence of overall correlations between co-pollutants, I then analysed the relationships among those variables in individual locations. To do this, I looked at the correlation of $PM_{2.5}$ with CO in each location (Figure 4.15). $PM_{2.5}$ concentration showed a significant positive correlation with CO in all four locations.

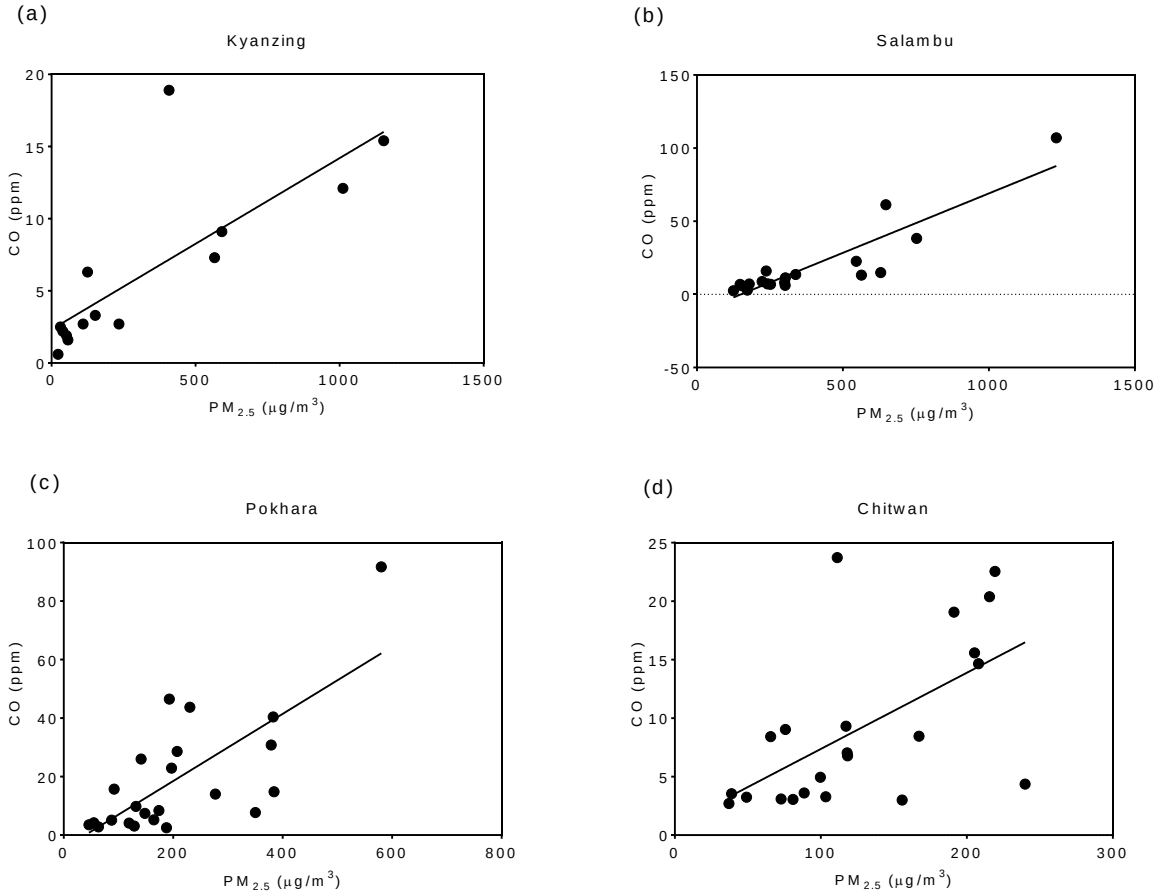

Figure 4.15 $PM_{2.5}$ correlated with CO in all four locations. (a) Kyanzing, (b) Salambu, (c) Pokhara and (d) Chitwan. Kyanzing: $PM_{2.5}$ correlation with CO: r^2=0.6, p=0.001. Salambu: $PM_{2.5}$ correlation with CO: r^2=0.8, p=0.0001. Pokhara: $PM_{2.5}$ correlation with CO: r^2=0.5, p=0.0001. Chitwan: $PM_{2.5}$ correlation with CO: r^2=0.35, p=0.003. Each data point represents values obtained during a single cooking episode.

4.4.10 Exhale Breath CO Concentration

The above data show high concentrations of indoor pollutants $PM_{2.5}$ and CO during cooking in the households using biomass fuel in the different locations. In those households, I also measured the concentration of CO in exhaled breathe of the individual involved in the cooking activity. The overall results indicate higher levels of exhaled breathe CO and % COHb in individuals using biomass fuel as a cooking source. The exhaled breathe CO variations in a different cooking phase were monitored only in Salambu, Pokhara and Chitwan. In all three locations, variations in exhaled breath CO followed a similar pattern (Figure 4.16 (a-c)) with the highest concentration noted during the cooking phase followed by the post-cooking phase and least high during the pre-cooking stage. The differences between pre-cooking and cooking period CO levels seen were significant ($p<0.0001$ in Salambu and Pokhara, and $p=0.02$ in Chitwan). The CO levels reduced again during a post-cooking phase in all three locations, however, the differences observed between cooking and post-cooking were significant in Salambu ($p<0.001$) and Pokhara ($p=0.003$), but not in Chitwan ($p=0.34$). Post-cooking levels were lower than during the cooking period levels; however, the levels were still higher, but not significant, than the levels noted in pre-cooking observations.

Although we were only able to obtain values during the cooking period in Kyanzing, differences in CO concentration between individuals in households with TCS and ICS were compared. In addition to that, CO

concentration in exhaled breath in individuals using LPG in Chitwan was also monitored. The levels of exhaled breath CO using LPG in Chitwan and using TCS and ICS in Kyanzing, are presented in Figure 4.16 (d) and (e) respectively. Exhaled breath CO levels in individuals using ICS in Kyanzing were significantly lower (75% lower, $p<0.0001$) than the individuals using TCS. Similarly, the use of LPG in Chitwan showed that exhaled breath CO levels were not significantly elevated during cooking.

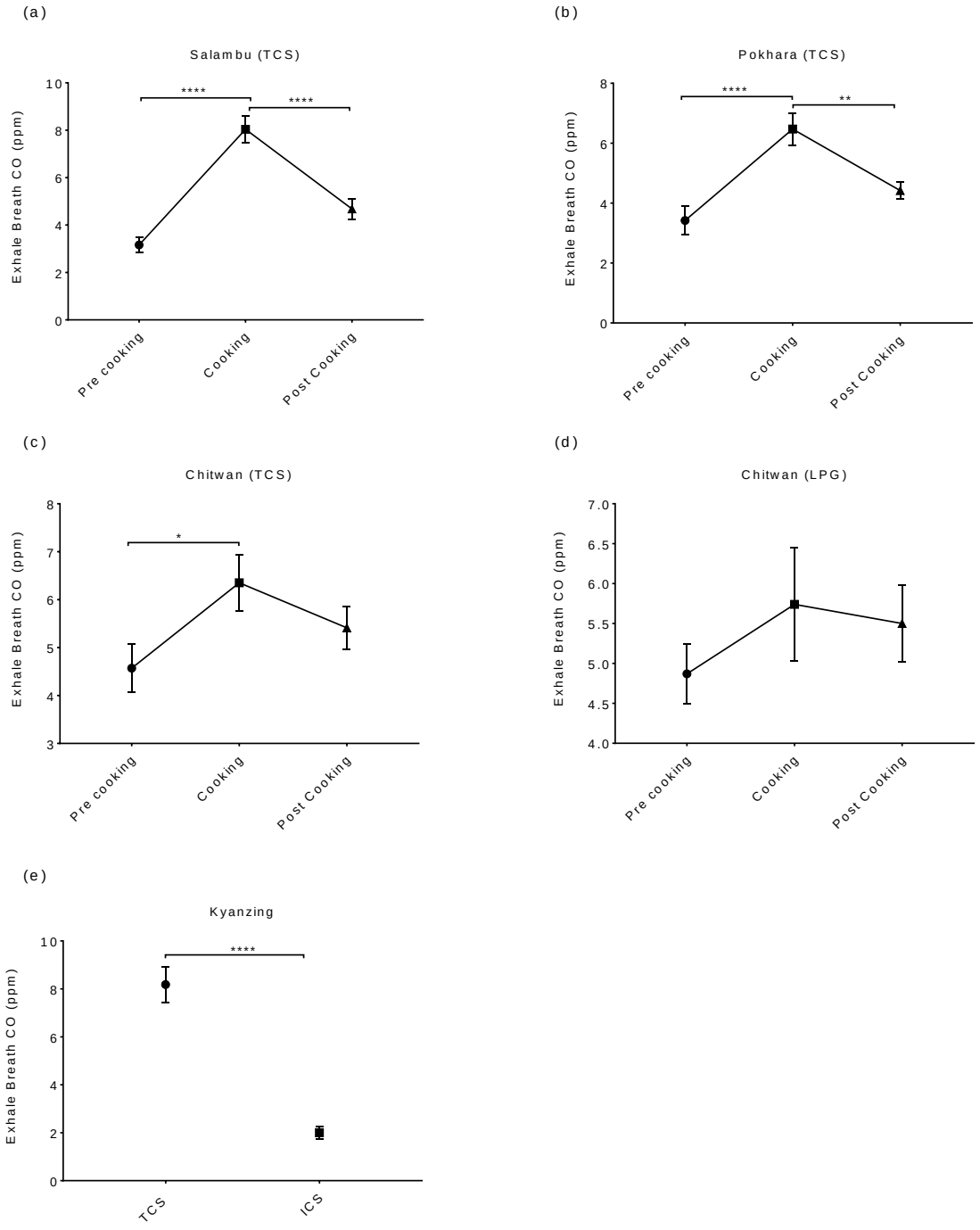

Figure 4.16 Variations in exhaled breath CO concentration. Exhaled breath CO levels in pre-cooking, cooking and post-cooking phase in (a) Salambu, (b) Pokhara and (c) Chitwan, (d) Exhaled CO levels using LPG in Chitwan, (e) Exhaled CO levels using TCS and ICS in Kyanzing. Values across the groups were compared using Kruskal-Wallis, followed by Dunn's multiple comparison test for more than 2 groups (figure a-d) and Mann-Whitney t-test to compare two variables (figure e). (*=$p<0.05$, **=$p<0.01$, and ****=$p<0.0001$).

4.4.11 Pulse and SpO2

A pulse and SpO2 data were obtained from a total of 45 individuals actively involved in cooking using biomass fuel in Salambu and Pokhara. There were no significant changes in pulse rate before and after cooking exposure (Figure 4.17(a)). However, a significant decrease in mean SpO2 levels was observed during cooking (-1.2%, 95% CI -1.85 to -0.59, $p<0.0001$) than the pre-cooking levels (Figure 4.17(b). The mean SpO2 levels increased but not significant (mean difference between cooking and post-cooking, 0.77, 95% CI 0.01 to 1.54) after cooking.

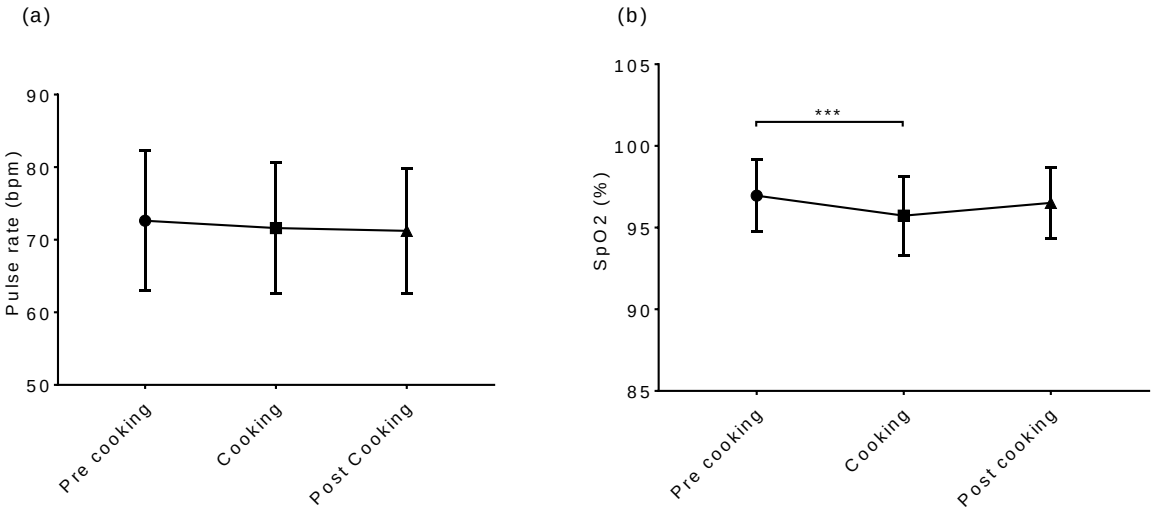

Figure 4.17 Comparison of pulse and SpO2 level in different cooking phase. The data shown are mean (±SD) (a) pulse rate and (b) SpO2 for a total of n= 45 individuals. The variation across the groups was checked with One way ANOVA followed by Tukey's multiple comparison test. (***=$p<0.001$).

4.5 **Discussion**

The main aim of the study described in this chapter was to further investigate the pattern of real-life personal exposure to HAP in rural areas of Nepal in a range of geographical settings. The key aims were to (i) quantify real-life exposure to $PM_{2.5}$ and CO in households using biomass fuel in four different Nepalese villages, (ii) assess the variation in exposure pattern in those settings using more efficient biomass combustion cook stove, (iii) assess the variation in exposure pattern in households using clean fuel, and (iv) assess physiological effects due to short term exposures to indoor pollutants. I studied 4 rural to semi-rural Nepalese villages in different locations at altitudes ranging from ~100 m to 4000 m above sea level. The villages were chosen because they were in different altitude and vegetation zone, and they represent all three ecological regions of the country. Combustion of biomass fuel on low efficient traditional cook stove (TCS) was the primary source of cooking in all four villages, though efficient improved cook stove (ICS) and clean fuel like LPG were also used as a cooking source in some households in these settings.

The main findings that can be drawn from the work described in this chapter are that in general high levels of $PM_{2.5}$ and CO exposures were seen in households cooking with biomass fuel on traditional stoves, in the range that would be considered harmful to human health by WHO. Personal exposures were measured for both cooking and non-cooking

period and found that cooking period exposures were significantly higher than non-cooking period exposures in all regions. The observed temporal variation in $PM_{2.5}$ and CO exposure pattern is consistent with the variations presented in the previous chapter, and the cooking period variations reported in other studies elsewhere [20, 269]. The exposure patterns were similar, but the overall mean personal exposures are lower in magnitude than those seen in households at Phoolbari presented in the previous chapter. The differences in exposures between two studies could be due to high levels of exposures seen in households using agricultural residue and *cow dung* as a cooking source.

Similarly, there were some differences in the absolute exposures observed between different households and in different geographical regions, suggesting possible differences in social and cultural cooking practises. The exposures observed in this study in different locations were between 5-29 fold higher than WHO standards for indoor $PM_{2.5}$ (25 $\mu g/m^3$) [18]. In general high levels of $PM_{2.5}$ were seen in households using traditional stoves at a higher altitude, whilst as altitude decreases, so do the levels of $PM_{2.5}$, and seen lowest at lower altitude regions. A similar pattern of overall exposures to CO was seen; however, higher level of exposures was observed in Salambu than in Kyanzing. The differences in exposures between regions are probably due to incomplete combustion, use of different biomass fuels or due to difference in cooking practises followed in different households in different regions. It has already been shown in previous studies that emissions from biomass combustion

depend on many factors like types of fuel and its specific properties, use of combustion devices and individuals cooking habits [249, 250]. In addition to this, overall exposures in Kyanzing would have been much higher because stoves were in use for much longer.

The measurement of $PM_{2.5}$ concentrations in ambient air in each region showed that the levels were higher at lower altitude regions. The higher concentration of ambient $PM_{2.5}$ levels in Chitwan is likely to be due to various anthropogenic activities like vehicular emissions, agricultural burning in the field, waste burning outside homes for heating purposes and forest fires. In comparison, these types of environmental pollutions producing activities are minimum at Kyanzing. Various previous studies have shown that ambient air pollution contributes to poor indoor air quality [270-272]. This suggests that levels of ambient $PM_{2.5}$ concentrations in each monitoring site might also have contributed to indoor $PM_{2.5}$ concentrations. However, defining the actual contribution of ambient air pollution to indoor air quality in each of these settings will require additional work to provide comprehensive data on the interactions between indoor and ambient air quality.

The majority of previous studies conducted in Nepal [20, 117, 119] and other developing countries [102, 269] have monitored indoor $PM_{2.5}$ and CO concentrations over a fixed period of time, and only a few previous studies have attempted to measure personal exposure to indoor $PM_{2.5}$ and CO [115, 233]. However, previous studies, conducted in Nepal and

elsewhere, using both real-life exposure and using gravimetric sampling have also shown high concentrations of indoor $PM_{2.5}$ and CO in households using biomass fuel consistent with our findings. Personal exposures to $PM_{2.5}$ and CO reported in previous studies have also shown high levels of indoor pollutants in line with our findings. However, these studies generally reported absolute exposures over a 24 hour, including both cooking and non-cooking period. Hence absolute exposures observed in this study were generally higher than exposures reported in the previous studies. For instance, the absolute exposure to $PM_{2.5}$ monitored in Chitwan and Kyanzing were ~2.5 fold and ~7.5 fold higher than the absolute exposure reported in a study conducted by Phillips et al.[115]. However, the concentrations reported in the study by Phillips et al. was the absolute exposure over a 24 hour, including both cooking and non-cooking period exposure. Hence it is obvious to see high exposure levels in our study where the absolute values were taken only for cooking period exposures. Though there were some differences in the absolute exposures between different studies, the main conclusion that can be drawn is that people cooking with biomass fuel on traditional stoves are exposed to high levels of indoor pollutants.

Having demonstrated that people using biomass fuel on TCS are exposed to a higher concentration of $PM_{2.5}$ (overall mean 276.1 $\mu g/m^3$) and CO (overall mean 16.3 ppm) in these settings, I then investigated whether or not exposure concentration is reduced using ICS. I found that the overall

$PM_{2.5}$ and CO exposures were reduced by 51%, and 72% respectively in households using ICS to combust biomass fuel than in households using TCS. The findings of this study are in line with the results presented in the previous chapter, where I showed overall $PM_{2.5}$ and CO exposures reduction of 65% and 50% respectively in households using ICS. In addition to this, several other studies conducted elsewhere have shown the exposure reductions using ICS consistent with our findings [102, 115-117, 188]. A study conducted in three rural districts in Nepal [116] had shown an overall reduction of 63.2% and 60% for indoor $PM_{2.5}$ and CO concentration in households after installation of ICS. Similarly, another study conducted by Parajuli et al.[117] had found 39% and 29.9% reductions in indoor $PM_{2.5}$ and CO in households using ICS. Similarly, personal exposure to $PM_{2.5}$ in households using ICS was found 68.4% lower than in household using TCS in a recent study conducted in Sri Lanka [115]. Though there were some differences in percentage reduction of indoor $PM_{2.5}$ and CO between our and various other studies, the main conclusion that can be drawn is that use of ICS reduces exposures in comparison with using TCS. However, the exposures seen with ICS in this study remained well above the threshold level recommended by WHO and other organization. Similar observations were reported in all previous studies that $PM_{2.5}$ concentrations remained well above the safe level recommended by WHO [188, 273] even after the installation of ICS. Hence, whilst it is highly likely the reductions seen will produce benefit further changes may be needed to reduce the levels below the safe limit.

Similar to the reduction seen with ICS, I found that personal exposures to $PM_{2.5}$ and CO were significantly reduced in households using LPG as a cooking source. A study conducted by Pokhrel et al. in Bhaktapur, Nepal [119] had reported 86% reduction in indoor $PM_{2.5}$ in households using LPG as compared in households using TCS. The percentage reduction reported in this study is in line with the percentage reduction seen in our study. However, the absolute exposure to $PM_{2.5}$ in our study (53 $\mu g/m^3$) is 50% less than the exposure reported in this study. Exposure to $PM_{2.5}$ in households using LPG reported in these studies is still 2-4 fold higher than WHO safe levels.

Measurement of CO concentration in exhaled breath is an easy non-invasive technique, considered as a sensitive biomarker of CO exposure [274, 275]. Exhaled breath CO concentrations during cooking and non-cooking period were measured in individual involved in cooking. The result showed a high concentration of CO during cooking period consistent with the findings from previous studies [276, 277]. The mean cooking period exhaled breath CO concentration in three monitoring regions were ranged from 6-8 ppm. The mean CO concentration in the present study is comparable to the levels reported by earlier studies [276, 278]. A study conducted by Vinod et al. reported that burning of biomass fuel enhanced exhaled breath CO levels of the cooks and reported concentrations of 6-9 ppm measured after cooking exposure using different biomass fuels. Similarly, another study conducted in Guatemala [278] also reported exhaled breath CO levels of 9ppm in women using biomass fuel. I also

found that exhaled breath CO levels were higher in an individual using biomass fuel in the traditional cook stove. The exhaled breath CO levels were reduced to about 75% in individual using biomass fuel in ICS in Kyanzing consistent with the findings from previous studies [279]. The study conducted by Adam et al. has shown that the exhaled breath CO concentrations were reduced from 6.71 ppm to 3.14 ppm after installation of chimney stoves in Peru [279]. The overall result that can be drawn from this study is that cooking exposures enhanced exhaled breath CO levels and increases the burden of CO exposure indicating physiological effects of exposure. The differences seen in exhaled breath CO concentration in cooking and non-cooking period indicate that the resulted breath CO concentration is due to the exposed CO resulting from the cooking activities. The comparatively higher concentration of CO during the post-cooking phase suggests that after exposure to a higher concentration of CO, some residue CO remained in the lung for some time. More follow up studies and continuous measurement of exhaled breath CO needs to be carried out to elucidate whether the presence of CO before and after exposure is from the residue CO or the concentration endogenously produced in the body [280]. The reduced level of exhaled CO while using ICS indicates improved physiological response using efficient technology.

In conclusion, this chapter has shown that people from different regions using biomass fuel for cooking are exposed to high concentrations of HAP. These exposures concentrations were varied in households in different

altitude regions. The high levels seen in households are because of using traditional stove designs with no flue and cooking indoors in poorly ventilated rooms. It has also found that the combustion of these biomass fuels in the more efficient stove and use of more clean fuel in these regions can reduce the exposure concentration significantly. However, further modifications or changes in cook stove design and other interventional strategies may be needed as the exposure concentration with existing ICS are not able to reduce the levels below WHO recommended safe levels. The data generated in this study could help in the development of HAP mitigation policies and help to assess health effects of particle exposures. Prolonged exposures to these high levels of indoor pollutants are thought to be a significant cause of chronic respiratory and cardiovascular disease in Nepalese populations. It is essential to know the immediate effect of short term exposure on human lungs or human airway cells. Hence, research assessing the pro-inflammatory effect on human airway cells or in lung tissue needs to be carried out to define the potential *in vivo* effects of these exposures.

Chapter 5: Inflammatory profiles of human lung explant tissue in response to smoke extracts from biomass burning.

5 Inflammatory profiles of human lung explant tissue in response to smoke extracts from biomass burning.

In the previous chapters, I demonstrated that combustion of biomass fuel produced high concentrations of indoor air pollutants and people using biomass fuel for cooking in indoor environments were exposed to up to 29 fold higher concentrations of those pollutants than the levels recommended by WHO. I also demonstrated that the exposures were varied among the different regions, and also depended on fuel and type of stove used for cooking. The exposures were reduced when clean fuel such as LPG was used or when biomass fuel was combusted in more efficient stove design. In this chapter, I describe work designed to assess the possible consequences of those short term exposures of harmful pollutants from cooking on responses in a human lung. This work took advantage of an *ex vivo* human lung tissue model by measuring a range of pro-inflammatory cytokines in the isolated supernatant after tissue stimulation. Also, I explored the potential effects of exposure reduction using clean fuel and efficient biomass combustion stoves on inflammatory cytokine profiles in this tissue.

5.1 **Introduction**

Inflammation is a defence mechanism which can be triggered by many different factors including harmful pathogens, inhaled toxic compounds such as cigarette smoke, or environmental pollution or biomass smoke (see Introduction for more details). These infectious or non-infectious

agents interact and activate resident tissue cells and inflammatory cells, and initiate inflammatory signalling cascades. These signalling molecules act on a range of inflammatory cells and help activate and recruit monocytes/macrophage to the site of injury. These activated resident tissue cells, activated monocytes/macrophages and other immune cells produce inflammatory cytokine, chemokines and other inflammatory mediators [167, 281]. Cytokines also act on various downstream inflammatory cells leading to the production of more inflammatory cytokines. In this way, a complex cytokine network is formed which in turn leads to acute and chronic inflammation. Though an inflammation process may be beneficial to health, prolonged exposure to toxic substances may result in the continuous production of cytokines which in turn leads to excess tissue damage.

Whilst there are various kinds of stimulus that initiate inflammation in the lung, inhalation of smoke produced from the combustion of biomass fuel in cooking is thought to be an essential factor for lung inflammation. A number of studies have already shown that biomass smoke exposure is an influential factor for the development of COPD [51, 282, 283]. In addition to this, WHO has reported that more than 50% of deaths attributed to HAP due to solid biomass fuel use are associated with lung inflammation [48]. A wide range of pro-inflammatory cytokines including IL-8, IL-6, TNF-α, IL-1β, GM-CSF are secreted from airway epithelial and other immune cells during lung inflammation [122, 284].

In this chapter, the inflammatory response of exposure to biomass smoke extract in human *ex vivo* lung tissue was assessed by incubating lung tissue with smoke extract samples. The smoke extract samples were collected in households during real-life cooking, and human parenchyma lung tissues were obtained from patients undergoing surgery with written consent through Papworth and Nottingham tissue biobanks. The tissue culture supernatant was collected after 48 hours of stimulation and stored at -80°C until its use. The pro-inflammatory mediators were assessed in the supernatant using Luminex Assays. A panel of 17 different analytes was initially chosen which was then cut down to 10 analytes after initial results were analysed. Lipopolysaccharide (LPS) was used as pro-inflammatory stimuli as a positive control throughout the experiments.

Lung tissue fragments have previously been used in studies defining airway inflammation of various stimuli. We have already shown in our previous studies that *ex vivo* lung tissue explant model can suitably be used to demonstrate airway inflammation [15]. In addition to this, the use of *ex vivo* human lung tissue can provide more relevant models in these kinds of study than using *in vivo* animal model or *in vitro* single cell stimulation [285]. For example, other studies have also shown an up regulation of inflammatory cytokines from human lung tissue explants stimulated with cigarette smoke extracts [286]. Hence, the use of *ex vivo* lung tissue in this study may mimic real inflammatory effects of biomass smoke exposure in human airway/lungs.

5.2 **Aims**

The overall aim of this current chapter was to elucidate the inflammatory profiles produced in *ex vivo* human lung tissue in response to short-term exposure of biomass smoke extracts.

5.2.1 Specific aims

- Assess the range of pro-inflammatory mediators that are released from cultured *ex vivo* human lung tissue following stimulation with biomass smoke extracts prepared from traditional cooking stoves.
- Identify the potential changes in effects seen with the samples collected from different geographical regions.
- Investigate the effects of using improved low emission cooking stoves on inflammatory responses from cultured human lung tissue.
- Investigate the effects of using other fuels than biomass on pro-inflammatory signatures produced from *ex vivo* human lung tissue.

5.3 **Methods**

5.3.1 Human parenchymal lung tissue

A total of 20 donor human parenchyma lung tissue samples collected from Nottingham Health Science Biobank, Papworth Hospital Tissue Bank and Arden Tissue Bank were used in this work. The ethical approval to use and process these human tissues for the research work was obtained before the commencement of the work from each biobank (refer section 2.7.1 for ethics reference numbers). The patient demographic information and the lung function test results of each donor which was conducted before a surgical operation is presented in Appendix II. In brief, the tissue samples were obtained from both male and female donors, and we used samples from 12 male donors and 7 female donors. The gender of one donor was unknown. The mean age of all donors was 64.7±10.4 years. The smoking history among the patient donors showed that the majority of donors, 14 out of 20, were ex-smokers (quit smoking≥ 5 years), 2 individuals were current smokers, and other 4 individuals were never smokers. The lung function test of those donors showed that the FEV_1 % predicted varied between 26.6% and 121.4 %.

5.3.2 Smoke sample collection

All cook stove exhaust samples used for this experiment were collected in a rural household in different regions of Nepal. The samples were collected in households using biomass fuel combusted in both TCS and ICS, and also in households using other fuels, including LPG. The samples were collected throughout the cooking period in each household. The sampling unit was started when the cooking process commenced and stopped when the cooking process completed. The sampling unit consists of a pump (set flow rate at 3 lpm) and impingers set connected in such a way that smoke exhaust passes through the solution placed in the impingers set when it is turned on. Cell and tissue culture media (DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich) was used as a solution to collect the sample, and the volume used was 10 ml. The sampling unit, previously assembled with adjusted pump flow rate, was placed in the kitchen before the commencement of the fire for cooking. The pump started when the fire was lit and pulled the smoke produced from the combustion of fuel and channelled it through the media continuously throughout the cooking period. The pump was stopped at the end of the cooking process, and the collected sample was immediately transferred to a 15 ml Eppendorf tube. The sample was then tightly sealed and immediately frozen down using a temporary field freezer maintaining the temperature below -10°C. After collection of all samples from each site, samples were transferred to the laboratory freezer (-20°C) at the International Centre for Integrated Mountain Development

(ICIMOD) and stored there prior to transfer to the laboratory at the University of Nottingham. All frozen samples from all sites were transferred to the laboratory by keeping them in a thermos flask filled with ice. This transfer method in a flask with ice was checked with dummy samples before the actual sample transfer. The samples remained frozen for more than 24 hours, which is greater than the maximum number of hours (~20 hours) it took to transfer samples from ICIMOD to Nottingham, UK. The sample collection process is presented in Figure 5.1.

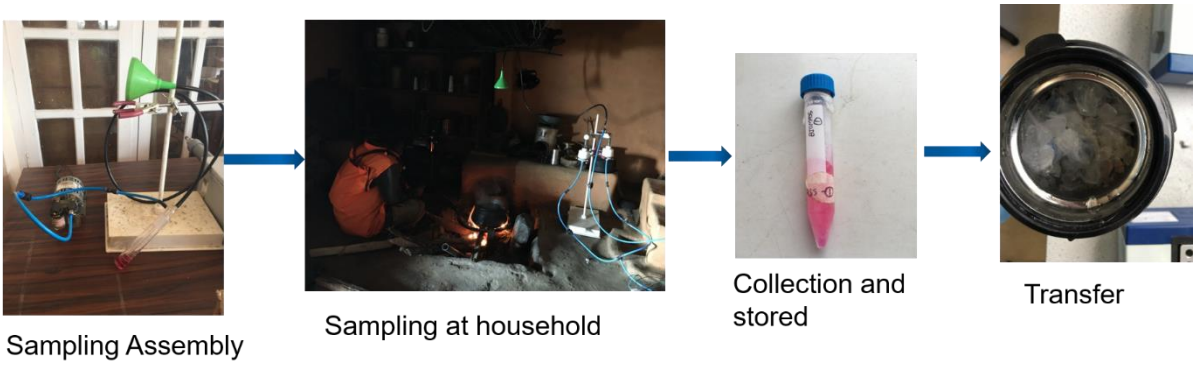

Figure 5.1 Smoke sample collection, storage and transfer process.

During the sample collection in the field, care was taken in order to minimize contamination of the samples. The media to collect the samples was taken from a fresh unopened media bottle (500 ml) in each monitoring site. Any remaining unused media from each site was discarded and was not used for other sites. Also, the media temperature was maintained at 4°C (supplier recommended temperature) during transfer as well as during the sampling. The sampling unit and the pump flow rate was checked and maintained each time before using the unit. The impinger used in each household was first washed with pure water

and then with fresh media before it was used in another household. The Eppendorf used to transfer the samples was sterile.

5.3.3 Lung tissue Culture

The tissue was transferred from the donor biobank in DMEM + 1 X PSF maintaining the temperature at 4°C. Following the protocol established by the group (respiratory department, UoN) for the tissue culture work, a piece of tissue was first taken for pre-culture expression analysis. For this, once the whole tissue weight was taken, two pieces of parenchymal tissue (50-100 mg) were cut, washed in Tyrode's buffer and transferred to separate microfuge tubes. A sufficient volume (1 ml) of RNA later was added to each tube and incubated at 4°C overnight. Following 24 hours of incubation, the tissue was removed from the RNA later and transferred to a new microfuge tube. The tissue sample was then transferred to storage at -80°C for subsequent RNA extraction. Similarly, two pieces of tissue (50-100 mg) were transferred to separate microfuge tubes and submerged in 4% formaldehyde (1 ml volume). The tubes with the tissue were then stored at 4°C for subsequent paraffin embedding and sectioning to allow expression analysis by immunohistochemistry (IHC).

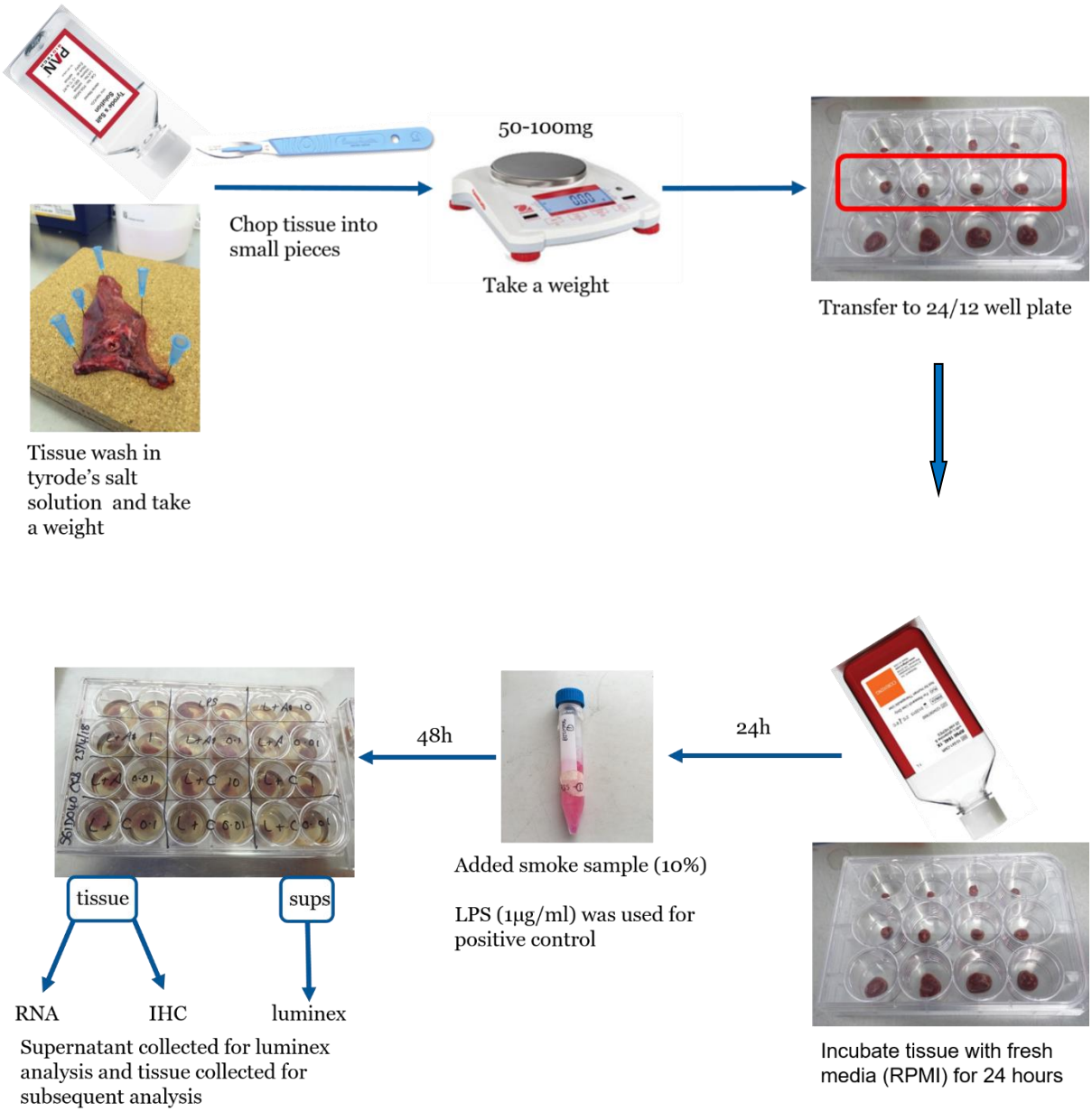

Figure 5.2 Tissue processing and stimulation.

The remaining tissue was used for tissue culture experiments and processed as shown in Figure 5.2. All tissue culture work was performed in a 24-well tissue culture plate. The tissue was dissected into pieces, weighed accurately (tissue weight from 50-100 mg), and transferred into each well of a plate. The tissue samples were then submerged in 2ml RPMI-1640 + 1X PSF in each well and the plate was incubated at 37°C,

5% CO_2 for 24 hours. Following the 24 hour pre-incubation, the spent media was replaced with 2 ml of fresh media in each well. Lipopolysaccharide (LPS) was used as a known stimulus for a pro-inflammatory response and the samples collected from the cooking exhaust were used to detect inflammatory effect of smoke exposures. LPS (1 µg/ml) or smoke sample (10% v/v) was added to the well as per the plate layout design. The plate was then incubated at 37°C, 5% CO_2 for 48 hours. After 48 hours the supernatants from the cultures were removed from each well of the plate and stored as 5 X 200 µl aliquots at -80°C for subsequent analysis by Luminex. The duplicate tissue from each condition was used for further expression analysis; one piece of tissue was stored in RNA later at 4°C for 24 hours and then transferred to -80°C in a separate tube for subsequent total RNA extraction for post-stimulation expression analysis. The other piece of tissue was stored in 4% formaldehyde at 4°C for subsequent paraffin embedding and sectioning to be used for further expression analysis by IHC.

5.3.4 Pro-inflammatory mediator quantification

The isolated tissue culture supernatants following 48 hours of incubation were analysed using a custom Magnetic Luminex Screening assay, Human Premixed Multi Analyte kit (R&D systems) to detect the presence of various cytokines, chemokines and other factors. The working principle of Luminex assay is described in section 2.8.2. A Luminex plate was customized with the pre-selected panel of analytes including cytokines,

chemokines and other factors. The list of analytes measured and assay ranges are presented in appendix III. The initial results from the plate showed that some analytes such as IL-8 and IL-6 concentrations were higher than the detection level of the assay. However, the concentration of some other analytes was in the range of the detection limit of the assay. Hence, the samples needed to be diluted to detect the levels of IL-8 and IL-6, whereas for the other analytes to be detected, samples needed to be used in neat. Hence we processed the supernatant from the same experiment in two different plates including IL-8 and IL-6 in one plate and other analytes in the second plate. The dilution factor used for the detection of IL-8 and IL-6 was 1000 fold, and the final result was calculated multiplying the recorded value by the dilution factor.

The assay was conducted in 96-well microplates provided by the kit supplier. All samples and standard were assayed in duplicate. In brief, 50 µl of diluted micro particle cocktail was added to each well of the plate followed by adding 50 µl of sample and standard as per the plate layout. Plates were sealed and incubated for 2 hours at room temperature on a plate shaker at 800 rpm. Following 2 hours incubation, the plate was washed three times with wash buffer using a microplate magnet (Bio-Plex handheld Magnetic Washer, Bio-Pad). After completion of washes, 50 µl of diluted Biotin Antibody Cocktail was added to each well. The plate was then sealed and incubated on the plate shaker at 800 rpm for 1 hour at room temperature. The plate wash was repeated three times with 100 µl of wash buffer and then 50 µl of diluted Streptavidin-PE was added to

each well. The plate was incubated for 30 minutes at room temperature on the plate shaker at 800 rpm. Following a final three washes, micro particles were resuspended by adding 100 µl of wash buffer to each well. The plate was sealed and incubated for 2 minutes, and then the plate was read using the Bio-Rad Bio-Plex analyser with 50 events per bead.

The data in each well were normalized with the wet tissue weight for each plate, and the level of analytes measured in duplicate wells for each condition was averaged. In all Luminex plates, two samples from each condition were assayed, and the mean value of those two samples was used for further analysis.

5.4 **Results**

5.4.1 Inflammatory response to LPS

The pro-inflammatory cytokine profile release from *ex vivo* lung tissue following LPS stimulation was first analysed (Figure 5.3). All 10 inflammatory analytes including cytokines, chemokines and other growth factors assayed in a multiplex Luminex assay generated quantifiable signals across all donors following LPS stimulation. The fold increase in the induced level of all analytes was significant ($p<0.05$ for all) over the baseline (unstimulated) condition. There was obvious variation in the absolute value of cytokines across different donors due to the variation seen in each donor samples. However, reasonably reproducible fold

stimulations across all donor samples for all analytes were observed. The mean fold increase was 7.56 fold for IL-8, 5.86 fold for IL-6, 196.8 fold for TNF-α, 49.45 fold for IL-1β, 4.62 fold for MCP-4, 12.93 fold for MIP-1α, 6.8 fold for MCP-1, 13.6 fold for GM-CSF.

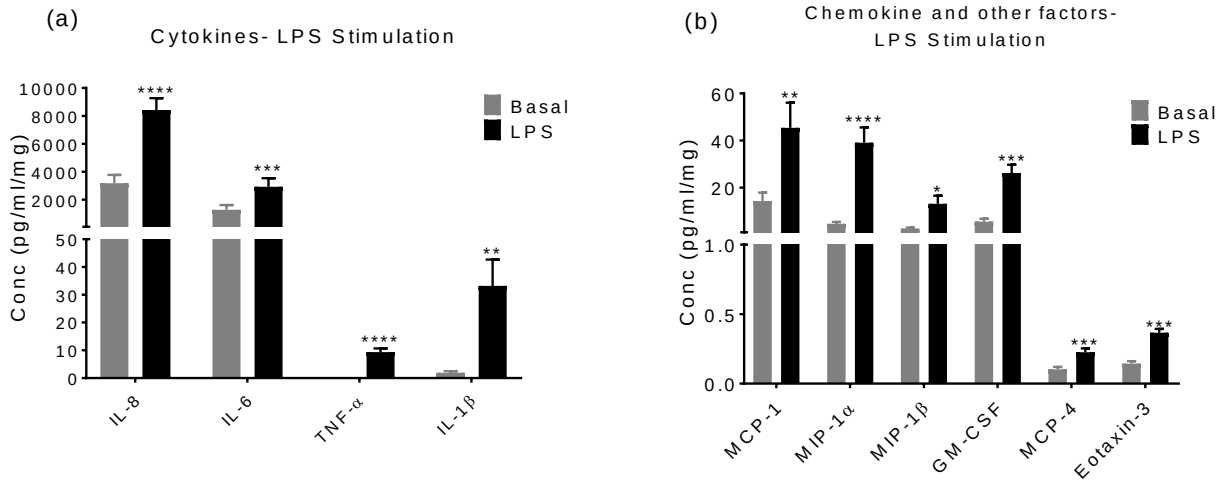

Figure 5.3. **The inflammatory profile of *ex vivo* human lung tissue following LPS stimulation.** LPS (1 μg/ml) stimulation showed an effect on cultured human tissue explants by inducing the release of all 10analytes assayed including (a) cytokines (IL-8, IL-6, TNF-α and IL-1β) and (b) chemokines and other factors (MCP-1, MIP-1α, MIP-1β, GM-CSF, MCP-4 and Eotaxin-3). Data were normalized using tissue mass for each analyte. The data shown are the overall mean (± SEM) of n=20 donors. Elevated levels between LPS treated and untreated basal condition were checked using Wilcoxon matched paired test (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001). IL: Interleukin, TNF-α: Tumor Necrosis factor-alpha, MIP: Macrophage inflammatory proteins, GM-CSF: Granulocyte-macrophage colony-stimulating Factor, MCP: Monocytes chemoattractant protein.

The result observed in this work was in line with our previously published data, where 11 of the 21 inflammatory analytes assayed were significantly elevated following LPS stimulation [15]. These results show that tissue was viable and able to respond to a standardised stimulus by producing a range of pro-inflammatory cytokines. I also undertook an analysis to see whether or not the response to LPS was related to underlying lung function. For this, I also used the data from similar experiments performed by other group members (unpublished data) to increase the donor number. The results from this analysis showed that the responses seen with the donor samples were independent of the lung function and age (Figure 5.4 and Figure 5.5). The linear regression analysis was performed between FEV_1/FVC values, FEV_1 % predicted value and age with responses seen for 4 analytes. There was no significant correlation between any variables tested with any of the analytes measured.

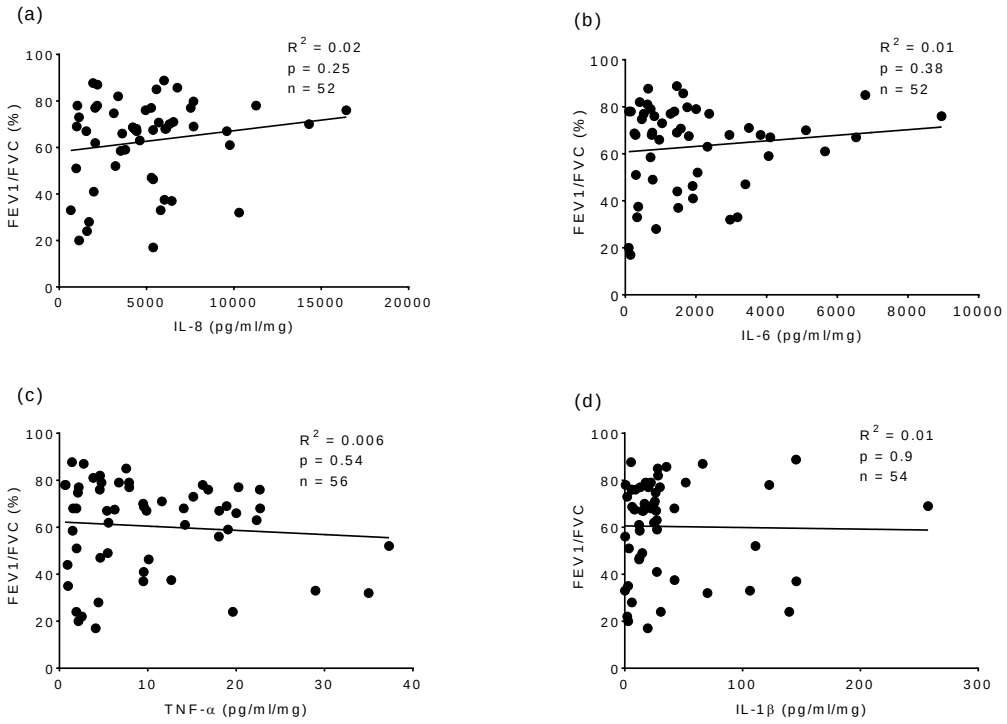

Figure 5.4 Lack of correlation between induced cytokine levels in tissue culture supernatant following LPS stimulation and correspondingpatient lung function (FEV_1/FVC). Induced cytokine levels for (a) IL-8, (b) IL-6, (c) TNF-α and (d) IL-1β were independend of patients lung function. Data are from lung tissue donors where n represents an individual donor for each analyte.

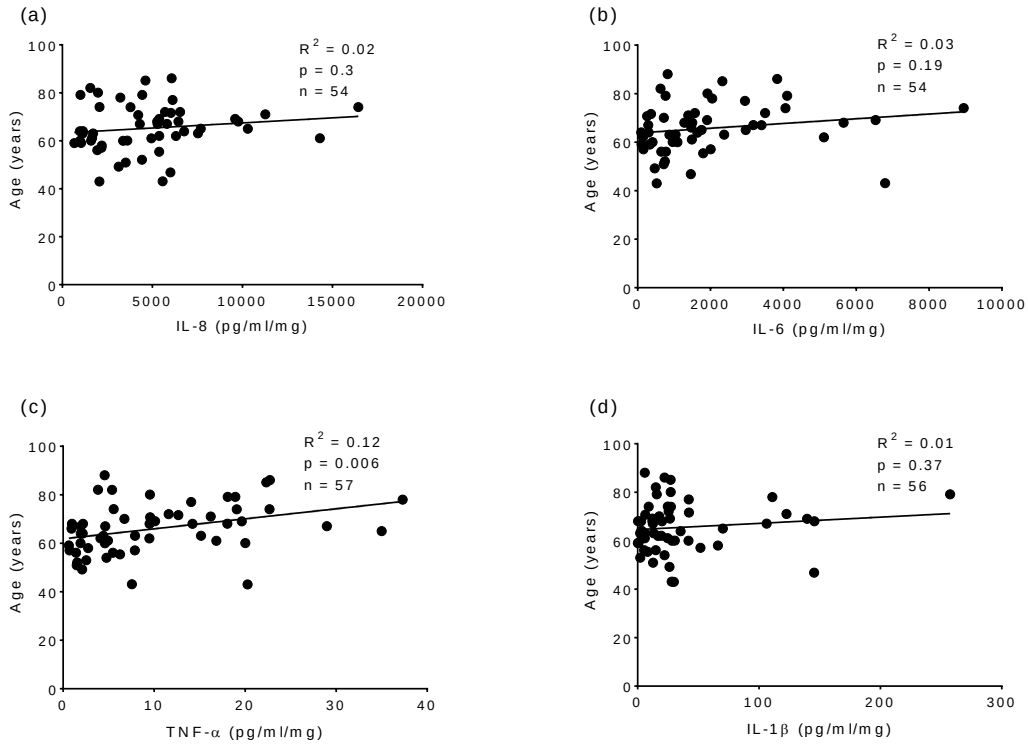

Figure 5.5 Lack of significant correlation between induced cytokine levels in tissue culture supernatant following LPS stimulation andcorresponding patient age (years). The donor sample age was not related to the induced cytokine levels for (a) IL-8, (b) IL-6, (c) TNF-α, and (d) IL-1β. Data are from lung tissue donors where n=individual donor for each analyte.

5.4.2 Inflammatory Response to biomass smoke extract

Having established the secretory profile of *ex vivo* human lung tissue following LPS stimulation I then investigated the effect of biomass smoke extracts on the tissues. The tissues were stimulated with the biomass smoke sample, and the levels of pro-inflammatory cytokines including IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α, MCP-4, MIP-1β, GM-CSF and Eotaxin-3 in isolated supernatants from the culture were measured. The

supernatants were collected after 48 hours and cytokine levels were measured using Luminex assay (for method see section 2.8.2).

Elevated levels of pro-inflammatory cytokines were detected, which were reproducible for most (8 of the 10) of the analytes assayed. The levels of MIP-1β and Eotaxin-3 were below the lower detection limit of the Luminex assay for most of the donors (>3 donors) and hence not included in the further analyses. The secretory levels of all 8 analytes are shown in Figure 5.6. The data in the figure represents the mean level of analytes from 5 different donors stimulated with the samples from four different regions.

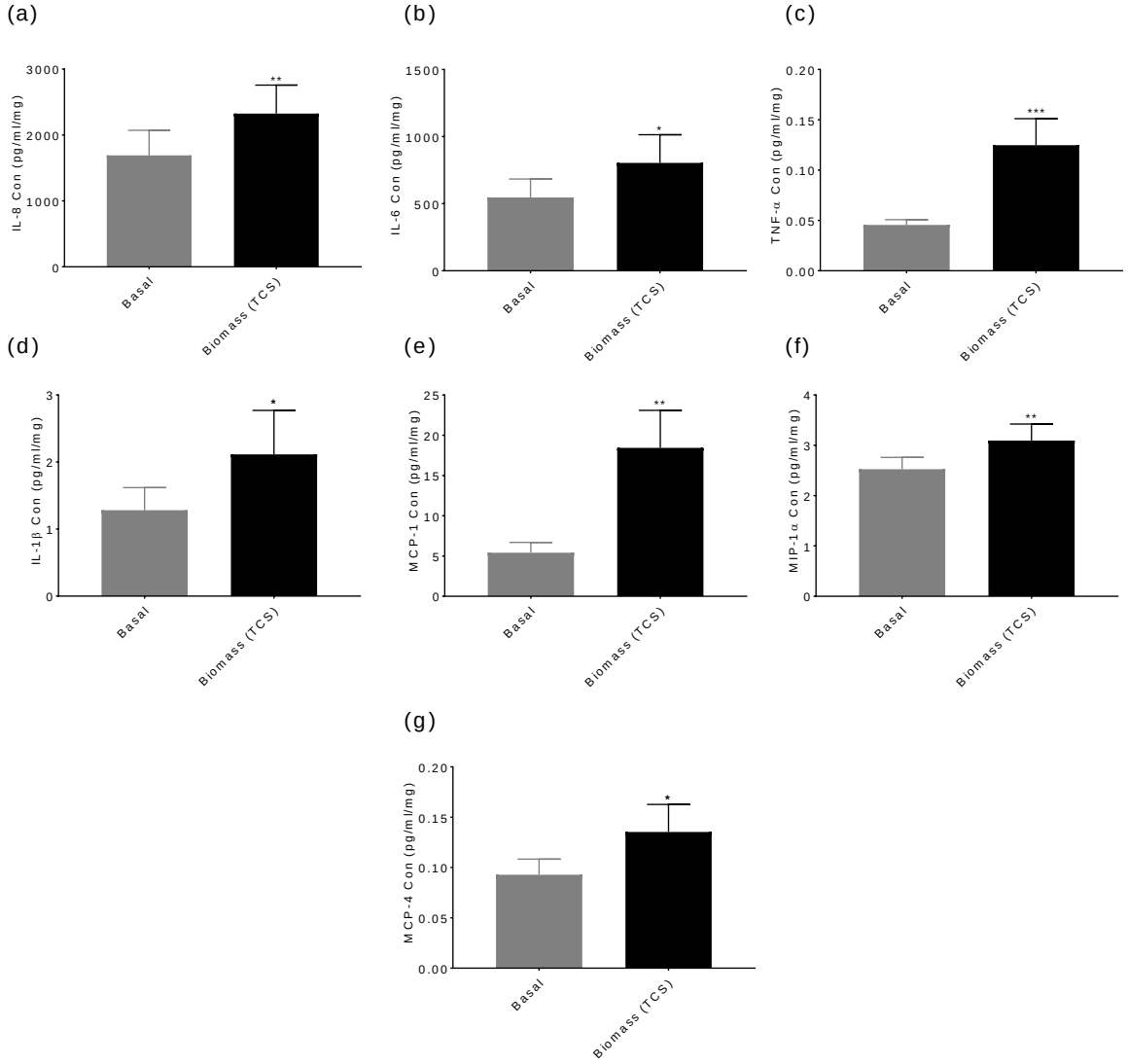

Figure 5.6 The inflammatory profile of *ex vivo* human lung tissue following Biomass stimulation. Biomass smoke extract collected in TCS induced production of pro-inflammatory cytokines including (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MCP-1, (f) MIP-1α, and (g) MCP-4. Data were normalized using tissue mass for each analyte. Data shown are the overall mean (± SEM) of 20 data points (n=5 donors, each donor stimulated with 4 different samples from four different sites). Elevated levels between smoke extract treated and untreated basal condition were checked using Wilcoxon matched paired test (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)

The levels of 7 analytes, except GM-CSF, were significantly higher than the baseline levels (unstimulated). The fold increase under the baseline conditions with these donors is shown in Table 5.1.

The relative levels of cytokine responses seen with LPS and Biomass smoke stimulation varied for each analyte. For example, whilst the fold stimulation for IL-8 was 7.56 with LPS and 1.89 with biomass (i.e. 25% of the LPS response), it was 196.8 fold for TNF-α with LPS and 2.97 fold for biomass (1.5% of the LPS response). The differences seen are presented in Table 5.1.

Table 5.1 Biomass smoke response in *ex vivo* human lung tissue as a percentage of LPS response. The data in the table shows the mean (SEM) fold increase for each analyte following LPS and biomass stimulation (n=5 independent donors).

Analytes	**IL-8**	**IL-6**	**TNF-α**	**IL-1β**	**MCP-1**	**MIP-1α**	**MCP-4**
LPS stimulation fold change	7.56 (2.05)	5.86 (1.73)	196.8 (65.94)	49.45 (9.2)	6.8 (2.03)	12.93 (3.35)	4.62 (1.56)
Biomass stimulation fold change	1.89 (0.21)	2.15 (0.35)	2.97 (0.36)	1.88 (0.35)	2.9 (0.57)	1.23 (0.05)	1.47 (0.14)
% of LPS response	**25**	**36.7**	**1.5**	**3.8**	**42.6**	**9.5**	**31.8**

5.4.3 Inflammatory response to site-specific biomass smoke extracts

The inflammatory response to *ex vivo* human lung tissue was measured with the smoke extracts collected from four different regions of rural

Nepal. By using smoke extract samples from all sites, I demonstrated that the biomass smoke extracts induced inflammatory cytokines in the lung tissue samples. I then investigated whether or not the responses seen varied by the sites. For this, the levels of all 7 analytes were checked with the samples from each site.

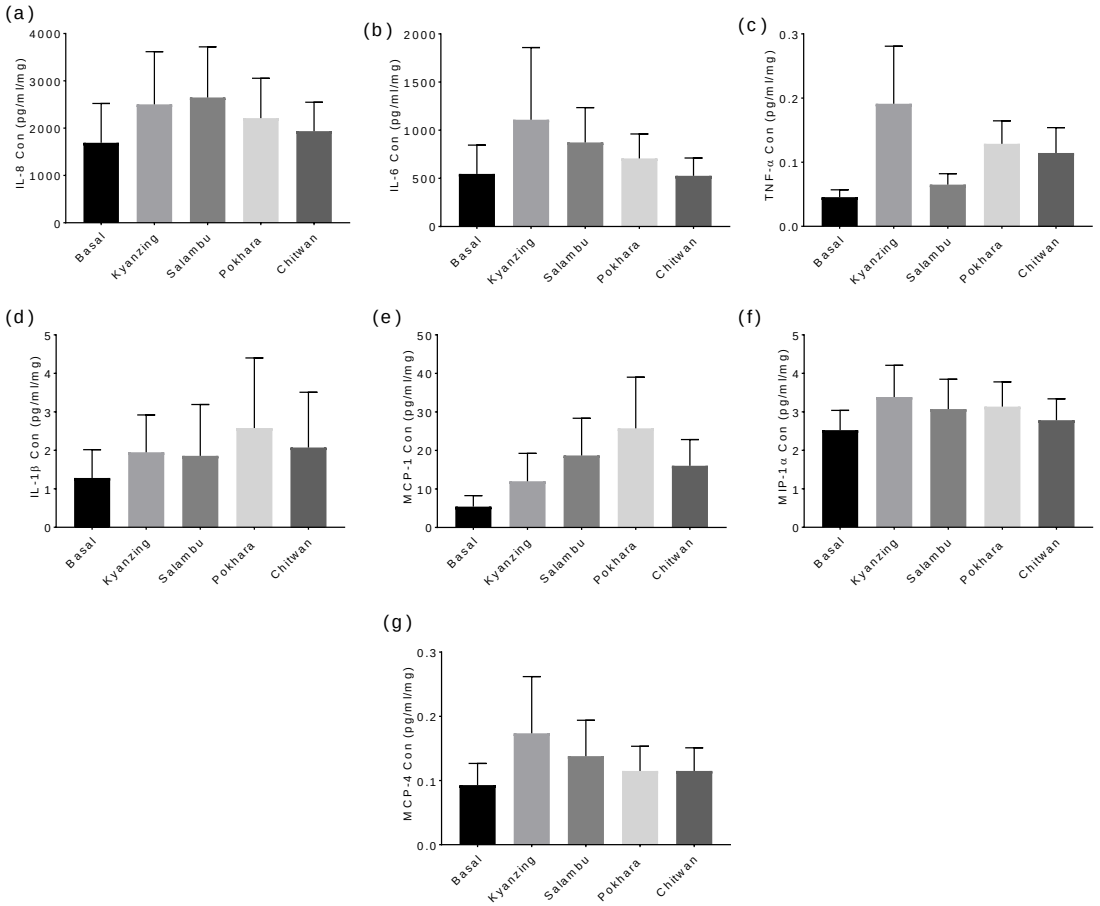

Figure 5.7 Biomass smoke extract collected from different sites showed a similar response for each of the analytes. A quantifiable response for all 7 analytes was measured with samples from each site and remained quantitatively similar across all sites. The analytes are (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MCP-1, (f) MIP-1α, and (g) MCP-4. The data represents mean (± SEM) elevation of each analyte for n=5 donors. Variations were checked using the Kruskal-Wallis test followed by Dunn's multiple comparison test (none of the variables tested was significant).

The inflammatory profile observed in lung tissue stimulated with site specific biomass smoke sample remained quantitatively similar across all sites (Figure 5.7). A quantifiable increase in levels of all 7 cytokines was observed with the samples from all sites. Probably, due to inherent variability in responses across different donor samples and variability in exposure among the households, the mean fold increase for each cytokine appeared to vary to some extent across different sites (Table 5.2). However, the variations seen for each of the cytokine response across sites were not statistically significant.

Table 5.2 Inflammatory profiles fold stimulation across four different sites. The data represents the mean (SEM) fold increase for each of the analytes from n=5 independent donor experiments for each site.

Site/Cytokine	IL-8	IL-6	TNF-α	IL-1β	MCP-1	MIP-1α	MCP-4
Langtang	1.88 (0.49)	2.13 (0.54)	2.52 (1.15)	2.45 (1.37)	3.26 (1.75)	1.32 (0.14)	1.59 (0.22)
Salambu	1.88 (0.23)	2.15 (0.45)	2.59 (0.85)	1.29 (0.21)	2.3 (0.17)	1.2 (0.1)	1.61 (0.45)
Pokhara	1.74 (0.24)	1.98 (0.38)	3.07 (0.42)	1.78 (0.24)	3.05 (1.13)	1.26 (0.08)	1.3 (0.24)
Chitwan	2.1 (0.69)	2.34 (1.31)	3.1 (0.87)	2.02 (0.51)	2.76 (1.07)	1.15 (0.15)	1.38 (0.26)
p value	0.86	0.91	0.94	0.52	0.96	0.7	0.62

5.4.4 Inflammatory response with samples from ICS

The increased levels of inflammatory cytokines in *ex vivo* lung tissue following biomass smoke stimulation reported above were all from the samples collected from biomass combustion using TCS. I have already

demonstrated (in chapter 4) that use of a more efficient stove reduced PM exposure by ~70%. Hence I investigated the effect of using ICS on pro-inflammatory responses by stimulating *ex vivo* human lung tissue samples with the smoke extract samples collected with ICS.

For this, the smoke extracts samples collected in households using ICS from two monitoring sites were used for lung tissue stimulation. The levels of cytokines that were significantly elevated than the levels seen in unstimulated basal condition are presented in Figure 5.8. Similar to the responses seen with extracts with TCS, the levels of 8 of the 10 cytokines were reasonably reproducible across all donors. However, the type of cytokine response and fold change for each cytokine varied between the two extracts samples. The levels of MCP-4 were below the lower detection limit of the assay for >3 donors for ICS sample whilst the levels were significantly higher for TCS sample. Similarly, the elevated levels of Eotaxin-3 (1.17 fold, p=0.01) were significant for ICS samples while for TCS samples, the levels were below the detection limit of the assay. In both samples, MIP-1β were not included as the levels were not in a detectable range of lower limit of the assay. The elevated levels of IL-8 (1.3 fold, p=0.05), IL-6 (1.8 fold, p=0.01), TNF-α (4.7 fold, p=0.01), IL-1β (2.3 fold, p=0.01) and MIP-1α (1.82 fold, p=0.01) were significant than the levels seen in basal which is similar to the response seen for TCS sample. Unlike the TCS sample, however, there was a significant elevation of GM-CSF (1.17 fold, p=0.01) for ICS sample and not significant for MCP-1 (1.45 fold, p=0.09). Overall, human lung tissue

sample responded and secreted different pro-inflammatory cytokines in response to smoke extracts samples generated from both TCS and ICS stove designs.

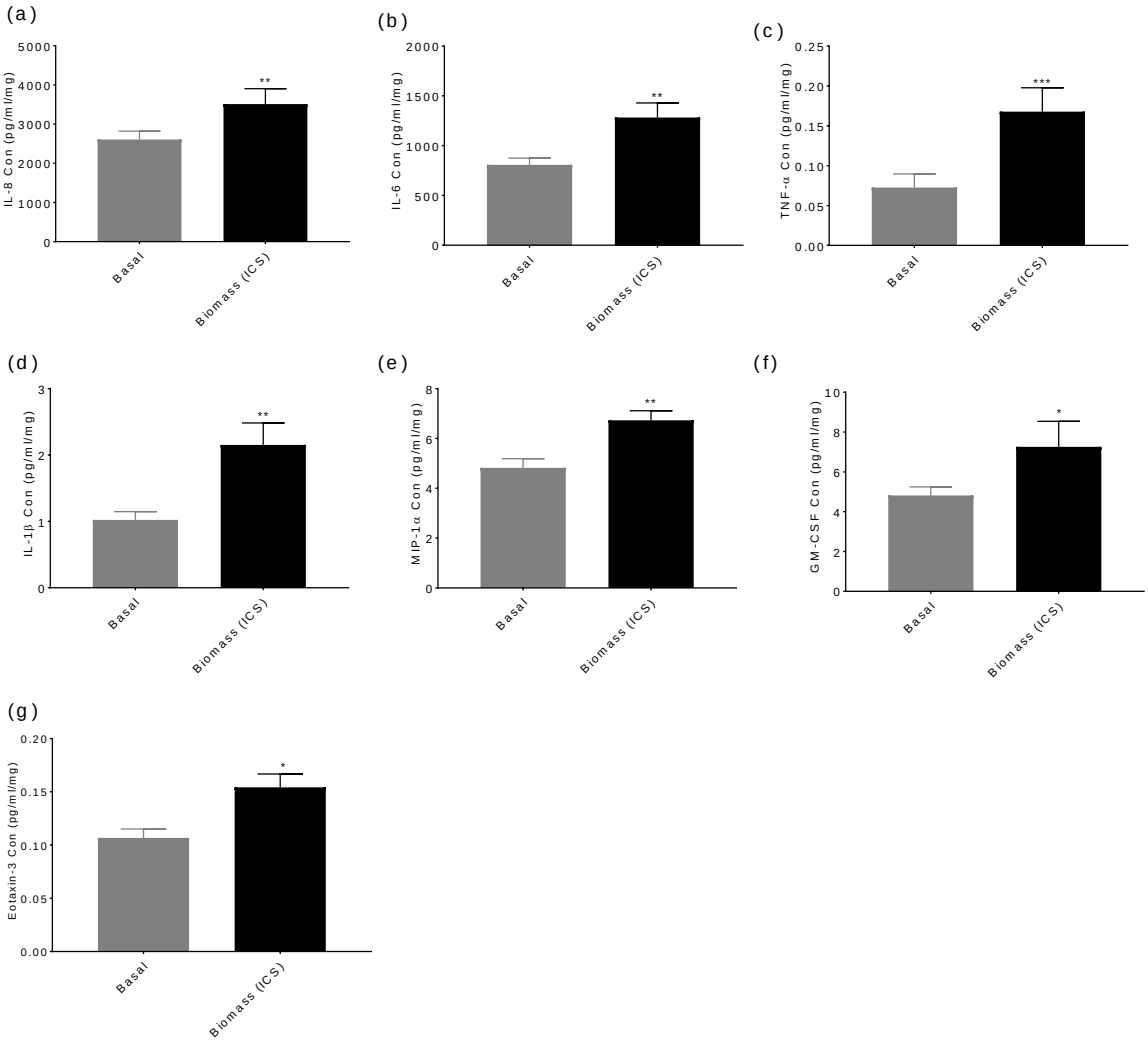

Figure 5.8 The inflammatory profile of *ex vivo* human lung tissue following biomass smoke extract collected in ICS. Biomass smoke extract in ICS induced production of pro-inflammatory cytokines including (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MIP-1α, (f) GM-CSF, and (g) Eotaxin-3. Data shown are the overall mean (±SEM) of 20 data points (n=5 donors, each donor stimulated with 4 different samples from 2 monitoring sites). Elevated levels between smoke extract treated and untreated basal condition were checked using Wilcoxon matched paired test (*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001).

5.4.5 Inflammatory response to site-specific biomass smoke extracts from ICS

The pro-inflammatory secretory profile with the samples from biomass combustion using ICS was conducted with the samples collected from two different monitoring sites. Despite the significant reduction of exposure using ICS, the inflammatory effect of biomass smoke extracts on human lung tissue remained similar to that seen with TCS samples. I then investigated whether or not the responses seen varied by different sites and for this, I checked the 7 cytokine levels providing significantly elevated responses with the samples from each site.

As expected, quantifiable increased levels of all 7 cytokines were observed with the samples from both sites. The mean fold stimulation with the samples from two sites varied to some extends (Figure 5.9). The fold increase of IL-8, IL-6, MIP-1α, GM-CSF and Eotaxin-3 remained similar for both sites while the samples from Salambu showed higher fold stimulation of TNF-α and IL-1β than the samples from Langtang. However, the differences seen across sites were not statistically significant.

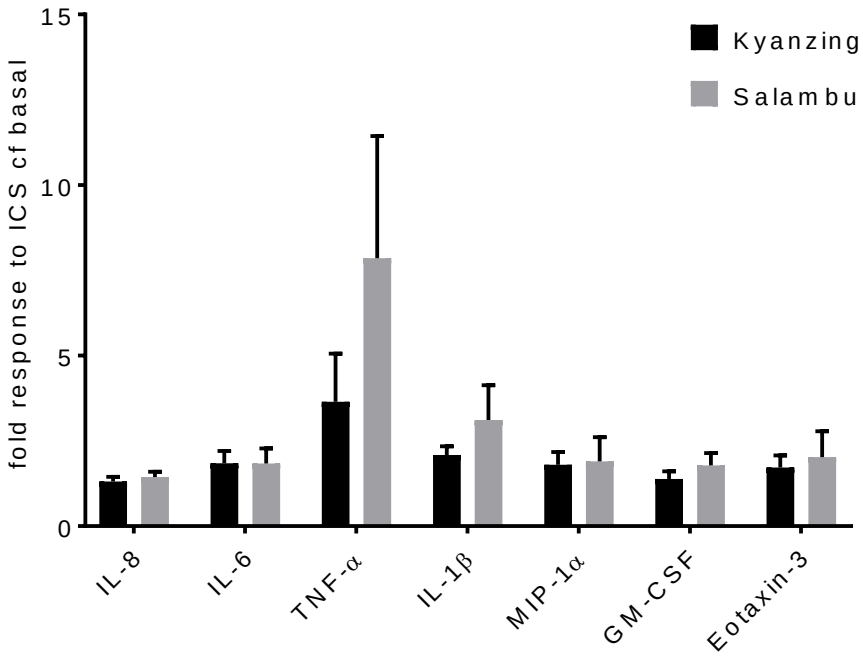

Figure 5.9 Comparison of elevated levels of analytes with ICS samples from two monitoring sites. The data represents mean (±SEM) fold stimulation over the unstimulated basal condition for each analyte from n=5 independent donor experiments. None of the analytes showed significant variation between two sites (Kruskal-Wallis test followed by Dunn's multiple comparison test).

5.4.6 Inflammatory response to cook stove exhaust from clean fuel

Having established the inflammatory profile seen in a human lung following exposure to biomass smoke extracts produced during cooking, I was interested to see if there were any potential benefits of using clean fuel on these responses. For this, I collected samples from two sites in households using LPG as cooking fuel. The samples were used to

stimulate *ex vivo* lung tissue donor samples and pro-inflammatory cytokine release was measured as before.

Though LPG is considered a clean fuel compared to biomass, there was still significant production of cytokines following stimulation with the samples generated from the cooking using LPG. However, only 3 cytokines showed significant fold stimulation over the unstimulated basal condition. The cytokines with the significant fold increase were IL-8 (1.47 fold, $p<0.05$), TNF-α (6.3 fold, $p<0.05$) and IL-1β (4 fold, $p<0.01$) (Figure 5.10).

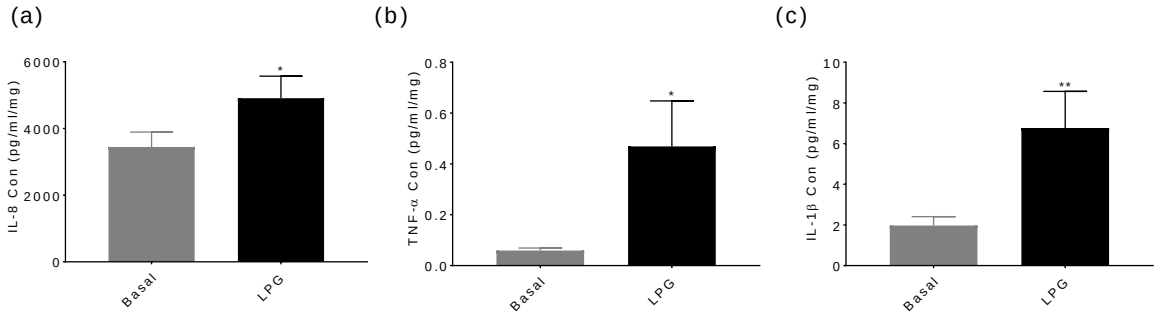

Figure 5.10 The inflammatory profile of *ex vivo* human lung tissue following stimulation with smoke extract samples generated from LPG. LPG smoke extract induced production of pro-inflammatory cytokines including (a) IL-8, (b) TNF-α and (c) IL-1β. Data were normalized using tissue mass for each analyte. Data shown are the overall mean (± SEM) of 12 data points (n=6 donors stimulated with each sample from 2 monitoring sites. Elevated levels between smoke extract treated and untreated basal condition were checked using Wilcoxon matched paired test (*=$p<0.05$, **=$p<0.01$).

I also investigated for these three cytokines to see if the responses seen were different between the sites. Quantifiable increased levels of all 3 cytokines were observed with the samples from both sites Figure 5.11. However, some variation in mean fold stimulation was observed among the samples. The mean fold stimulation of IL-8 remained similar for both sites while the sample from Chitwan showed higher fold stimulation of TNF-α (10.5 fold cf 2 fold) and IL-1β (6 fold cf 1.9 fold).

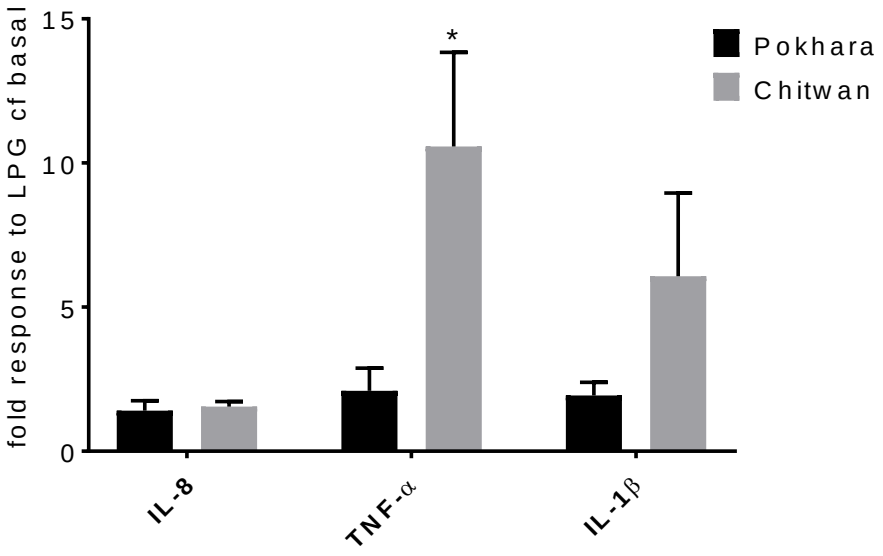

Figure 5.11 Comparison of elevated levels of analytes with LPG samples from two monitoring sites. The data represents mean (±SEM) fold stimulation over the unstimulated basal condition for each analyte from n=6 independent donor experiments. Significant variation in fold stimulation between sites was seen only for TNF-α (*=$p<0.05$). (Kruskal-Wallis test followed by Dunn's multiple comparison test).

5.4.7 Inflammatory response to an ambient air sample

Ambient air can also contribute to HAP. I, therefore, measured ambient $PM_{2.5}$ concentration at the time of field visits in all four sites. At the same time, I also collected ambient air sample in the same way indoor biomass smoke samples were collected using the same equipment and methods. Ambient air samples from each of the 4 sites were collected twice at two different time points. The collection unit was placed in a central location in the village and the sample was collected between 12 and 2 pm. This collection time was selected because during this period the use of biomass fuel for cooking in households was minimum and therefore, the direct contribution of HAP to ambient air was minimum.

These ambient air samples were used to stimulate lung tissue, and as expected, the ambient air sample did not produce much response. However, quantifiable levels of TNF-α and IL-1β were observed with the air samples from Chitwan and Pokhara (Figure 5.12), although the fold increase over basal conditions for these cytokine levels was not statistically significant.

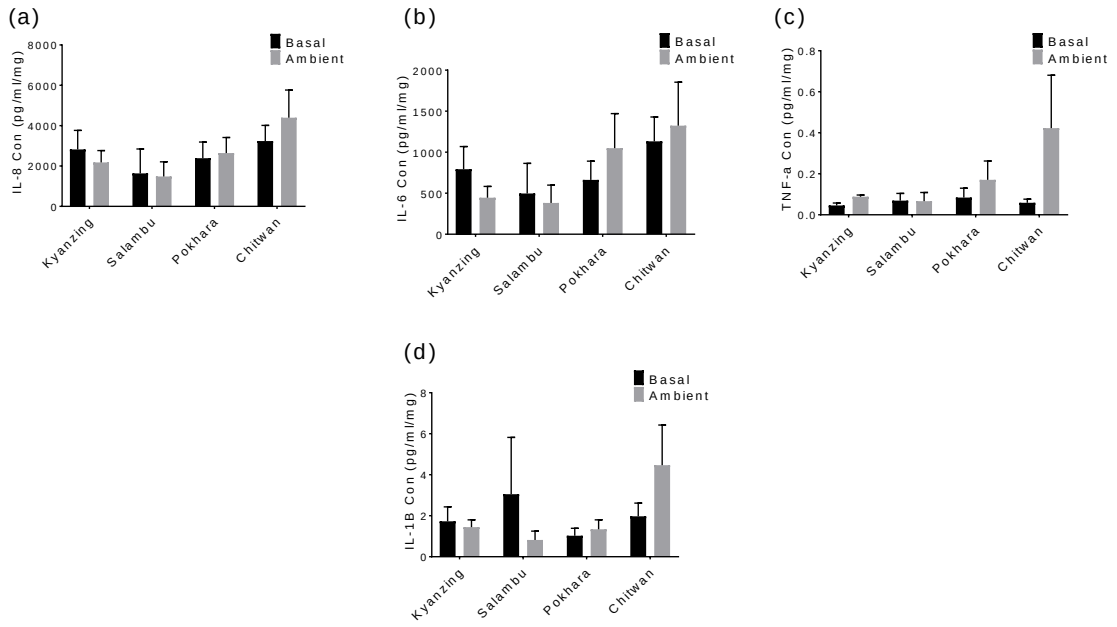

Figure 5.12 Inflammatory response seen in human lung tissue stimulated from ambient air samples. Data are shown for (a) IL-8, (b) IL-6, (c) TNF-α and (d) IL-1β as the concentration of other analytes were not higher than the levels seen in basal condition at any site. The data represents mean (±SEM) concentration of n=5 for Kyanzing and Chitwan samples and n=4 for samples from the other two sites.

5.4.8 Inflammatory profile from different sources

Finally, the inflammatory responses seen in each monitoring site with the samples from different sources were compared. Among the cytokines assayed, only four cytokines (IL-8, IL-6, TNF-α and IL-1β) showed levels detectable by the Luminex assay for all the samples used. Hence, I considered only these cytokines for the analyses. The fold stimulation over the basal condition of each the 4 cytokines for the sample used in each monitoring site is presented in Table 5.3.

Among all the sites, the fold increase of cytokines IL-8 and IL-6 were higher following stimulation with the biomass smoke samples generated from TCS than from the other sources. In Salambu, the levels of TNF-α and IL-1β were higher with the samples from ICS than from the TCS. Similarly, the levels of TNF-α and IL-1β with LPG sample from Chitwan were higher than the level seen with TCS samples. However, it should be noted that no correction for multiple testing has been used in these comparisons and hence some apparently significant differences may be false positives.

Table 5.3 Inflammatory cytokine fold stimulation among various sources in each of the four sites. The data represents the mean (SEM) fold increase over basal (unstimulated) condition. The data are from n=5 independent donor experiments for all samples from all sites except n=4 for ambient samples from Salambu and Pokhara. TCS: Traditional Cook stove, ICS: Improved Cook stove.

		Kyanzing		
	TCS	ICS	Ambient	p-value
IL-8	1.88 (0.49)	1.3 (0.13)	0.87 (0.42)	0.08
IL-6	2.13 (0.54)	1.84 (0.35)	0.63 (0.13)	0.01
TNF-α	2.52 (1.15)	3.64 (1.4)	3.3 (1.3)	0.19
IL-1β	2.45 (1.37)	2.08 (0.25)	1.4 (0.54)	0.06
		Salambu		
IL-8	1.88 (0.23)	1.43 (0.15)	1.4 (0.38)	0.03
IL-6	2.15 (0.45)	1.83 (0.44)	1.35 (0.5)	0.18
TNF-α	2.59 (0.85)	7.85 (3.58)	0.84 (0.13)	0.08
IL-1β	1.29 (0.21)	3.11 (1.02)	1.26 (0.96)	0.11
		Pokhara		
	TCS	LPG	Ambient	p-value
IL-8	1.74 (0.24)	1.4 (0.34)	1.12 (0.21)	0.18
IL-6	1.98 (0.38)	1.84 (0.73)	1.58 (0.3)	0.45
TNF-α	3.07 (0.42)	2.09 (0.29)	1.86 (0.33)	0.07
IL-1β	1.78 (0.24)	1.93 (0.45)	1.08 (0.48)	0.19
		Chitwan		
IL-8	2.1 (0.69)	1.55 (0.17)	1.46 (0.56)	0.35
IL-6	2.34 (1.31)	1.48 (0.2)	1.86 (1.16)	0.68
TNF-α	3.1 (0.87)	10.57 (3.26)	4.49 (2.43)	0.02
IL-1β	2.02 (0.51)	6.07 (2.88)	1.4 (0.54)	0.14

5.4.9 Cytokine profile correlation with $PM_{2.5}$ concentration

In order to help identify any association of exposure to particulate matter and the inflammatory profile seen in *ex vivo* human lung tissue, I performed linear regression analysis of $PM_{2.5}$ concentration and levels of

cytokines measured using data from each cooking period and matched samples. For this, mean fold increase of each of the cytokine measured from the tissue donors were calculated and compared with the $PM_{2.5}$ concentration observed. For all the cytokines assayed, no significant correlation between the two variables was observed. The linear regression R^2 value was generally low ranging from 0.004 to 0.07.

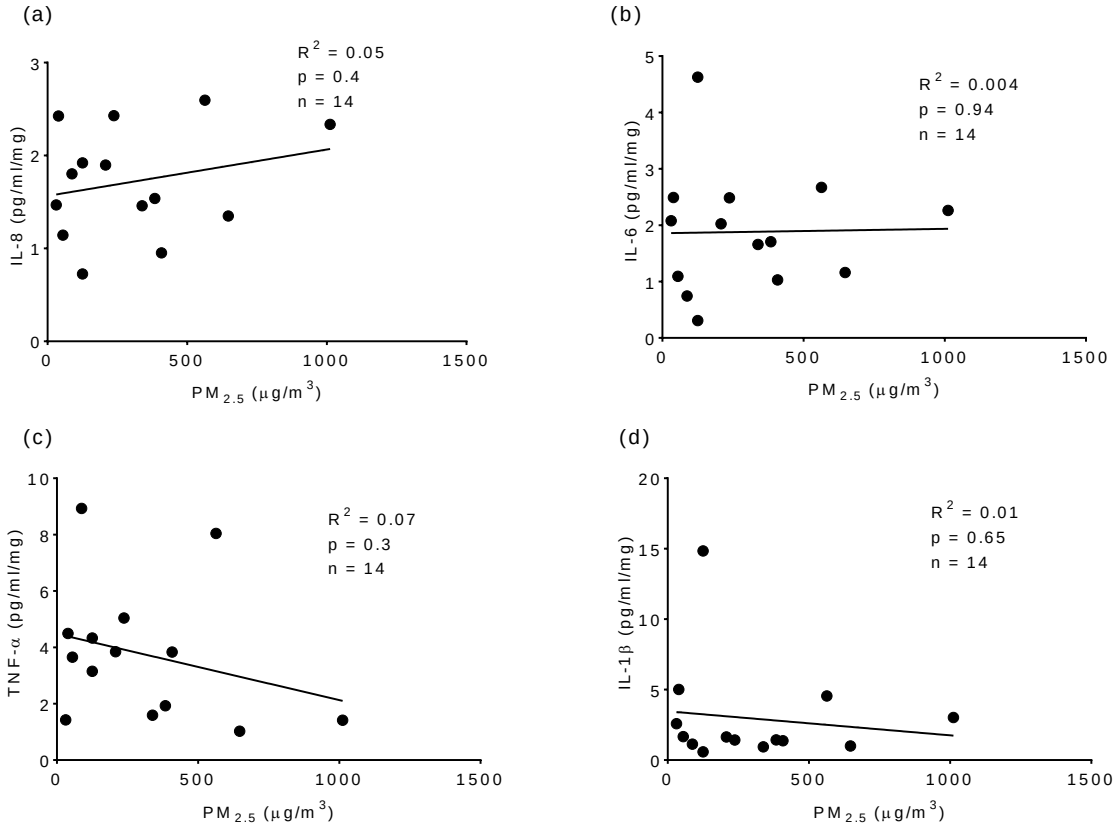

Figure 5.13 Lack ofcorrelation between the concentration of $PM_{2.5}$ exposure and the fold stimulation of each of the analytes. The cytokine expression is not significantly related to the concentration of the PM. The linear regression R^2 value generated from n= 14 $PM_{2.5}$ levels with the corresponding levels of (a) IL-8, (b) IL-6, (c) TNF-α and (d) IL-1β were analyzed.

5.4.10 Inflammatory profile from extracted PM from filter sample

In order to further explore the mechanism underlying the inflammatory effect of PM from biomass combustion during cooking, I also used PM samples from filters to stimulate human lung tissue. The inflammatory profile following PM stimulation was measured using the Luminex assay. The particulate matter ($PM_{2.5}$) resulting from cooking activities using biomass fuel was collected in a filter paper using gravimetric sampling (refer section 2.4.1). The sampling was performed in each household simultaneously with the sampling for media based smoke sample collection. The PM sample extraction method described in section 2.7.2 was used to generate a biomass $PM_{2.5}$ extract. Briefly, one half of the filter sample was pooled in a glass beaker with 4 ml of pure methanol and PM was extracted by sonication for 1 hour in a water bath sonicator. The solutions from both halves of the filter were then transferred into a previously weighed sterile tube, and the solution was evaporated under a gentle stream of nitrogen gas. The total weight of the remaining extracts was taken and stored at -80° C until use. On the day of stimulation PM extract was suspended in 1 ml of phosphate buffer solution (PBS) and the final concentration of 200 $\mu g/m^3$ was prepared in cell and tissue culture media (RPMI). In the multiplex Luminex assay, the levels of 8 of the 10 inflammatory cytokines assayed were within the detection limit of the assay. However, the induced levels of all these cytokines were not higher

than the unstimulated basal condition. This suggests that the extracted PM sample did not increase the production of any of the cytokine assayed from the human lung tissue (Figure 5.14).

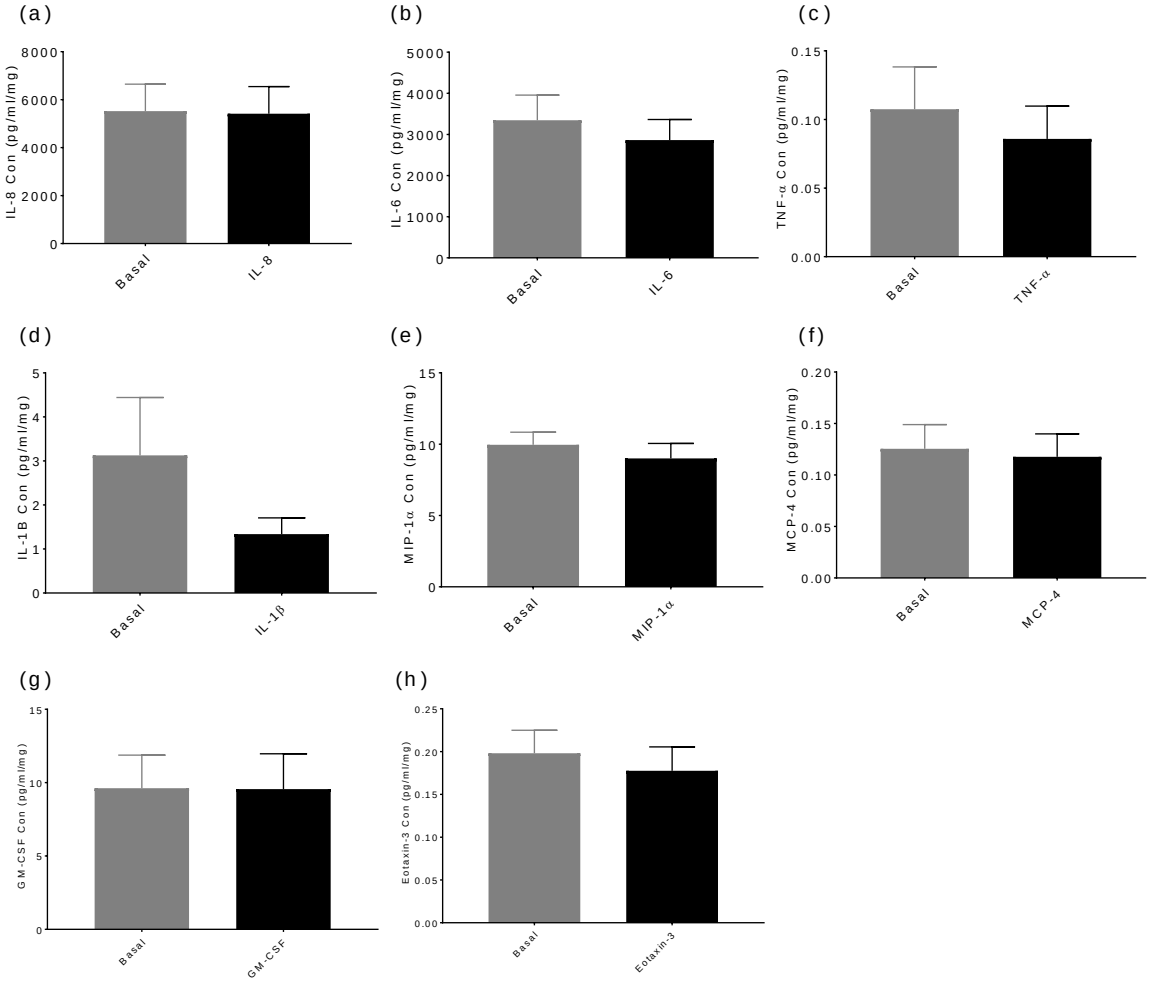

Figure 5.14 Inflammatory cytokine expression from *ex vivo* human lung tissue following particulate matter sample generated and extracted from the biomass combustion. The data represents the mean (±SEM) concentraion of each analyte (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MIP-1α, (f) MCP-4, (g) GM-CSF, and (h) Eotaxin-3. The data represents mean of 12 data points (n=5 donors, each donor stimulated with 3 samples from 3 monitoring sites). None of the analytes were significantly elevated (Elevated levels between smoke extract treated and untreated basal condition were checked using Wilcoxon matched paired test).

5.4.11 Endotoxin Concentration in the indoor air sample

I also measured the presence of possible endotoxin level in the collected biomass smoke extracts sample. The endotoxin concentration present in the biomass smoke sample collected from different locations was quantified using a Limulus Amebocyte lysate (LAL) assay (Pierce^{TM}LAL Chromogenic Endotoxin Quantification Kit, ThermoFisher Scientific, US). The LAL is a highly sensitive, quantitative endpoint assay to detect and measure endotoxin concentration based on the activation of a proenzyme in the modified lysate. The activation rate is proportional to the amount of endotoxin present in the sample. The experimental procedure for the quantification of endotoxin level was performed as per the protocol suggested by the manufacturer (ThermoFisher Scientific, US) under sterile conditions. Dilutions of endotoxin standard (Escherichia coli 011:B4) were prepared as specified in the protocol using endotoxin-free water and a four points standard curve was generated (R^2 = 0.996, Figure 5.15). LAL reagent containing lyophilized lysate and chromogenic substrate was prepared by reconstituting the given amount with the required amount of endotoxin-free water. After all the reagents were prepared, 50 µl of each standard and unknown samples were dispensed into each well of a 96 well plate previously maintained at 37°C±1°C. 50 µl of LAL reagent to each well was added at the same time and incubated at 37°C±1°C for 10 minutes. This was followed by adding 100 µL of pre-warmed (37°C±1°C) Chromogenic Substrate Solution to each well and

incubated for a further 6 minutes at 37°C±1°C. After 16 minutes, 100 µL of stop reagent (25% acetic acid) was added to each well, and the plate was read at 405nm on a plate reader. All the samples were used in 50 fold dilution prepared using endotoxin-free water, and neat endotoxin-free water was used as a blank. All the samples in the assay plate were ran in duplicate. The final concentration of endotoxin for each sample was calculated subtracting mean concentration in the blank from the mean concentration of each sample.

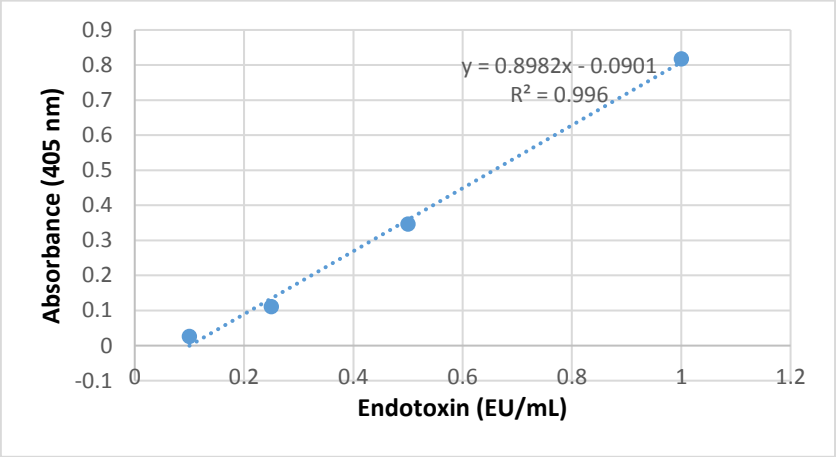

Figure 5.15 Standard curve generated from the endotoxin plate.

The overall results indicated the presence of endotoxin in the indoor air sample generated during cooking period. Biomass combustion for cooking was the main source of indoor endotoxin exposure in all four monitoring sites (Figure 5.16). The mean endotoxin concentration in households of all locations indicated highest levels of endotoxin in a kitchen at a higher altitude, and subsequently lower in a kitchen at lower altitude locations. However, no significant differences were seen in the levels of endotoxin in the different samples from different sites.

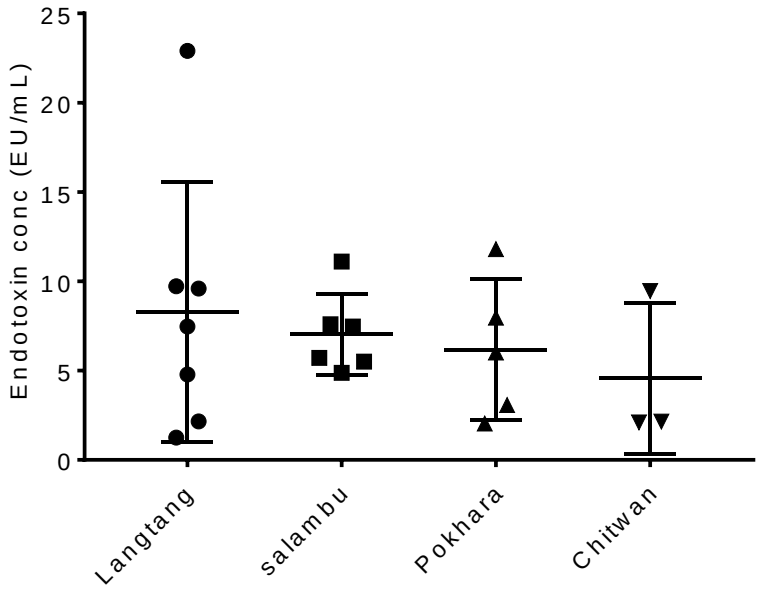

Figure 5.16 Endotoxin concentration present in samples from four different monitoring sites. The data represents the mean (±SD) from the samples from biomass combustion in respective sites. Each point represents a different sample.

Results also indicated highest levels of endotoxin in a kitchen using biomass fuel than in a kitchen using clean fuel like LPG. It was observed that endotoxin concentrations were highest in kitchen using traditional cook stove for biomass combustion followed by kitchen using biomass fuel combusted in an improved cook stove and least in a kitchen using LPG (Figure 5.17).

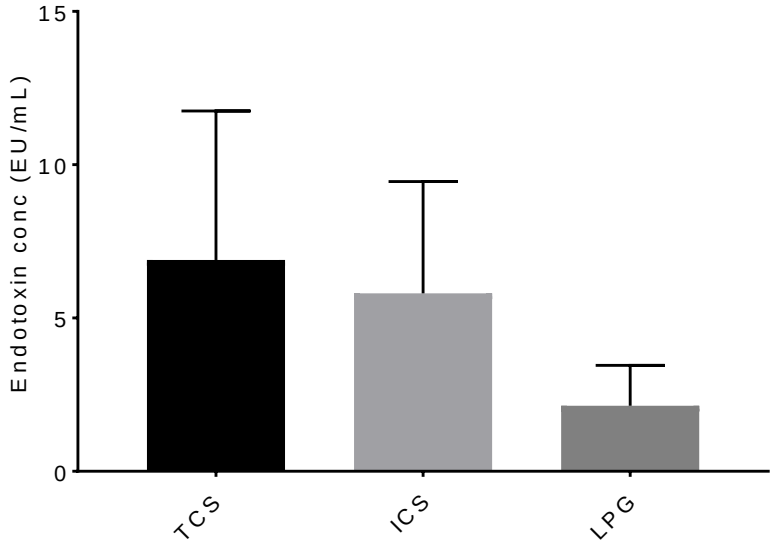

Figure 5.17 Endotoxin concentration present in indoor air samples generated from different cook stove and fuel sources. The data shown are the mean (±SD) endotoxin concentration in samples from all monitoring sites using TCS (n=21), ICS (n=4) and LPG (n=2). TCS: Traditional Cook stove, ICS: Improved cook stove, and LPG: Liquefied petroleum gas.

Having demonstrated the presence of endotoxin level in indoor air sample from different cooking sources, I then investigated the potential correlation between the level of endotoxin present in samples and cytokine expression from stimulated *ex vivo* human lung tissue. For this, the fold increase of cytokine expression from the tissue with the biomass smoke sample was compared with the endotoxin level in the respective sample. The data from all locations and all cooking sources showed only the expression of IL-8 ($r=0.46$, $p=0.01$) and MCP-1 ($r=0.38$, $p=0.04$) were moderately correlated with the endotoxin level. A weak correlation was formed between the expression of IL-6 ($r=0.25$, $p=0.18$), IL-1β

(0.35, p=0.06), and MIP-1α (r=0.24, p=0.2), with the endotoxin levels (Figure 5.18).

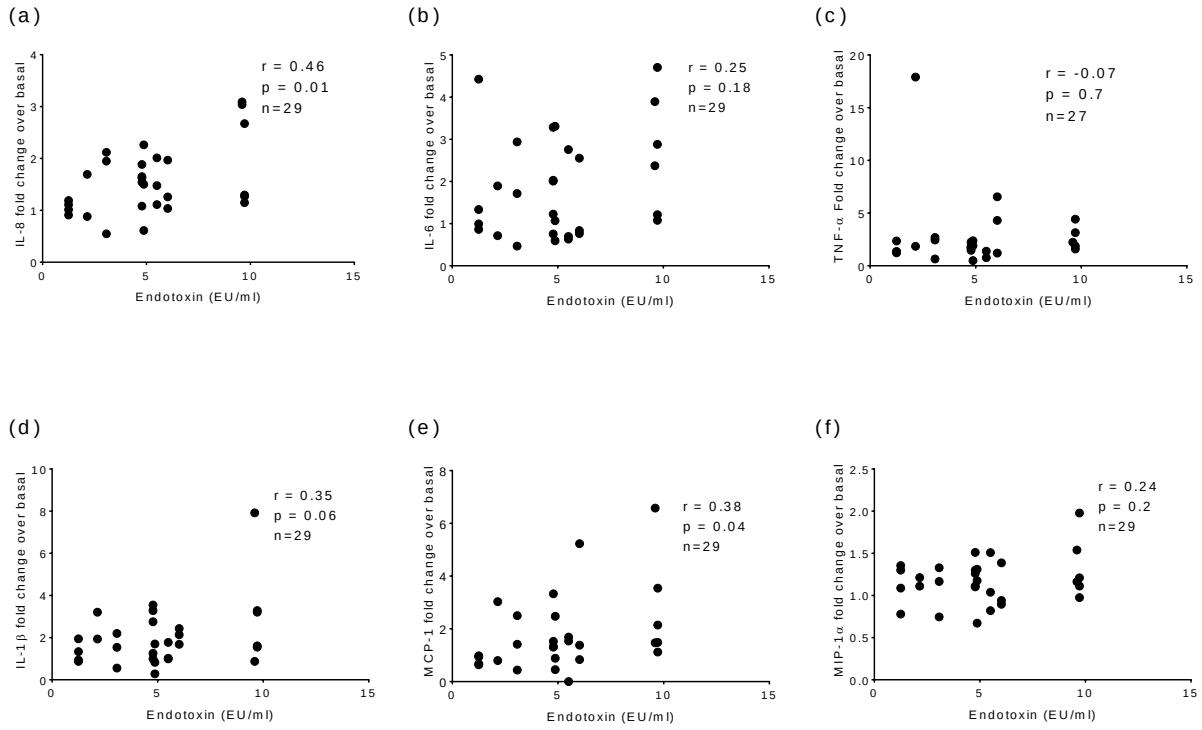

Figure 5.18 Correlation between levels of endotoxin in the biomass smoke sample and the expression of cytokines from the respective smoke samples. The endotoxin level was correlated with the cytokine fold stimulation over basal, (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MCP-1, and (f) MIP-1α. Data shown are an expression of each cytokine from all tissue donor with a sample from respective endotoxin concentration where n= individual data points. The correlation was checked with Peasron r value, and significance was checked with p-value <0.05.

5.5 **Discussion**

The study in this chapter set out to assess potential inflammatory effects of exposures in human *ex vivo* lung tissue to respirable products generated from biomass combustion for indoor cooking. The key aims were to (i) assess the secretion of a range of pro-inflammatory mediators in human *ex vivo* lung tissue with the smoke extract samples generated from biomass combustion in TCS, (ii) examine the potential variations in inflammatory effects observed with smoke extracts collected from different geographical regions, (iii) investigate the potential benefits of using more efficient biomass combustion cook stove or clean fuel LPG on inflammatory responses in *ex vivo* human lung tissue. For this, a range of pro-inflammatory mediators was measured in human *ex vivo* lung tissue following stimulation with biomass smoke extracts collected in the real-life settings of geographically different locations. Overall results show that smoke extracts obtained in the field during 'real-life' cooking produce an inflammatory response in human lung tissue. Due to inherent differences between donors, there was some variation in levels of inflammatory mediators in both unstimulated basal conditions and after stimulation with biomass smoke extract. Hence, for comparisons, fold stimulation was used rather than absolute values.

In these experiments, lipopolysaccharide (LPS) was used as a positive control because of its known potent inflammatory effects on human cells, a strong activator of monocytes/macrophages and its ability to produce

key inflammatory mediators including cytokines and chemokines [287]. Several previous studies have already shown potent effect of LPS on inflammatory response in various cell lines [288-291], and these responses were activated by TLR4 signalling pathway [292, 293]. The effect seen with LPS stimulation in this study is consistent with the findings of those previous studies, although unlike other studies, I used human lung tissue instead of single-cell lines in this study. In addition to that, we have already shown in a previous study that LPS induces production of a range of pro-inflammatory cytokines in human *ex vivo* lung tissue [15], which is in line with the findings of this study. Moreover, a study assessing the inflammatory effect of LPS in explanted lung tissue from donors with and without lung disease has been performed (Thakker et al. abstract submitted to ATS 2020). The result of this unpublished work showed that there was no differences in the fold stimulation seen with the core set of inflammatory mediators by disease states.

Biomass smoke extract generated during cooking in real-life showed a significant response in human lung tissue for IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α and MCP-4. To the best of our knowledge, this is the first study of its kind to study the effect of smoke extract samples from real cooking environment on human lung tissue fragments. However, previous studies have assessed and showed the pro-inflammatory effect of biomass smoke extract in different cell lines consistent with our findings. Significantly increased levels of IL-8 and MMP-1 in human small airway epithelial cells treated with biomass smoke extract were shown in a study

conducted by Mehra et al.[13]. The effect of biomass smoke extract sample in that study, however, did not mimic the potential effect of using biomass fuel in actual cooking as extracts were prepared in a laboratory setting using dung biomass fuel combusted for only 9 minutes. Another similar study conducted by McCarthy et al. also showed that smoke extract generated from the combustion of dung biomass fuel induces IL-8 and GM-CSF in small airway epithelial cells [294].

I also measured endotoxin levels in extract samples, and as would be expected, these were moderately elevated consistent with the findings from other studies [295, 296]. The effect seen in human lung tissue with biomass smoke extract could be driven by the presence of endotoxin in the extract samples, suggesting the responses seen were driven directly through TLR activation. However, as biomass smoke contains a wide range of potentially active compounds, including carbon monoxide, VOCs, PAHs, aldehydes, free radicals, sulphur and nitrogen oxides, benzene, and particulate matter [17], the inflammatory responses can be driven by more than a single mechanism. Qualitative differences observed between LPS responses and biomass smoke extract responses in this study also supports the idea of the involvement of a range of pathways. In addition to this, findings from previous studies also support that endotoxin is not the only active component in biomass smoke extracts that produces inflammatory effects. A study has shown that dung biomass smoke extract contain endotoxin levels, but in the same study, it has also shown that the inflammatory effects remained the same after removal of

endotoxin from the extracted sample [13]. It has also shown in another study that pro-inflammatory cytokine release from human small airway epithelial cells was through the activation of activator protein (AP)-1 and arylhydrocarbon receptor (AhR), but not through nuclear factor-kB pathways [294].

The inflammatory response in human *ex vivo* lung tissue was also monitored with the smoke extract sample collected in households using more efficient biomass combustion stoves. I found that the inflammatory effect remained the same despite using ICS and found a significant response in human *ex vivo* lung tissue for IL-8, IL-6, TNF-α, IL-1β, MIP-1α, GM-CSF and Eotaxin-3. Though there was a significant reduction of HAP in households using ICS, the extracts still produced an inflammatory effect and hence promote lung inflammation from occurring. The study to look into the effect of using a more efficient stove on inflammatory effects using cell lines *in vitro* or animal model in *in vivo* has not been conducted in any of the previous studies. However, the overall impact of using ICS on respiratory health has been performed in various part of the world. In a board spectrum, findings of this study can be related to the finding of a cluster randomised controlled trial study conducted in Malawi and Guatemala [297]. Both studies have shown that intervention of improved cookstove did not reduce the risk of pneumonia in young children [22]. Similarly, the findings from other studies conducted elsewhere have also shown no significant improvement in lung function after installation of ICS [23, 24]. In contrast to these earlier findings, however, few studies have

found improvement in lung function and other respiratory symptoms using ICS [298].

Another surprising yet interesting findings in this study was extract samples of LPG combustion for cooking also showed an inflammatory response and significantly induced the levels of IL-8, TNF-α and IL-1β in human *ex vivo* lung tissue. Though particle emissions in households using LPG were reduced by about 80%, it is interesting to know the potential contributing factors present in the extracts producing those responses in human lung tissue. One possible explanation for this is that ambient air pollution in these regions may be contributing to these responses as certain levels of particle concentrations has been observed in ambient air in these regions. However, we found no significant responses in lung tissue to ambient air samples collected in these regions. This further indicates that extracts generated from LPG combustion might contain additional gaseous pollutants or VOCs that contributed to these responses. In addition to this LPG combustion and cooking activities may also be producing ultrafine particles which are contributing to these responses. This explanation further requires in-depth field and laboratory studies to identify actual contributing factors of these responses seen with extracts from LPG combustion in real-life settings.

As smoke extract samples were collected from different locations, I attempted to look for differences in inflammatory response based on locations. There were some differences in fold stimulation of analytes

among different locations, probably due to different fuel type or different cooking practises followed in each location. The variations in altitude among these locations could also be a contributing factor to observe differences in an inflammatory response. However, due to a small number of samples, overall, these differences were difficult to study in-depth and hence, further studies need to be carried out.

Taken together, the main conclusion that can be drawn from this chapter is that smoke extract collected in real-life cooking produced an inflammatory response in human lung tissue. The responses remained the same despite using improved biomass combustion stove. This suggests that inhaling biomass smoke during cooking activities is likely to induce lung inflammation in the real-life settings, and interventions currently being trialled are not able to reduce the inflammation from occurring in these settings. Hence, in order to improve respiratory health and to prevent lung inflammation, exposures need to be reduced, and additional methods other than relying on existing improved cook stove may be needed which will reduce exposure to levels that will prevent the inflammation from occurring. However, whilst I have demonstrated the inflammatory effect of biomass smoke extract in a lung tissue fragments, it is still unclear which cell types contribute to these responses from the different cell types present in the tissue. Hence, I repeated key experiments using human bronchial epithelial cells (HBECs) to try and estimate if the lung epithelium contributed to these responses.

Chapter 6: Inflammatory profiles of human bronchial epithelial cells in response to smoke extracts from biomass burning.

6 Inflammatory profiles of human bronchial epithelial cells in response to smoke extracts from biomass burning.

As discussed in chapter 4, it is clear that the combustion of biomass fuel for cooking generates higher concentration of air pollutants including $PM_{2.5}$, CO and other harmful chemicals. A considerable number of studies have already shown the association of biomass smoke exposure and risk of developing various pulmonary and cardiovascular diseases. In the previous chapters, I demonstrated that people using biomass fuel in rural Nepal for cooking are exposed to a high levels of indoor $PM_{2.5}$ and CO. I also demonstrated that biomass smoke extracts generated in rural households during 'real-life' cooking produced an inflammatory response in human lung tissue. A variety of pro-inflammatory mediators have been measured from cultured *ex vivo* human lung tissue stimulated with biomass smoke extracts. Alveolar type I epithelial cells, alveolar type II cells, type I pneumocyte, type II pneumocyte and alveolar macrophages are major cell types in the lung parenchyma [299]. These cells take part in the inflammatory response in the lung to inhaled harmful pathogens or pollutants and release a variety of cytokine and mediators [122]. The profile of inflammatory cytokines released from lung tissue to biomass smoke extract will be the combined effect of all cells present in the tissue. However, the contributions of each cell type on the production of these cytokines are not clear. Hence, in this chapter, I tried to estimate if lung epithelium contributes to these inflammatory responses, as epithelial cells in the lung contribute the largest surface area of the lung [122] and form

the first point of contact for inhaled substances. For this work, lung bronchial epithelial cells were stimulated with biomass smoke extract *in vitro*, and the secretion of pro-inflammatory cytokines was measured.

6.1 **Introduction**

Airway epithelium serves as the initial barrier against a wide range of inhaled environmental insults and pollutants including cigarette smoke and biomass smoke. Airway epithelium comprises of specialised cell types that play an important role in regulating airway homeostasis [300]. It also plays a vital role in host defence and actively participates in both innate and adaptive immune responses to allergens and environmental concomitants [130]. The airway epithelial cells regulate an inflammatory process by secreting a variety of inflammatory cytokine and other mediators in response to inhaled irritants. Studies have demonstrated that the human airway epithelial cells can secrete cytokines including interleukin (IL)-8, IL-6 and tumour necrosis factor-α (TNF-α) which may have profound effects on neutrophil activity in the site of inflammation [301, 302]. Studies have also shown that airway epithelial cells can produce large variety of cytokines and chemokines including interleukins (IL)-1, -3, -6 and -8, GMCSF (granulocyte macrophage-colony stimulating factor), G-CSF (granulocyte-colony stimulating factor), TNF-α, Eotaxin-1-2, RANTES, MIP-1α (macrophage inflammatory proteins) [303]. The activation of these inflammatory mediators plays a key role in growth, differentiation, migration and activation of various inflammatory cells

including eosinophils, neutrophils, mast cells, macrophages and lymphocytes [302]. Airway epithelial cells have been shown capable of generating a wide range of cytokines, and hence it plays a vital role in the pathogenesis of inflammatory airway disorders such as asthma, cystic fibrosis and chronic obstructive pulmonary disease [304, 305].

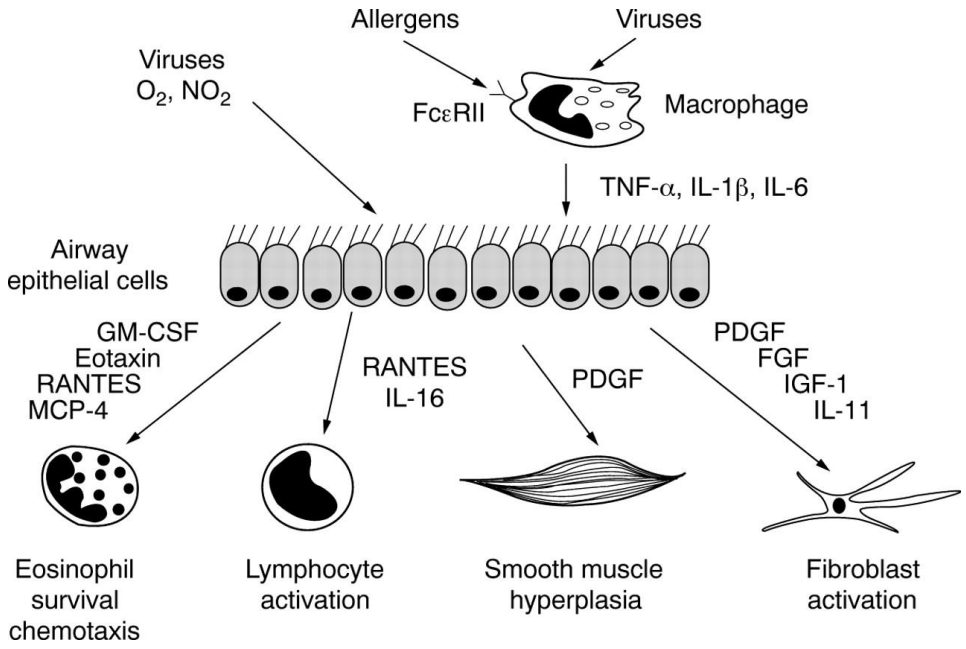

Figure 6.1 Cytokine release from airway epithelial cells in response to various stimuli [303].

Several studies have demonstrated that exposure of human airway epithelial cells to cigarette smoke results in the production of a wide range of pro-inflammatory cytokines. A study conducted by Mortaz et al. found that exposure of HBECs to cigarette smoke resulted in the production of IL-8 and IL-1β through the activation of Toll-like receptors (TLRs)-4 and 9 [306]. Similarly, a recent study conducted by Hulina et al. showed that cigarette smoke extracts elevated IL-8 and IL-6 secretion in primary bronchial epithelial cells from both healthy and COPD donors

[307]. Similar to this, several studies have also shown the association of bronchial epithelial cells in the pathogenesis of airway disease in response to atmospheric pollutants, including pollutants from residential biomass combustion. HBECs produced inflammatory cytokines like IL-17A, IL-8, IL-6, IL-1β, MMP-9 in response to particulate matter generated from different sources like ambient urban particles, diesel exhaust particles or sandstorm particles [308-310]. Other *in vitro* studies have also demonstrated that PM activates airway epithelial cells to produce cytokines like TNF-α, IL-6, IL-8, GM-CSF and IL-1β [311]. The cytotoxicity of exposure to wood smoke generated from the combustion of fuelwood for cooking has been measured in different in vitro and human exposure studies. Significant increase in epithelial CD3+ cells and epithelial CD8+ cells were measured in bronchial wash collected from healthy subjects underwent controlled exposures to wood smoke from incomplete combustion [183]. Similarly, an *in vitro* study showed that human lung A549 cells exposed to PM from biomass fuel combustion increased the release of IL-8 and IL-6 [12].

Airway epithelial cells are capable of producing a wide range of pro-inflammatory cytokines and can be used in mechanistic studies of air pollution-induced airway disease. I, therefore, sought to investigate the pro-inflammatory effect of biomass smoke extract on HBECs. Hence, I repeated key experiments using HBECs to try and investigate if the lung epithelium contributes to the responses seen in human lung tissue.

6.2 **Aims**

The overall aim of this chapter was to investigate the contribution of bronchial epithelial cells to the inflammatory response observed in *ex vivo* lung tissue stimulated with biomass smoke extracts.

6.2.1 Specific aims

- Assess the range of pro-inflammatory mediators that are released from the cultured HBEC *in vitro* following stimulation with biomass smoke extracts prepared from traditional cooking stoves.
- Identify the potential changes in effects seen with the samples collected from different geographical regions.
- Investigate the effects of using improved low emission cooking stoves on inflammatory responses from HBECs.
- Investigate the effects of using other fuels than biomass on pro-inflammatory signatures produced from HBECs.

6.3 **Methods**

6.3.1 Human bronchial epithelial cells

Human bronchial epithelial cells (HBECs) were sourced from Lonza® (Castleford, UK, Product code CC-2540), which were initially obtained from the post-mortem lungs of healthy, Caucasian male subjects. Two primary donors were used throughout the experiment; D355687 and D369144. The information of the donors used for this work is presented in table 2.5 of the general method section 2.5.2. In brief, Donor355687 was a 38 year old male Caucasian and donor 369144 was a 43 year old male Caucasian. Both donors were a non-smoker and non-alcoholic. Cryopreserved cells from two donors were recovered from frozen vials and cultured for subsequent analyses.

6.3.2 Cell Culture

HBECs were cultured in bronchial epithelial basal media (BEBM™, Lonza) supplemented with growth factors and additives (2 ml Bovine Pituitary Extract, 0.5 ml Insulin, 0.5 ml Hydrocortisone, 0.5 ml retinoic Acid, 0.5 ml Transferrin, 0.5 ml Triiodothyronine, 0.5 ml Epinephrine and 0.5 ml Epidermal Growth factor in 500 ml BEBM) to form bronchial epithelial growth media (BEGM). Initially cryopreserved cell stocks from two donors at passage 1 were recovered from frozen vials, cultured in T75 flask with BEGM and frozen to a temperature of -180°C by placing them in a cryogenic tank filled with liquid nitrogen. For this, the cells were initially

expanded in a T75 cell culture flask (Corning®) containing 15 ml of pre-warmed (37°C) fresh media. Media was replaced every 48 hours until cells reach ~85% confluence. The cells were then frozen at passage 2 in a freezing medium of BEGM+10%v/v DMSO at a concentration of $2.5x10^6$cells per/mL in each vial and stored in liquid nitrogen for the further use. DMSO acts as a cryo protective agent preventing cell dehydration by reducing the rate of cooling. It also stops the formation of ice crystals and hence stops lyse of the cells. A total of 8 vials (1mL) were obtained from one confluent T75 flask.

Cells at passage 2 were used for this work, and each vial was used for one experiment. On the day of the experiment one vial is taken out and expended in a T75 cell culture flask with 15 mL of fresh pre-warmed culture media. The cells were cultured as per the methods described in section 2.5.2. In brief, cells from a confluent T75 flask were detached by adding trypsin and with trypsin de-activated using trypsin inhibitor. The cells pellet were then recovered from the solution after centrifugation and suspended in 1 mL of fresh media. The cells after counting were then seeded into 24 well plate for the stimulation at a density of 20,000 cells per well in 1mL of fresh media. The cells were then incubated at 37°C, 5% CO_2 until they become ~85% confluent for the stimulation. All cell culture works were conducted in a 24 well plate where all experimental conditions were prepared in triplicate.

6.3.3 Cell stimulation

In order to observe the inflammatory effect of biomass smoke extracts on HBECs, the cells *in vitro* were stimulated with the extracts. The smoke extracts were collected in rural households using biomass fuel for cooking. The detailed collection method has already been described in section 5.3.2. The HBECs were stimulated with the same smoke extracts that were used in human lung tissue stimulation experiments in the previous chapter. The cells on each well were observed for the confluence under the microscope. The stimulation was performed when cells in all wells become about 85% confluent. On the day of stimulation, the spent media was replaced with the fresh pre-warmed media and 10% v/v biomass smoke extracts were added in each well to make a final volume of 1 mL. The plate was then incubated for 48 hours, followed by a collection of supernatant for further protein analysis.

In these experiments, cells were also stimulated with polyinosinic:polycytidylic acid (poly (I:C)) to observe cells response to a known stimulus. Poly (I:C) is structurally similar to double-stranded RNA (dsRNA) and is known to interact with toll-like receptor 3 (TLR3) [312]. It can be considered a synthetic analog of viral dsRNA and can be used to mimic the immune system in scientific research. It induced the characteristic inflammatory response associated with a viral infection and increased the production of inflammatory cytokine [313]. A study conducted by Lever et al. had shown that primary normal human

bronchial epithelial cells stimulated with poly(I:C) induced increased production of IL-6, IL-8, TNF-α and RANTES [313]. Another study conducted BY Hirotaka et al. had also presented that induction of poly(I:C) to human bronchial epithelial cells resulted in secretion of pro-inflammatory cytokines [314]. Increased level of cytokine profile has also been measured in bronchoalveolar lavage fluid (BALF) of different animals treated with poly(I:C) [315, 316]. A working concentration of 25 μg/mL was used for the stimulation which was prepared from 1 mg/mL stock concentration in PBS. Same incubation period of 48 hours was used for poly(I:C) stimulation as well.

6.3.4 Smoke sample collection

As already mentioned smoke extracts collected in households using biomass fuel for cooking were used for cell stimulation. Refer previous section 5.3.2 in chapter 5 for further details of sample collection, storage and transfer.

6.3.5 Pro-inflammatory mediatory quantification

A custom Magnetic Luminex Screening assay, Human Premixed Multi Analyte kit (R&D systems) (refer section 2.8.2 for working principle) was used to detect the levels of pro-inflammatory cytokines in cell cultured supernatant. The analytes selected for this experiment were IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α, MIP-1β, MCP-4, GM-CSF and Eotaxin-3. The Luminex assay was used to measure the induction of various cytokine

using the same protocol and methods that have been used to quantify cytokine from human lung tissue. The detailed protocol and methods were described in section 5.3.4 in the previous chapter 5. In brief, all supernatant samples were run in Luminex plate following the same protocol, and analytes were measured in duplicate wells for each condition and the values were averaged. In all Luminex plates, two samples from each condition were assayed and the mean values of these two samples were used for the further analyses.

6.4 **Results**

6.4.1 Inflammatory response to Poly (I:C)

Inflammatory cytokine expressions to Poly(I:C) induction in normal human bronchial epithelial cells were measured in cell culture supernatant by Luminex assay. The increased level of 8 analytes including cytokines, chemokines and other growth factors 48-hour post-stimulation is presented in Figure 6.2. The induced level of MIP-1β and MCP-4 were below the detection limit of the assay and hence not included in analyses. The levels of all analytes to poly(I:C) stimulation were compared with the levels of each analyte in baseline (unstimulated) conditions. The level of all analytes increased with Poly(I:C) stimulation compared to unstimulated baseline levels; however, the overall fold increase varies among the analytes. The greatest level of induction was observed for IL-8, TNF-α and IL-6 with the fold stimulation >5 fold relative to baseline

levels seen with untreated controls. The fold increased for other analytes were relatively smaller with ~2 fold for MCP-1 and GM-CSF and ~1.5 fold for IL-1β, Eotaxin-3 and MIP-1α. Though the fold increase was relatively low for some analytes, induced levels were significantly higher for all analytes than the levels observed in the baseline condition.

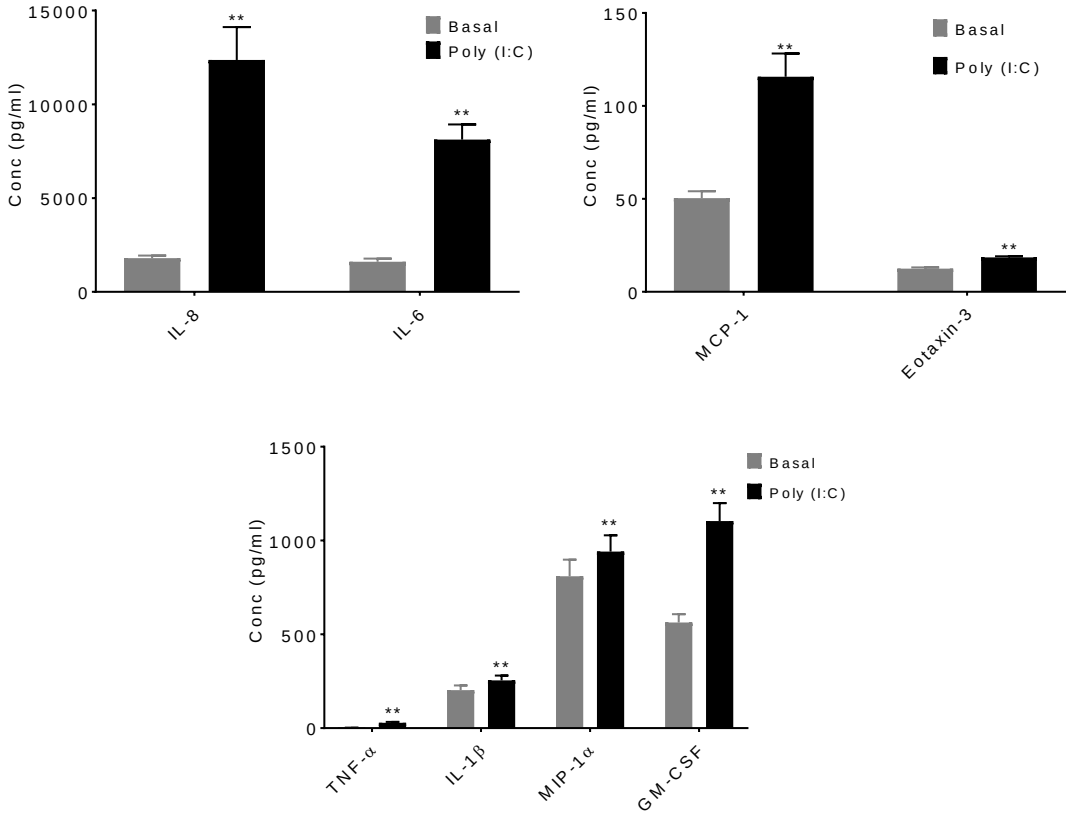

Figure 6.2. Poly(I:C) induced the release of pro-inflammatory cytokine and chemokine in HBECs. Poly(I:C) (25μg/mL) stimulation induced the release of 8 of 10 analytes assayed including IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α, GM-CSF and Eotaxin-3. The data shown are the mean (±SEM) concentration of each analyte. The variations in induced level for each analyte over baseline levels were assessed using the Wilcoxon paired test (**=$p<0.01$). IL: Interleukin, TNF-α: Tumor Necrosis factor-alpha, MIP: Macrophage inflammatory proteins, GM-CSF: Granulocyte-macrophage colony-stimulating Factor, MCP: Monocytes chemoattractant protein.

6.4.2 Inflammatory response to Biomass Smoke extracts

Having demonstrated that poly(I:C) stimulated human bronchial epithelial cells produced a range of pro-inflammatory cytokines, I then investigated the effect of biomass smoke extracts on HBEC. For this, a range of pro-inflammatory cytokine expression from cultured HBECs was measured following stimulation with biomass smoke extracts *in vitro*. Similar to the expression seen with poly(I:C), levels of both MIP-1β and MCP-4 were below the lower detection limit of the assay in most of the repeated experiments and hence not included in further analyses. Figure 6.3 presents the mean concentration of induced expression of all 8 analytes including IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α, GM-CSF and eotaxin-3. The data in the figure represents mean concentration from two donors with four biological repeated experiments for each donor.

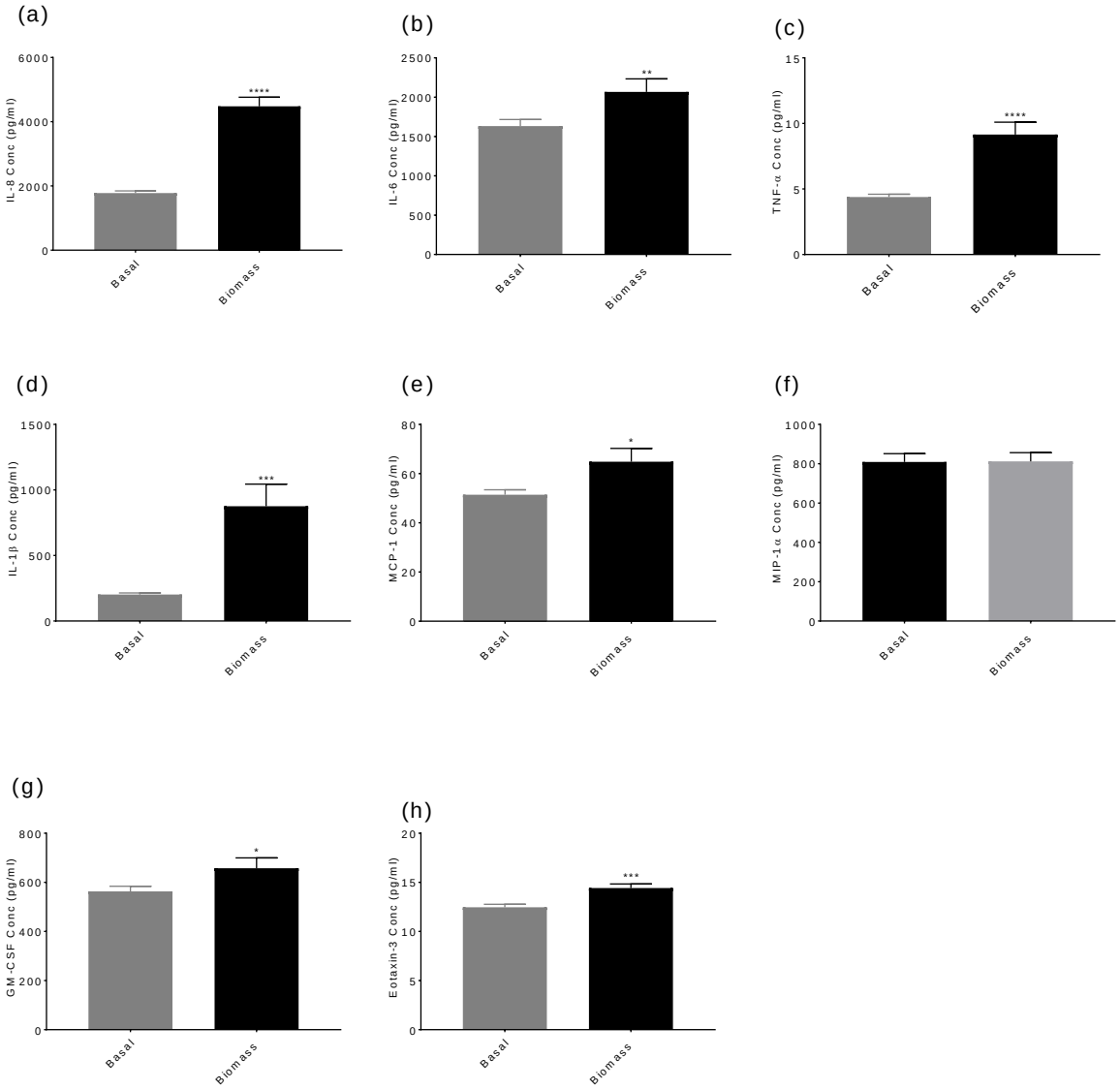

Figure 6.3 Biomass smoke extract generated from TCS induced release of pro-inflammatory cytokines in HBECs. HBECs stimulated with biomass smoke extract secreted increase levels of (a) IL-8, (b) IL-6, (c) TNF-α, (d) IL-1β, (e) MCP-1, (f) MIP-1α, (g) GM-CSF, and (h) Eotaxin-3. The data shown are the mean (±SEM) concentration of each analyte from n=8 independent experiments. The variations in induced level for each analyte over baseline levels were assessed using Wilcoxon matched paired test (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$). IL: Interleukin, TNF-α: Tumor Necrosis factor-alpha, MIP: Macrophage inflammatory proteins, GM-CSF: Granulocyte-macrophage colony-stimulating Factor, MCP: Monocytes chemoattractant protein.

Biomass smoke extracts increased levels of all 8 analytes assayed in HBECs. Except for MIP-1α, induced levels of all other analytes were significantly higher compared to baseline levels seen in untreated basal condition. However, the overall fold increased relative to baseline levels vary among the analytes and were comparatively less than the mean fold stimulation with poly(I:C) for each analyte, except for IL-1β (Table 6.1). The induced level of IL-1β to biomass smoke extracts was ~3.5 fold higher compared to poly(I:C). The greatest level of induction was observed for IL-8 and TNF-α with the fold stimulation >2 fold relative to baseline levels. The fold increased for other analytes were relatively small, with < 1.5 fold increase compared to baseline levels. The relative levels of cytokine response seen with poly(I:C) and biomass smoke extracts stimulation are shown in Table 6.1. The relative levels of cytokine responses seen with poly(I:C) and biomass smoke extract varied to some extent for each analyte. For example whilst the fold stimulation for IL-8 was 7.01 with poly(I:C) and 2.62 with biomass (i.e. 37% of the poly(I:C) response), it was 5.43 fold for IL-6 with poly(I:C) and 1.29 fold for biomass (23.7% of the poly(I:C) response).

Table 6.1 Biomass smoke extracts response to HBECs as a percentage of poly(I:C) response. The data in the table shows the mean (SEM) fold increase for each analyte following poly(I:C) and biomass smoke extracts stimulation (n=8 independent experiments).

Analytes	IL-8	IL-6	TNF-α	IL-1β	MCP-1	GM-CSF	Eotaxin-3
Poly(I:C) stimulation fold change	7.01 (0.92)	5.43 (0.86)	6.54 (0.99)	1.29 (0.06)	2.26 (0.15)	2.0 (0.21)	1.5 (0.08)
Biomass stimulation fold change	2.62 (0.19)	1.29 (0.09)	2.08 (0.16)	4.85 (1.01)	1.25 (0.09)	1.17 (0.06)	1.17 (0.04)
% of poly(I:C) response	**37.3**	**23.7**	**31.8**	**375**	**55.3**	**58.5**	**78**

6.4.3 Inflammatory response to Biomass smoke extract generated from ICS

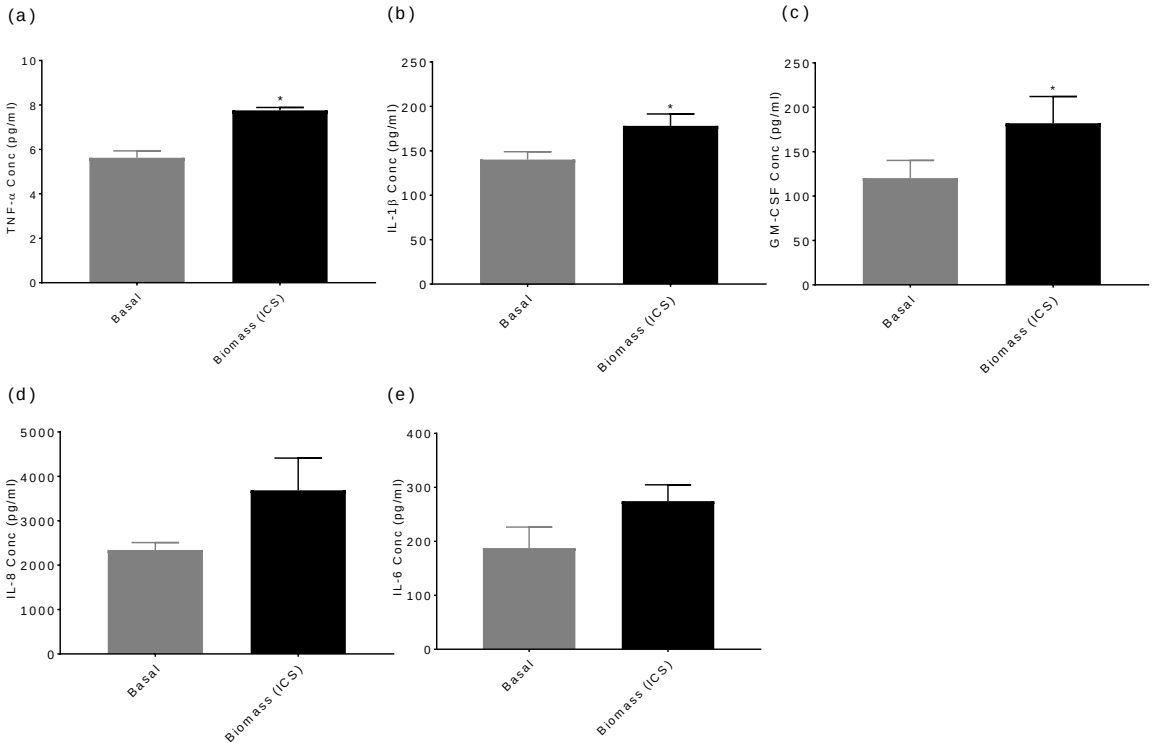

Figure 6.4 Biomass smoke extract generated from ICS induced release of pro-inflammatory cytokines in HBECs. HBECs stimulated with smoke extract secreted increase levels of (a) TNF-α, (b) IL-1β, (c) GM-CSF, (d) IL-8, and (e) IL-6. The data shown are the mean (±SEM) concentration of each analyte from n=6 independent experiments. The variations in induced level for each analyte over baseline levels were assessed using Wilcoxon matched paired test (*=$p<0.05$).

Having demonstrated that biomass smoke extract generated from TCS increased the production of pro-inflammatory cytokines in HBECs, I then attempted to see whether or not the same inflammatory effects would be seen on HBECs using smoke extracts generated from ICS. Similar to the findings seen with lung tissue samples, smoke extracts from ICS also induced production of pro-inflammatory cytokines in HBECs. The levels

seen of 5 of 10 analytes assayed are presented in figure 6.4. The levels of the other four analytes were either below the detection limit of the assay or not higher than the levels seen in basal condition for the respective analytes. The levels of all 5 analytes including IL-8, IL-6, TNF-α, IL-1β, and GM-CSF appeared higher than the levels seen in basal condition, however, only four analytes were significantly higher. The fold increased, but not significant, relative to baseline levels for IL-8 and IL-6 were 1.52 fold ($p=0.15$) and 1.46 fold ($p=0.21$) respectively. Small but significant fold increases over the baseline levels for TNF-α (1.37 fold, $p<0.05$), IL-1β (1.27 fold, $p<0.05$), and GM-CSF (1.52 fold, $p<0.05$) were observed.

6.4.4 Inflammatory response to LPG

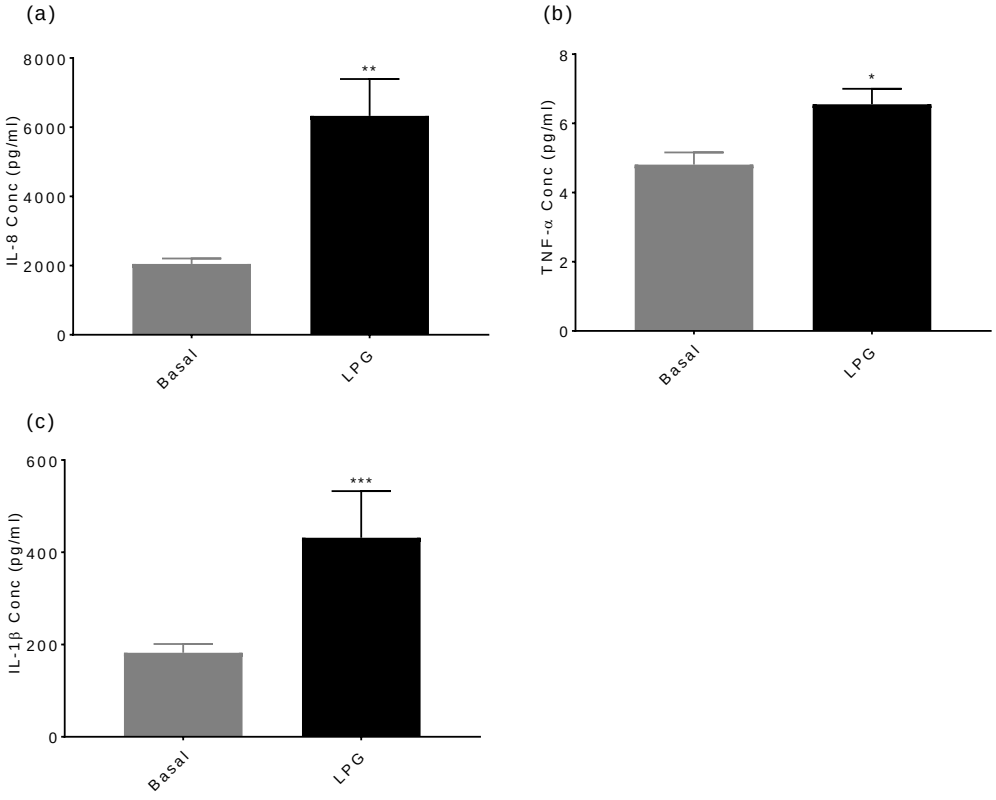

Figure 6.5 Smoke extract generated from LPG induced release of pro-inflammatory cytokines in HBECs. HBECs stimulated with smoke extract form LPG secreted increase levels of (a) IL-8, (b) TNF-α, and (c) IL-1β. The data shown are the mean (±SEM) concentration of each analyte from n=6 independent experiments. The variations in induced level for each analyte over baseline levels were assessed using Wilcoxon matched paired test (*=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$).

Despite a reduction of PM exposure by about 80% using LPG instead of biomass in TCS, a significant elevation of cytokines in lung tissue samples following stimulation with extracts generated from cooking using LPG was also observed. Having described these findings in the previous chapter, I attempted to see if HBECs stimulation with LPG extracts would show the same effect. LPG extract samples showed a similar effect in HBECs as

increased levels of cytokines were observed in cell culture supernatants following LPG extract stimulation (Figure 6.5). Similar to the response seen in lung tissue, levels of 3 cytokines were significantly elevated than the levels observed in unstimulated basal condition. The cytokines with significant fold increases were IL-8 (3.1 fold, p=0.002), TNF-α (1.5 fold, p=0.01) and IL-1β (2.4 fold, p=0.0005).

6.4.5 Site-specific inflammatory effect of biomass smoke extracts (TCS)

Table 6.2 Pro-inflammatory response in HBECs remained quantitatively similar across all sites for smoke extract collected in TCS. The data shown are mean (range, min-max) of n=8 independent experiments.

	Kyanzing	Salambu	Pokhara	Chitwan
IL-8	2.06 (0.83-3.75)	3.29 (2.53-5.55)	2.72 (1.25-4.08)	2.6 (0.82-3.63)
IL-6	1.08 (0.64-1.49)	1.32 (0.4-2.14)	1.36 (0.62-2.27)	1.44 (0.81-2.01)
TNF-α	1.51 (0.95-2.33)	2.77 (1.47-3.94)	2.04 (1.2-3.01)	2.25 (1.22-3.75)
IL-1β	1.68 (1.03-2.41)	7.34 (0.76-18.2)	4.68 (1.2-15.49)	5.72 (1.86-22.19)
MCP-1	1.51 (0.76-2.17)	0.9 (0.6-1.26)	1.49 (0.6-1.9)	1.07 (0.49-1.92)
MIP-1α	1.02 (0.92-1.12)	1.03 (0.87-1.16)	1.0 (0.77-1.125)	0.98 (0.63-1.125)
GMCSF	1.06 (0.88-1.33)	1.19 (0.49-2.14)	1.07 (0.42-1.5)	1.35 (0.88-1.66)
Eotaxin-3	1.05 (0.94-1.2)	1.18 (0.8-1.56)	1.15 (0.9-1.57)	1.3 (0.9-1.85)

Having demonstrated that smoke extract from cooking in the rural kitchen produced an inflammatory response in HBECs, I then investigated the response seen in HBECs from the smoke extracts generated in each monitoring site using TCS. The overall mean fold increase of each of 8 analytes for smoke extract samples from each monitoring site is presented in Table 6.2. The fold stimulation of all analytes remained quantitatively similar across all sites. A quantifiable increase in levels of all cytokines was observed with samples from all sites. Probably due to variability in exposure among different houses in each monitoring site, the mean fold increase for each cytokine appeared to vary to some extent across different sites. However, the variations seen for each analyte across each site were not statistically significant.

6.4.6 Inflammatory response of smoke extract samples in different sources in each monitoring site

In Kyanzing, smoke extract samples were collected in households using biomass fuel in both TCS and ICS. The data in the above section and previous chapter showed that smoke extracts generated in a real kitchen environment using biomass fuel either in TCS or ICS enhanced the production of pro-inflammatory cytokines from both lung tissue samples and HBECs samples. Though smoke extract from both sources increased cytokine production, there were quite marked differences between cytokine responses among those extract samples. Here I attempted to observe the fold stimulation of each of 8 analytes with the extract

samples collected in households of Kyanzing using either TCS or ICS. The fold stimulation normalised with the levels seen under basal conditions for each of the analytes is presented in Figure 6.6.

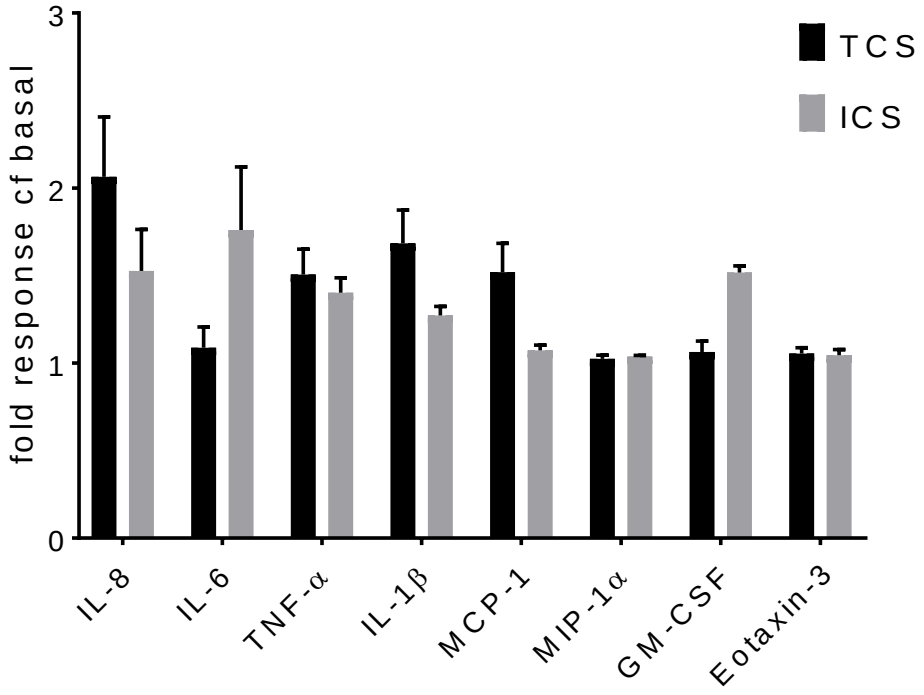

Figure 6.6 Smoke extract samples collected in Kyanzing from TCS and ICS all enhanced the production of pro-inflammatory cytokines in HBECs. Overall mean fold increase of all 8 analytes was observed for both TCS and ICS samples. The data shown are mean (±SEM) fold increase for each analyte with two smoke extract samples. Variations in each analytes with two stove designs were checked for significance using Mann-Whitney t-test.

Whilst the fold stimulations for the majority of cytokines including IL-8, TNF-α, IL-1β, MCP-1, were higher in extracts from TCS than in ICS, levels of IL-6 and GM-CSF were higher in extracts from ICS then in TCS. The mean fold stimulation for MIP-1α and Eotaxin-3 remained similar for both extract samples. However, the differences seen in the mean fold increase

of each cytokine among two smoke extract samples were not statistically significant

Exposures to HAP in households using LPG were significantly lower than in households using biomass fuel. However, increased levels of some pro-inflammatory cytokines were seen in both human lung tissue and HBECs in response to smoke extract samples collected in households using LPG. The overall mean fold increase of IL-8, TNF-α and IL-1β were significantly higher in HBECs stimulated with extracts from LPG cooking. The LPG extracts samples were collected from two monitoring sites Pokhara and Chitwan. Hence I attempted to see if the fold stimulations of analytes vary with the extract samples collected in households of Pokhara and Chitwan using either TCS or LPG samples. As there were responses seen with ambient air samples from Pokhara and Chitwan in human lung tissue samples, the inflammatory responses in HBECs with ambient air samples from these two sites were also compared. The fold stimulation normalised with the levels observed in basal for 3 analytes (those were significantly higher with LPG in overall) in Pokhara and Chitwan are presented in Figure 6.7 and Figure 6.8 respectively.

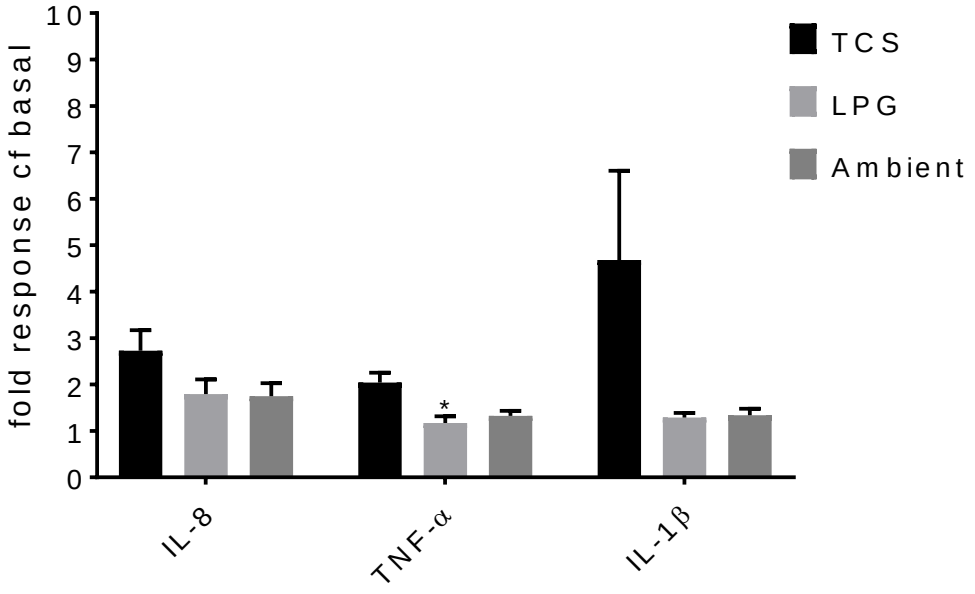

Figure 6.7 Smoke extract samples collected in Pokhara from TCS and LPG, and ambient air sample all enhanced production of pro-inflammatory cytokines in HBECs. Overall mean fold increase of IL-8, TNF-α, and IL-1β was observed for LPG samples. The data shown are mean (±SEM) fold increase for each analyte with different extract samples. The overall fold increase with TCS was compared with the overall mean fold increase with LPG and ambient using Kruskal-Wallis test followed by Dunn's multiple comparison tests.

The data from Pokhara (Figure 6.7) showed that the mean fold increase of all three analytes was higher with TCS samples than with LPG and ambient air samples, TNF-a being significantly high in TCS than in LPG ($p<0.05$). The ambient air sample also induced the production of all three analytes with the fold stimulation of 1.74 fold for IL-8, 1.32 fold for TNF-α and 1.33 fold for IL-1β. The fold increase of all three analytes with LPG samples was similar to that seen with ambient air samples. The fold stimulation of all 8 analytes with TCS, LPG and ambient air samples is presented in Table 6.3. Except for GM-CSF and IL-6, fold stimulation of all

other analytes were higher with TCS samples than with LPG and Ambient samples in Pokhara. Also, except for IL-6, fold stimulation for both LPG and ambient samples were relatively similar for all other analytes. LPG showed higher fold increase of IL-6 than with TCS and ambient samples.

Table 6.3 Fold stimulation of all 8 analytes in Pokhara with different extract samples. The data shown are mean (range, min-max) fold increase with each extract samples than the levels seen with the basal condition for each analyte (n=8 independent experiments).

	TCS	LPG	Ambient
IL-8	2.72 (1.25-4.08)	1.79 (1.24-2.39)	1.74 (1.1-2.47)
IL-6	1.36 (0.6-2.27)	1.78 (0.65-2.92)	1.19 (0.42-1.96)
TNF-α	2.04 (1.2-3.01)	1.16 (0.94-1.56)	1.32 (1.09-1.57)
IL-1β	4.68 (1.2-15.49)	1.28 (1.0-1.44)	1.33 (0.98-1.64)
MCP-1	1.49 (0.6-1.91)	1.06 (1.01-1.11)	1.04 (1.0-1.07)
MIP-1α	1.001 (0.77-1.12)	1.03 (0.96-1.07)	1.03 (0.99-1.06)
GM-CSF	1.07 (0.42-1.5)	1.38 (1.19-1.52)	1.57 (1.22-1.84)
Eotaxin-3	1.15 (0.9-1.15)	0.95 (0.88-1.01)	0.98 (0.9-1.08)

The data from Chitwan (Figure 6.8) showed that the mean fold increase of TNF-α and IL-1β were higher with TCS samples than with LPG and ambient air samples. However, unlike the responses seen in samples from

Pokhara, the mean fold increase of IL-8 was higher with LPG samples than with TCS and ambient sample. The ambient air sample also induced the production of all three analytes with the fold stimulation of 2.38 fold for IL-8, 1.53 fold for TNF-α and 1.91 fold for IL-1β. The ambient air samples from Chitwan showed higher response for all three analytes than the response seen with ambient samples from Pokhara. Also, unlike responses seen with LPG samples from Pokhara, the mean fold increase with LPG samples in Chitwan were higher than the mean fold increase seen with ambient air sample for all three analytes.

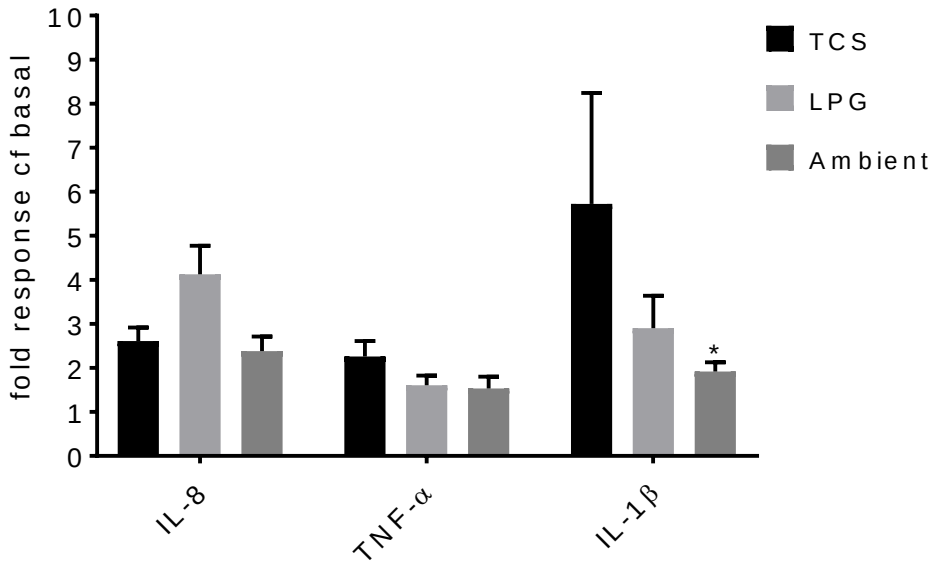

Figure 6.8. Smoke extract samples collected in Chitwan from TCS and LPG, and ambient air sample all enhanced production of pro-inflammatory cytokines in HBECs. Overall mean fold increase of IL-8, TNF-α, and IL-1β was observed for LPG samples. The data shown are mean (±SEM) fold increase for each analyte with different extract samples. The overall fold increase with TCS was compared with the overall mean fold increase with LPG and ambient using Kruskal-Wallis test followed by Dunn's multiple comparison test.

The fold stimulation of all 8 analytes with TCS, LPG and ambient air samples is presented in Table 6.4. Both LPG and ambient air sample did not produce levels of MCP-1 and MIP-1α higher than the levels seen in basal condition for respective analytes. Except for GM-CSF, a similar pattern of higher fold stimulation with TCS followed by LPG and ambient air samples was observed for all other analytes. LPG sample did not produce levels of GM-CSF higher than the levels seen in basal, however, ambient air sample showed an increased level.

Table 6.4 Fold stimulation of all 8 analytes in Chitwan with different extract samples. The data shown are mean (range, min-max) fold increase with each extract samples than the levels seen with the basal condition for each analyte (n=8 independent experiments).

	TCS	LPG	Ambient
IL-8	2.61 (0.82-3.63)	4.12 (2.71-6.31)	2.38 (1.16-3.69)
IL-6	1.44 (0.81-2.01)	1.04 (0.39-1.76)	0.94 (0.66-1.28)
TNF-α	2.25 (1.22-3.75)	1.6 (0.74-2.61)	1.53 (0.85-2.72)
IL-1β	5.72 (1.86-22.19)	2.91 (1.2-7.47)	1.91 (1.51-3.09)
MCP-1	1.07 (0.49-1.92)	0.86 (0.48-1.51)	0.81 (0.53-1.3)
MIP-1α	0.98 (0.63-1.12)	0.89 (0.57-1.08)	0.94 (0.56-1.16)
GM-CSF	1.35 (0.88-1.66)	0.72 (0.19-1.44)	1.16 (0.39-1.64)
Eotaxin-3	1.3 (0.92-1.84)	1.27 (0.92-1.78)	1.3 (0.77-1.75)

6.4.7 Inflammatory response comparison between different fuel sources

Having demonstrated the response of smoke extract samples collected from different sources in different monitoring sites, I then investigated the overall variation in pro-inflammatory cytokine response in HBECs with smoke extract samples from TCS, ICS and LPG. The overall fold stimulation of 4 of 8 analytes was higher than the levels seen in basal condition of respective analytes for all three smoke extract samples. The fold stimulation of these four analytes with all three smoke extract samples is presented in Figure 6.9. The fold increase of all other analytes with all three smoke extract is shown in Table 6.5.

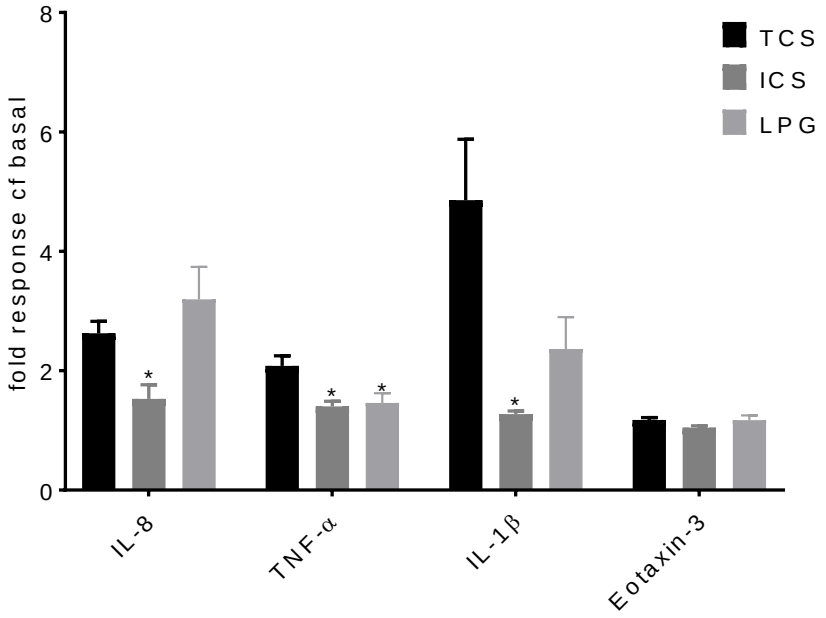

Figure 6.9 Smoke extract samples from TCS, ICS and LPG all enhanced the production of pro-inflammatory cytokines in HBECs. Overall mean fold increase of IL-8, TNF-α, IL-1β and Eotaxin-3 were observed for all three smoke extract samples. The data shown are mean (±SEM) fold increase for each analyte with different extract samples. The overall fold increase with TCS was compared with the overall mean fold increase with LPG and ambient using Kruskal-Wallis test followed by Dunn's multiple comparison test.

Except for fold increase of IL-6 and GM-CSF, fold stimulation of all other analytes were higher with TCS sample than with ICS and LPG samples. The fold stimulation was higher with ICS sample than TCS and LPG sample for IL-6 and GM-CSF. The fold stimulation of IL-8, IL-1β was significantly higher in TCS than in ICS ($p<0.05$); however the induced level of GM-CSF was significantly higher in ICS than in TCS. Similarly, the fold stimulation of TNF-α was significantly higher in TCS than in ICS and in LPG. Though the fold increase of other analytes showed higher levels

with TCS than both ICS and LPG, none of the variations was significant. This might be because of a small number of samples for each case.

Table 6.5 Overall fold stimulation of all 8 analytes with extract samples from different sources. The data shown are mean (range, min-max) fold increase with each extract samples than the levels seen with the basal condition for each analyte (n=8 independent experiments).

	TCS	ICS	LPG
IL-8	2.62 (0.82-5.55)	1.52 (0.97-2.15)	3.19 (1.24-6.31)
IL-6	1.29 (0.4-2.27)	1.76 (0.74-3.06)	0.93 (0.16-1.76)
TNF-α	2.08 (0.95-3.94)	1.4 (1.13-1.61)	1.46 (0.74-2.61)
IL-1β	4.85 (0.76-22.19)	1.27 (1.08-1.46)	2.36 (1.01-7.47)
MCP-1	1.25 (0.5-2.17)	1.07 (1.03-1.13)	0.91 (0.48-1.51)
MIP-1α	1.01 (0.6-1.16)	1.03 (1.01-1.06)	0.93 (0.57-1.08)
GM-CSF	1.17 (0.42-2.14)	1.52 (1.34-1.62)	0.94 (0.19-1.52)
Eotaxin-3	1.17 (0.8-1.84)	1.04 (0.92-1.14)	1.17 (0.88-1.78)

6.4.8 Comparison of Pro-inflammatory response from human lung tissue and HBECs

Finally, inflammatory responses seen in lung tissue and HBECs stimulated with smoke extract samples from different sources were compared. Figure 6.10 presents the overall mean fold stimulation of analytes that were significantly elevated in both lung tissue and HBECs with smoke extract samples from TCS, ICS and LPG respectively. It can be seen from the figure that only TNF-α and IL-1β were significantly elevated in both lung tissue and HBECs with all three smoke extract samples. Whilst the elevated level of IL-8 was only significant with TCS and LPG, the level of IL-6 was significant with only TCS samples. However, it should be noted that levels of both IL-8 and IL-6 were significantly higher in lung tissue with extract samples from ICS (data not shown here). Also, the elevated level of MIP-1α was significant with both TCS and ICS samples only in lung tissue, while GM-CSF level was significant with both TCS and ICS samples in HBECs, while it was significant with only ICS in lung tissue samples.

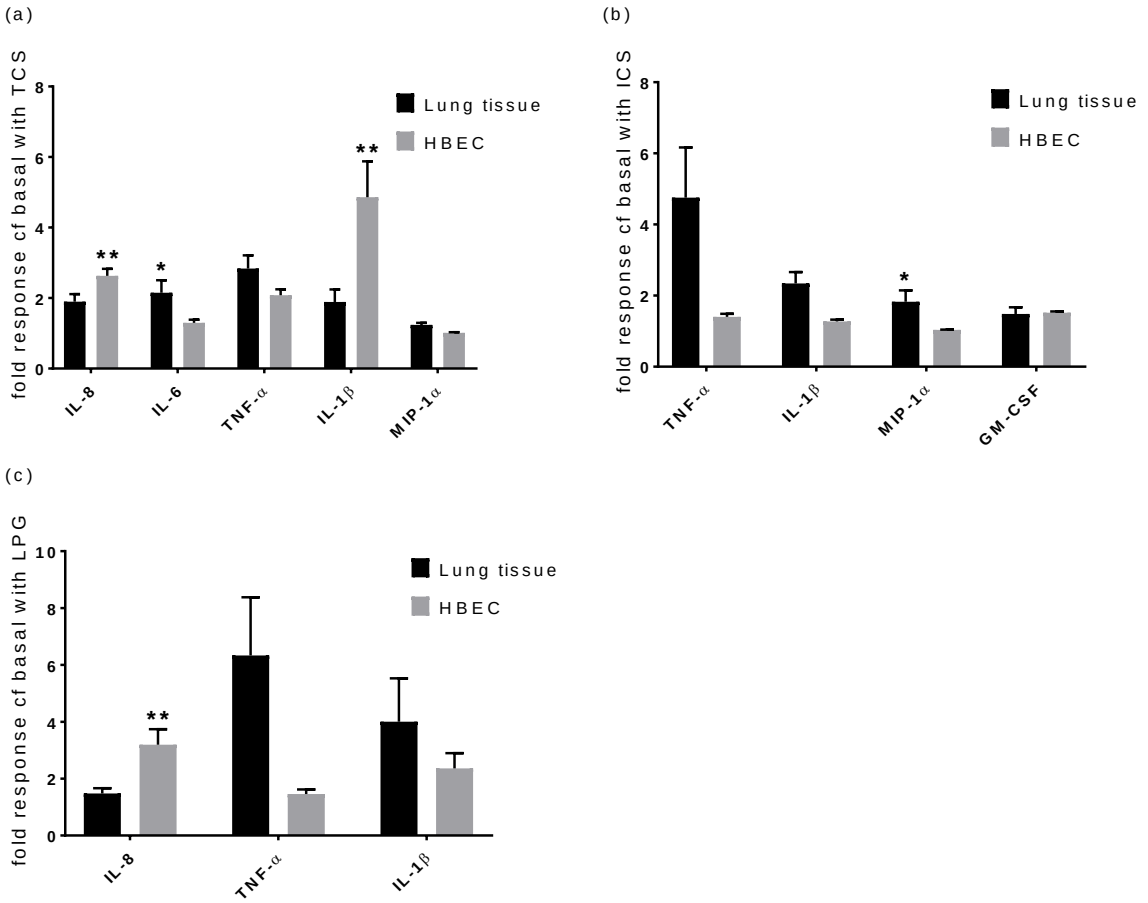

Figure 6.10 Comparison of pro-inflammatory response seen in lung tissue and HBECs with smoke extract samples from different sources (a) TCS, (b) ICS and (c) LPG. The data shown are the mean (±SEM) fold stimulation for each analyte. Variations between lung tissue and HBECs for each analyte was compared using Mann-Whitney t-test (*=p<0.05, **=p<0.01).

Comparing the results, it can be seen that the overall mean fold stimulation of IL-8 was higher in HBECs than in lung tissue with both TCS and LPG smoke extract samples. Similarly, the fold increase of IL-1β was also higher in HBECs than in lung tissue with TCS; however, the levels were higher in lung tissue with ICS and LPG. All other analytes presented in Figure 6.10 showed that the mean fold increase was higher in lung tissue than in HBECs for all smoke extract samples.

6.5 **Discussion**

The overall aim of this chapter was to assess potential inflammatory effects of smoke exposures in HBECs and to investigate the contribution of HBECs to the inflammatory response observed in *ex vivo* human lung tissue. The work presented in the previous chapter showed that smoke extract collected in real-life cooking produced an inflammatory effect in human *ex vivo* lung tissue. In this chapter, I set out an objective to repeat key experiments using HBECs to assess if the lung epithelium contributes to the inflammatory responses observed in human lung tissue. Overall results show that smoke extract samples generated in the field during cooking produce an inflammatory response in HBECs, suggesting a contribution of epithelial cells to the overall response seen in human lung tissue.

Poly(I:C) was used as a positive control in these experiments because of its known inflammatory effects on human cells [313, 317]. As expected, poly (I:C) increases the production of 8 of 10 analytes assayed, which is in line with the findings presented in previous studies [314, 318]. Significant increases in levels of IL-8, IL-6, TNF-α, IL-1β, MCP-1, GM-CSF and Eotaxin-3 were observed in HBECs stimulated with smoke extracts. This suggests that inhaling biomass smoke generated during cooking activities in real-life elicits an inflammatory response in lung epithelium. Previous studies have already shown that smoke from biomass combustion includes toxic products [17] and findings from this study also

suggest that smoke extract collected in real-life cooking is toxic. This can be further supported by the responses seen in HBECs with the extract samples as airway epithelial cells interact and produce a variety of inflammatory cytokine and chemokines in response to inhaled toxic irritants [130, 301]. In addition to this, there are convincing amount of data available showing the toxic effect of cigarette smoke on airway epithelial cells [306, 319]. Also, studies have shown that biomass smoke samples collected from different sources produce a pro-inflammatory response in human cells consistent with our findings. A biomass smoke extract collected from the combustion of different wood types showed increased levels of IL-8 and IL-6 from primary lung fibroblast [320]. Similarly, an increased level of IL-6 has been shown in a study conducted by Roscioli et al., where small airway epithelial cells were stimulated with wildfire smoke extract [321].

Effect of using an efficient stove and LPG stove in the inflammatory response in HBECs were also assessed by incubating cells with the extracts samples collected in those sources. Similar to the responses seen in human *ex vivo* lung tissue samples, using extracts from ICS and LPG also produced an inflammatory response in HBECs. Significant elevation of TNF-α, IL-1β, MIP-1α and GM-CSF were seen in HBECs stimulated with smoke extract samples from ICS, whereas IL-8, TNF-α and IL-1β were significantly elevated with extract samples from LPG. In comparison to the responses seen in human lung tissue, ICS induced significant production of more cytokines in human lung tissue than HBECs, while

cytokine responses were similar in both tissue and cell for LPG smoke extract. This suggests that epithelial cells are not the only cells responding to smoke extract samples and that other cells present in lung tissue samples also contributed to the responses. Taken together, despite using more efficient stoves and LPG stoves, the responses seen in both tissue and HBECs further suggest and support the need to intervene additional methods other than those currently being trialled to reduce exposure and to improve lung inflammation in this settings.

In general, cytokine responses were higher in lung tissue than in HBECs. This further suggests that the responses seen in human lung tissue were not only from lung epithelium and supports the idea that other inflammatory cells present in the lung also contributed to the response. The most likely additional contributions probably come from macrophages and neutrophils. However, more *in vitro* studies to look into the effect of biomass smoke exposure using different cell types need to be carried out to find the actual cytokine profile from each cell type and the relative contribution of each cell type in the overall response.

In conclusion, these data further support the findings presented in the previous chapter and show that smoke extract collected in real-life cooking also produced an inflammatory response in HBECs.

Chapter 7: General Discussion

7 General Discussion

The work described in this thesis set out to address the issue of solid biomass combustion in rural Nepal and the potential effects on the respiratory system caused by exposure to biomass combustion.

7.1 Problem statement and Aims

Solid biomass has been a primary source of fuel for cooking in low and middle-income countries for many years, and it is estimated that 1.8 billion people will still be using biomass for cooking by 2040 [30]. The combustion of these biomass fuels generates high concentrations of pollutants including particulate matter, carbon monoxide and other health damaging pollutants which have been predicted to result in about 3.8 million premature deaths worldwide [48]. A considerable number of studies have already linked biomass smoke exposure to increased risk of developing chronic lung diseases such as COPD [11, 322]. However, very little is known about the inflammatory response and the mechanisms underlying responses to short term exposures in a human lung. Several previous studies have monitored HAP in rural homes using biomass fuel for cooking; however, there is a lack of sufficient data on personal exposure to PM during cooking using biomass in a 'real-life' situation. ICSs with a flue system to vent smoke out of the room have been shown to be at least a partially effective intervention in reducing indoor exposures. In addition, a significant reduction in exposures in homes using LPG has been demonstrated in previous studies [119]. However,

studies assessing the health impact of using ICS or LPG have shown inconsistent findings, and none of the earlier studies has evaluated the effects of using ICS on inflammatory responses in a human lung.

Given a lack of exposure data and experimental data to show the inflammatory response to biomass smoke exposure in LMICs, this study set out to monitor real-life personal exposures during cooking in homes in rural Nepal and to gain insight into the potential effects of exposure of human tissue to respirable material generated from biomass combustion for cooking. Having seen inconsistent findings on the health impact of using ICS in previous studies, I also aimed to monitor exposure levels in homes using ICS and to assess potential consequences of reducing exposures using ICS on inflammatory response in human lung tissue. Furthermore, as it has been shown in a recent report published by Central Bureau of Statistics, Nepal that ~21% of total households in Nepal use LPG stoves as a cooking fuel [323], I also aimed to assess the effect of using LPG on personal exposure and inflammatory response in a human lung.

7.2 **Main Findings**

The data presented in this thesis show that biomass smoke extract generated in real-life cooking from rural households produce an inflammatory response in human *ex vivo* lung tissue. Elevated levels of a range of pro-inflammatory cytokines were observed in tissue culture

supernatants collected 48 hours post-stimulation with smoke extracts. This suggests that smoke extract generated in real-life cooking elicits an inflammatory response in lung tissue that may lead to lung inflammation in real-life settings. The inflammatory effect seen in human lung tissue would occur where high levels of exposures are present in households using biomass fuel for cooking. The exposure data presented in this thesis show that combustion of biomass fuels for cooking on traditional stoves generates high levels of indoor pollutants in the range that would be considered harmful to human health by WHO [18]. The overall mean exposure of $PM_{2.5}$, for example, in households in Nepal using traditional stoves was 276.1 $\mu g/m^3$ which is about 11 fold higher than the WHO safe recommended upper limit concentration of 25 $\mu g/m^3$ [18]. A significant reduction in exposure levels was observed in households using a more efficient biomass cook stove and in homes using LPG in these settings. However, significant elevation of inflammatory cytokines was still observed in human lung tissue stimulated with smoke extract samples collected from these more efficient stoves and LPG stoves. This suggests that exposure reduction levels may not be sufficient to prevent inflammatory effects in human lung, and it remains highly likely that lung inflammation will still occur in real-life settings, despite of using low emission cooking methods.

Concerning the previous studies showing emissions from the combustion of biomass fuel depends on fuel type [245, 247] and cook stove design

[19, 102], I also undertook work to monitor emission factors of commonly used biomass fuels in Nepal. Various biomass fuels collected from different parts of Nepal were combusted in a traditional stove in controlled laboratory settings, and resultant emissions from each of the fuels was measured. The findings of this study showed that burning of agricultural waste emits higher emissions than burning of fuelwood, consistent with the results presented in the previous studies [245, 247]. Further, the exposure from the combustion of different biomass fuels for real-life cooking in rural households of Nepal was conducted where real-life personal exposure to $PM_{2.5}$ and CO were monitored during the cooking period. This study was then extended to four different villages located in different ecological regions of the country partly because instead of using a single type of biomass fuel, a combination of all available biomass fuels are used in rural households in real-life cooking.

While there were some differences in the absolute exposures observed between different households and in different geographical regions, the overall findings show that generally high levels of pollutant exposures were seen in homes using biomass fuel for indoor cooking. The findings of this work are consistent with the exposure levels presented in previous studies conducted in different regions of the country [19, 20]. However, as mentioned, the overall magnitude of pollutants does vary to some extent in different ecological regions within this study. This suggests that possible variations in the social and cultural cooking practices followed, use of different kinds of biomass fuel and combustion devices, and also a

difference in altitude between the regions could all be influencing factors. Several previous studies examining the effectiveness of ICS [116, 117] and LPG [119] in the reduction of pollutant exposures have reported findings consistent with the results presented in this thesis. There was a significant reduction in exposure levels in households using ICS and LPG compared with households using TCS. However, the levels seen in houses with ICS and LPG in this study and other previous studies were well above the threshold level recommended by WHO. This further suggests that though the exposure reduction is likely to be of benefit for human health, additional measures to reduce the levels below the WHO recommended safe level would be needed to yield maximum benefit to human health [23, 24, 324].

Smoke extract samples collected during real-life cooking from rural households produced an inflammatory response in *ex vivo* human lung tissue for IL-8, IL-6, TNF-α, IL-1β, MCP-1, MIP-1α and MCP-4. Though this is a first study to use *ex vivo* lung tissue to investigate the inflammatory effect of biomass smoke extract that is collected in real-life settings in Nepal, the findings of this work can be related to other studies showing inflammatory effects in different cell lines [13, 325]. The presence of endotoxin in smoke extract samples potentially suggests that the inflammatory response in human lung from biomass smoke exposure could be driven through TLR activation. However, it is probable that the inflammatory response can also be driven by other pathways, given that biomass smoke contains other active compounds [17] that cause

inflammation. A study showing no significant changes in the inflammatory effect of biomass smoke extract on human small airway epithelial cells after removable of endotoxin from it further supports this argument [13].

A significant response in human *ex vivo* lung tissue for IL-8, IL-6, TNF-α, IL-1β, MIP-1α, GM-CSF and Eotaxin-3 was seen with the smoke extract samples generated in ICS. Similarly, smoke extract samples from LPG stove also showed elevated levels of IL-8, TNF-α and IL-1β, suggesting an inflammatory response in human *ex vivo* lung tissue occurs even when LPG is the fuel source. Though there is no evidence of *in vitro* and *in vivo* studies assessing the effectiveness of using ICS and LPG on lung inflammation, the findings of this study can be compared with studies showing no significant improvements in lung function after installation of improved cook stoves [23, 24]. The responses seen with ICS and LPG could be due to the exposure levels remaining higher than the WHO safe guidelines; however, further in-depth field and laboratory studies would be needed to identify the actual contributing factor in the smoke extract causing inflammation. The inflammatory effect of smoke extract samples was further examined in HBECs by repeating key experiments and measuring pro-inflammatory cytokines in cell culture supernatant *in vitro*. Similar to the responses seen in human lung tissue, elevated levels of a range of pro-inflammatory cytokines were seen in HBECs. However, the overall fold stimulation of cytokines was lower in HBECs than in lung tissue suggesting there is likely to be an involvement of other cells such

as macrophages and neutrophils in the production of inflammatory cytokines in lung tissue.

The pro-inflammatory cytokines produced in lung tissue may be secreted by different immune cells and structural cells and have a variety of effects in inflammation and pathogenesis of airway disease like COPD [155, 326, 327]. These pro-inflammatory cytokines play an essential role in mediating acute inflammation: IL-8, IL-6, IL-1β and TNF-α are all potent inflammatory mediators for acute inflammation [328]. The levels of these pro-inflammatory cytokines seen in culture supernatant suggest the initiation of an inflammatory response in *ex vivo* lung tissue which on prolonged exposure could lead to the lung inflammation and progression of inflammatory airway diseases.

Whilst a range of pro-inflammatory mediators were seen in human lung tissue in response to smoke extract samples, a key question is whether or not responses seen can be related to the real response in human lung *in vivo*. This is important as I have utilised lung tissue samples of a patient in the UK and smoke extract samples were collected in rural households in Nepal. Although plenty of people in the UK have wood burning stoves for heating (solid biomass fuel contributed 5.1% of total UK non-electric energy consumption in residential sectors [329]), it is highly likely that the patients donating tissue samples have never been exposed to high levels HAP from biomass combustion for cooking. So it is expected that the responses seen with these tissue samples may be different from the

real responses to human lung of indigenous people actually using biomass fuel for cooking, who might either be partly desensitised, or alternately sensitised by chronic long term exposure. It is not feasible to do bronchoscopy in the settings used in these studies and almost impossible to transfer human biological samples from Nepal to the UK, so the approach used was the best we could devise to utilise available tissue samples from the UK donors. It has also been shown in previous studies that the inflammatory effect of particulate matter was higher in exposed or inflamed tissue than in healthy unexposed tissue. A recent study conducted by Qing et al. had used uninflamed nasal tissue and nasal polyp tissue samples from patients with type-2 inflammations to study the inflammatory effect of ambient $PM_{2.5}$ [330]. The findings of this study showed that under the same *ex vivo* stimulation conditions, healthy tissue produced a significant elevation of IL-8, whereas inflamed tissue produced significant elevation of IL-1β, IL-5, IL-6 and TNF-α. This suggests that $PM_{2.5}$ initiate an inflammatory response in healthy tissue, whereas the inflammatory effect of $PM_{2.5}$ in inflamed tissue was even higher. These findings hence indicate that it is highly likely that the inflammatory effect of smoke exposure in real-life settings may be even higher than the responses seen in tissue samples in this study, because of already established inflammation due to prolonged biomass smoke exposure. In addition to that, the high prevalence of inflammatory lung disease like COPD in rural Nepal [331, 332] also implies the potential effect of biomass smoke exposure causing lung inflammation in these settings.

7.3 **Limitations of the study**

The findings of this thesis show clearly that high levels of exposures occur in households using biomass fuel for cooking and that these are likely to induce lung inflammation in real-life settings. However, there are some potential limitations of the current study. First, though the monitoring sites were selected to represent all three ecological regions of the country, the data only represents a few villages, and hence additional studies are required in other areas to have comprehensive exposure data in Nepal. Whilst the study also provides exposure data from a high altitude region (~4000 m above sea level), which proved to be very difficult to conduct in terms of difficult accessible to the site and difficult for research staff working in a lower oxygen setting, studies including more villages at higher altitude regions are required given the lack of HAP exposure data in these regions. Secondly, whilst the cooking period exposures were mainly contributed to from the combustion of biomass fuel, relatively high levels present during non-cooking period exposures in some sites suggest there may be a contribution from poor ambient air quality: this is likely to be a particular issue in villages in the Terai region. In these studies, although I was able to collect a few ambient air samples, the actual contribution of ambient air pollution in addition to the indoor pollutants is unclear. The potential sources of ambient air pollution will be different in different regions, and it has been shown in previous studies that ambient air pollution contributes to poor indoor air quality [270, 271]. Hence, defining the actual contribution of ambient air pollution to

indoor air quality in each of these settings will require additional work to provide comprehensive data on the interactions between indoor and ambient air quality.

Using low cost aerosol monitoring sensors for real-life personal exposure at a flow rate lower than the actual breathing volume of a normal healthy human may have resulted in underestimating true personal exposure. The real-life exposures were measured for cooking periods at a flow rate of 2.8 L/min, while the actual breathing volume is believed to be 6 to 10 L/min. Hence, the real personal exposure in these settings might be higher than the exposure levels presented in this thesis. In addition to this, while the data in this study only provides exposure data for a single cooking episode, this will underestimate total exposure throughout the day from all cooking activities (at least two main cooking sessions in all villages). The effect of long term exposure (as opposed to acute exposure) in these regions will also be an issue as people have been using biomass fuel for many years.

In this study, I tried to best mimic lung inflammation to biomass smoke exposure by incubating human *ex vivo* lung tissue fragments with biomass smoke extract samples generated in real-life cooking. However, it is difficult to accurately model true lung exposure even using the approach I have utilized because of some limitations. Samples were generated in real-life settings and stored at -20° C during transport from the field to the laboratory. Samples were handled carefully during storage

and transfer, and though all samples were in an initial frozen state when they reached the Nottingham laboratory, it is still possible that some active components may have been lost during sample storage and transfer. Furthermore, the dilution factors I estimated and used in experiments may have underestimated true inflammatory effects on the human lung. As mentioned samples were collected using a pump set at 3 L/min into a volume of 10mls of a medium, the final concentration human lung tissue exposed to was a 10% (v/v) dilutions of this extract. In the human lung, several factors including the dynamic equilibrium between inhaled and exhaled material, ventilation rates, the amount of lung lining fluid, the effective volume of distribution of inhaled material all come into play in estimating actual tissue exposures. The volume of the lung lining fluid is believed to be around 20-50mls [333] but the true volume of distribution of the active components of inhaled biomass smoke will vary depending upon the physicochemical properties of the constituent being considered. Hence it is a challenging task to calculate the actual dilution factor of the biomass smoke extract to be used to stimulate tissue samples so that stimulations can be made comparable to real-life exposure. It is possible, therefore that I may have underestimated the true local exposure in the lung using the experimental design I adopted. None the less I have tried to model real exposures as closely as possible. Future studies with similar approaches could be performed using various concentrations of smoke extract to obtain information on dose responses in tissue samples.

In addition, another limitation of this study is the lack of knowledge the chemical characterization of the smoke extract samples to identify the major active components responsible for the inflammatory response seen in lung tissue and HBECs. I have quantified endotoxin levels in the smoke extract samples; however, there may be other biologically active components present in the extract producing lung inflammation, providing the fact that endotoxin is not the only active component in biomass smoke extract that produces inflammatory effects [13]. Hence defining other active components in the smoke extract sample other than endotoxin that may contribute to the lung inflammation is something to consider.

The findings of this study would have been more relevant if I could use biological samples like tissue or cells from the indigenous people who are being exposed to biomass smoke in real-life. However, it would be challenging to conduct bronchoscopy in these real field settings and in addition to that it is nearly impossible to transfer biological samples from Nepal to the UK. Furthermore, due to the lack of proper infrastructure and laboratory facilities in developing countries like Nepal, studies utilizing biological samples from indigenous people seem to be impossible at present. However, in future studies using biological samples from the actual people being exposed in real-life settings need to be conceived and take into consideration.

7.4 **Future Directions**

Given the importance of personal exposure data and the fact that there is a lack of personal exposure data in Nepal, exposure monitoring studies need to be extended in other villages of the country, and other parts of the developing countries as well. Whilst cooking period exposures provide mean exposure for a single cooking episode, study monitoring personal exposures for at least 24 hours is important because that would help to predict more prolonged exposure and its health effect on a longer-term. The data lack in this study was the establishment of the interrelationship between indoor and ambient air quality in each of the monitoring site. Having understood the equal importance of ambient air quality and its contributions to indoor air, a future study assessing the health impact of air pollution should focus on both indoor and ambient air quality in each monitoring sites. Finding interrelationships and contribution of ambient air pollution on indoor exposure would help to estimate the actual burden of air pollution in respective villages and help to implement potential measures to reduce air pollution in a holistic approach.

The inflammatory response to short term exposure of biomass smoke extract samples in human lung tissue and HBECs suggest initiation of acute inflammation due to the exposure in the lung. Prolonged exposure to these pollutants in real-life could lead to chronic inflammation and eventually to chronic lung disease such as COPD. A mechanistic study to assess the long term effect of biomass smoke exposure on lung

inflammation needs to be designed and conducted. In addition to experimental studies, population-based observational studies to measure physiological changes such as lung function, blood pressure due to biomass smoke exposure is essential. Studies conducted in a different part of the world have shown lung function decline [23, 334] and an increase in systolic blood pressure [43] in a population using biomass fuel for cooking. A recent study conducted in Kyrgyzstan has shown that people living in high altitude region using biomass fuel were exposed to high levels of HAP and COPD prevalence were also higher in the highlands [335]. Whilst the findings of that study support the data presented in this thesis, it also suggests for further observational studies to see the changes in lung function due to exposures in these higher altitude settings. The inflammatory response in lung tissue with smoke extract sample from ICS observed in this thesis can be related to the findings of a cohort study conducted in rural Guatemalan women to see the effect of a chimney stove on lung function [23]. Population-based observational study to assess lung function in Nepalese population using biomass fuel in a traditional stove or improved cook stove would be an essential future direction of this current work, provided limited data on lung function measurements in rural areas in Nepal [336]. In addition to this, the prevalence of chronic lung disease estimate in Nepal is likely to be under estimated, it is of utmost importance to conduct longer follow up studies to estimate the prevalence of chronic lung disease in Nepal. This is not only necessarily include long term exposure to biomass smoke but also

include other air pollution sources in both rural and urban areas of the country.

The data presented in this thesis indicate that additional methods other than those currently being used in these settings are needed to reduce exposures below the level that would be considered safe by WHO. Alternative clean fuel sources other than biomass fuel like LPG, electricity or solar energy would be the ideal solution to reduce exposures and to improve respiratory health in rural settings like these [185]. However, due to lack of infrastructure, poor social and economic conditions of people living in these villages, switching to alternative fuel is not feasible. Biomass cook stove with improved design and better combustion efficiency is likely to be a viable alternative solution to reduce exposure levels [337]. Data showed the potential lung inflammation despite of exposure reductions using existing improved cookstove in this settings, and hence more research work would be needed to improve the stove design further or enhance the combustion efficiency of the stoves currently being used.

Finally, as the approach we have utilised of collecting samples in real-life to use in human lung tissue fragments is likely to be an effective method to observe lung inflammation, it can be further utilised to monitor inflammatory effect of source dependent air pollution other than biomass burning for cooking including garbage burning, brick kilns, vehicular emissions and agricultural burning.

7.5 **Final Conclusion**

The findings of this thesis provide further support the fact that biomass combustion generates pollutants, including particulate matter, carbon monoxide and people combusting biomass fuel for cooking inside homes are exposed to high levels of those pollutants. The real-life exposures to pollutants in homes using biomass fuel for cooking were different in different regions, suggesting potential variations in fuel type, stove designs, cooking practices followed and combustion characteristic due to altitude variations among the regions. A significant reduction observed in real-life exposure using more efficient stoves further clarify the effectiveness of improved cook stove being trailed in these villages. However, the levels of exposure seen in homes using efficient stoves were still several fold higher than the safe exposure levels recommended by WHO [257]. This further indicates that alternative measures other than those currently being used may be needed in these regions as a significant proportion of population are still exposed to a higher concentration of HAP despite using efficient stoves.

The findings presented in this thesis also provide further evidence that biomass smoke exposure is pro-inflammatory to human lung leading to lung inflammation, which could be the basis for further investigation in the development of the respiratory diseases. Similar inflammatory response in lung tissue and HBECs with the smoke extract samples generated in more efficient stove indicates that the exposure reductions

are not sufficient to prevent lung inflammation and people having efficient stove still remain likely to be at risk of developing airway disease. Furthermore, the potential causes of inflammation observed with the extract samples generated in LPG are a fundamental question for further investigation.

In summary, for the first time, I have shown that smoke extract collected in real-life settings produced an inflammatory response in human lung tissue and HBECs. These data further suggest that improvements on cook stove designs currently being used or further additional interventions may be needed to reduce exposures to safe levels which will prevent lung inflammation from occurring in real-life settings.

8 Appendices

8.1 **Appendix I: Equipment, material and reagent**

Exposure monitoring equipment and materials

Item	Supplier
Aerocet 831	Met one Instrument, Inc. USA
Aethalometer AE33-7	Magee Scientific Aethalometer Co, USA
E-Sampler	Met One Instrument, Inc, USA
GRIMM-EDM-180D	Grimm Aerosol technikGmbH&Co, Germany
Indoor air pollution meter	Aprovecho Research centre, USA
Indoor air quality meter	GrayWolf® Sensing solutions, USA
Laboratory emission monitoring system	Aprovecho Research Centre, USA
MicroAeth AE51	AethLansmicroAeth® AE51, USA
Micro CO meter	CareFusion, UK
Pulse Oximeter	Microlife® AG, Switzerland
Cyclone	URG Corporation, USA (URG-2000-30E-5-2.5-S)
Filter Holder	URG Corporation, USA (URG-2000-30RAF-1)

General materials

Item	Supplier
Dimethyl Sulphoxide (DMSO)	Sigma, 1304
Endotoxin free water	Sigma Aldrich
Ethanol	University of Nottingham stores, LH-M0120E
Flexstation 3 microplate reader	Molecular Devices
Human IL-8 Duoset ELISA	R&D Systems
Human IL-6 Duoset ELISA	R&D Systems

Sulpheric Acid (2N)	SLS, CHE3688
Trypsin-EDTA solution	Sigma-Aldrich
Trypsin inhibitor	Sigma Aldrich
Tween® 20	Sigma-Aldrich, P-1379
96 well assay plate	Coring, 9170
BEGM Basal Medium	Lonza, CC-3171
2 ml 13mg/ml bovin pituitary extract 0.5ml mg/ml hydrocortisone 0.5ml 0.5ug/ml human recombinant epidermal growth factor 0.5ml 0.5mg/ml epinephrine 0.5 5mg/ml insulin 0.5ml 0.1ug/ml retinoic acid 0.5ml 6.5ug/ml triiodothyronine 0.5ml 50ug/ml gentamicin/amphotericin B	Lonza, CC-3170
DMEM culture media	Sigma D5796, lot RNBD0057
Dulbecco's Phosphate buffered saline	Sigma-Aldrich
HBEC primary cells	Lonza
T75 tissue culture flasks	Corning
Limulus amebocyte lysate assay	ThermoFisher Scientific
Lipopolysaccharide (LPS)	Sigma L2654-1mG lot 043M4033V
Poly(I:C)	Sigma-Aldrich
Tyrode's duffer	Sigma T2397
RNA later	Sigma R0901 lot SLBG1157
Formaldehyde	Sigma 252549 lot SZBC2830V
Dichloromethane	Sigma-Aldrich 650463-1L-D
Nanodrop 2000c UV/IV Spectrophotometer	Thermo Scientific
Dexamethasone	Sigma Aldrich D4902

8.2 **Appendix II: Patient Demographics**

The donor sample ID represents study number and donor sample number: 'S' followed by the number represents study number and 'D' followed by the number represents donor number. BMI: Body Mass index, FEV_1: Forced expiratory volume in first 1 second, FVC: Forced Vital Capacity, PH: Pulmonary Hypertension, HP: Hypersensitive pneumonitis, IPF: Idiopathic Pulmonary fibrosis.

Donor Sample ID	Age	Gender	BMI	FEV1 % predicted	FEV1/FVC (%)	Smoking	Disease condition
S61 D034	68	M	20.83	26.6	37	Ex smoker	Severe COPD
S61 D035	50	F	N/A	54	58.5	Ex smoker	PH
S61 D036	49	M	30.6	55	74.7	Ex smoker	HP
S61 D037	46	F	21.3	121.4	88.8	Ex smoker	Cancer
*S54D107							
S61 D039	71	M	34.83	58.4	37.5	Ex smoker	COPD
S61D043	62	F	23.09	104.9	70	Ex smoker	Cancer
S61 D044	70	M	31.46	86.1	68.7	Ex smoker	Cancer
S61D045	56	M	21.88	98.9	87.7	Ex smoker	IPF
S61 D046	63	M	28	42	85.7	Never	IPF
S61D047	65	M	27	95.7	79.8	Ex smoker	Cancer
S61 D048	79	F	26.31	124.4	67	Ex smoker	Cancer
S61D049	60	F	28.9	96.7	66	Never	Cancer
S61 D051	74	M	N/A	82.2	59	Ex smoker	Cancer
S61 D053	69	F	23.93	52.4	46.3	Current	Cancer
S61 D054	N/A	N/A	24.9	105	69	Ex smoker	Cancer
S61 D055	72	F	31.73	107.8	70.7	Never	Cancer
S61 D057	55	M	32.92	80.2	67.5	Ex smoker	cancer
S61 D059	74	M	23.15	91.9	61.9	Current	Cancer
S61 D061	82	M	25.96	99	67	Never	Cancer

*clinical details not available.

8.3 **Appendix III: Assay detection ranges of analytes measured using Luminex**

Cytokine	*Standard Curve range (pg/mL)*
IL-8/ CXCL8	9-5020
IL-6	11.3-5640
TNF-α	9.3-6590
IL-1β	10.8-9220
MIP-1α	22.5-14850
GM-CSF	6.5-5380
Eotaxin-3/CCL26	1.5-13310
MCP-4/CCL13	19.3-6050
MIP-1β/CCL4	5-8740
MCP-1/CCL2	1-16600

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