INDOOR AIR POLLUTION FROM BIOMASS FUEL SMOKE AND ITS EFFECT ON RESPIRATORY HEALTH, IN A POPULATION AT RISK OF HIV RELATED PNEUMONIA

THESIS SUBMITTED IN ACCORDANCE WITH THE REQUIREMENTS OF THE UNIVERSITY OF LIVERPOOL FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

DUNCAN GORDON FULLERTON

MB ChB, MRCP, DTM&H

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DECLARATION

This thesis is the result of my own work, except where indicated below. Studies in this thesis were done in conjunction with other projects and in some instances work was shared with colleagues. My contributions for the reported work are shown below.

Activity	Responsibility
Endotoxin content analysis	Prof P Thorne. Environmental Health Science Research Centre, University of Iowa
Transition metal content	Institute of Occupational Medicine, Edinburgh.
Image analysis Software	Dr S Barrett, Dept of Physics, University of Liverpool
Bronchoscopy	Shared
Air sampling measurements	Shared
Spirometry measurements	Shared
Sample processing	Shared
Image analysis and acquisition	Shared
ELISA	Shared
Reporter bead assays	Shared
Flow cytometry analysis	Sole
Statistical data analysis and presentation	Sole
Thesis Preparation	Sole

This research was carried out at Liverpool School of Tropical Medicine and the Malawi-Liverpool-Wellcome Clinical Research Programme.

The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or other qualification.

Dr Duncan G Fullerton
University of Liverpool.

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ABSTRACT

Background: Three billion people use biomass fuel (BMF) as the main source of household energy. Indoor air pollution (IAP) from BMF smoke is associated with lower respiratory tract infections and low birth weight in children; COPD and TB in adults. 3 hypotheses were tested in this thesis: IAP levels in Malawian homes are high; BMF smoke exposure is associated with impaired lung function; Particulate matter exposure (PM) impairs alveolar macrophage function.

Methods: In urban and rural homes in Malawi, 4 different air sampling devices were located to measure levels of PM and carbon monoxide. Questionnaire data were collected to identify risk factors for COPD; Spirometry was measured. In Malawi and the UK adult volunteers underwent bronchoalveolar lavage (BAL). The *in vivo* human alveolar macrophage (HAM) appearance was reproduced *in vitro* by challenging macrophages with PM. Dose and time dependent experiments were performed *in vitro* to assess PM phagocytosis by HAM and monocyte derived macrophages (MDM). The effect on inflammatory cytokines (IL-6 and IL-8) secretion by HAM and MDM *in vitro* exposure to PM was measured by ELISA. HAM functions were assessed using a novel reporter bead flow cytometry assay. Oxidative burst, proteolysis and phagocytic activity were compared by HAM PM load, using a novel image analysis method.

Results: In 80% of 74 homes sampled, the PM levels measured were four times greater than the WHO safe level for indoor air quality. 374 individuals performed spirometry. Data showed that wood smoke and lower socioeconomic status are both associated with impaired lung function. PM load in HAM was associated with the type of BMF used. An increase in the dose and incubation time of PM, led to greater ingestion of PM by MDM and HAM. Secretion of IL-6 and IL-8 was increased by increasing the dose of PM. HAM produced more IL-6 and IL-8 compared to MDM. In HAM, impaired oxidative burst was observed with higher PM load.

Conclusion: This thesis is the first description of IAP and the effect it has on lung function in Malawi. It explores an *in vitro* model and increases the understanding of the mechanisms behind lung damage. It describes a biomarker that can be used to support epidemiological associations as well as to plan effective and appropriate interventions to reduce exposure.

RELATED PUBLICATIONS

Original papers

1. **Fullerton DG,** Rylance J, Tolmie H, Scriven J, Nasser A, Swann N, Baple K, Knott A, Barrett S, Glennie SJ, Russell D, Heyderman R, Gordon SB. Human macrophage models of biomass smoke exposure show impaired ingestion of S. pneumoniae but not M. Tuberculosis. *Sept 2011. Manuscript under preparation.*

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LIST OF ABBREVIATIONS

AIDS	=	*Acquired immune deficiency syndrom*
ALRI	=	*Acute Lower Respiratory Infections*
AM	=	*Alveolar Macrophage*
BAL	=	*Bronchoalveolar lavage*
BALT	=	*Bronchus-associated lymphoid tissue*
BMF	=	*Biomass Fuel*
CD	=	*Cluster of Differentiation (e.g.CD4 cells)*
CI	=	*Confidence Interval*
CO	=	*Carbon Monoxide*
COPD	=	*Chronic Obstructive Pulmonary Disease*
CPU	=	*Central Processing Unit*
CRP	=	*C-reactive protein*
DALY	=	*Disability Adjusted Life Years*
DNA	=	*Deoxyribonucleic acid*
EPA	=	*Environmental Protection Agency*
FCB	=	*Fine Carbon Black*
FDA	=	*Fluorescein Diacetate*
FEV_1	=	*Forced Expiratory Volume in 1 second*
FVC	=	*Forced Vital Capacity*
GACC	=	*Global Alliance for Clean Cookstoves*
GDP	=	*Gross Domestic Product*
GM-CSF	=	*Granulocyte-macrophage colony-stimulating factor*
HAM	=	*Human Alveolar Macrophage*
HIV	=	*Human Immunodeficiency Virus*
IAP	=	*Indoor Air Pollution*
IARC	=	*International Agency for Research on Cancer*
IFNγ	=	*interferon-γ*
Ig	=	*Immunoglobulin (e.g. IgG or IgA)*
IL	=	*interleukin (e.g. IL-1 or IL-6)*
IOM	=	*Institute of Occupational Medicine (Edinburgh)*
LBW	=	*Low Birth Weight*
LPG	=	*Liquefied Petroleum Gas*
LSTM	=	*Liverpool School of Tropical Medicine*
LPS	=	*Lipopolysaccharide*
MARCO	=	*Macrophage receptor with collagenous structure*
MBL	=	*Mannan-Binding Lectin*
MDM	=	*Monocyte Derived Macrophage*
MIF	=	*Mean Intensity Fluorescence*
MLW	=	*Malawi Liverpool Wellcome Clinical Research Programme*
NK	=	*Natural Killer*
PAFr	=	*Platelet activating factor receptors*
PAH	=	*Polyaromatic Hydrocarbons*
PAMPS	=	*Pathogen associated molecular patterns*
PBS	=	*Phosphate Buffered Solution*
PCP	=	*Pneumocystis jiroveci pneumonia*
PDGF	=	*Platelet-derived growth factor*
PEPFAR	=	*U.S. President's Emergency Plan for AIDS Relief*
pf	=	*Pyrogen free*
PM	=	*Particulate Matter*
RANTES	=	*Regulated on activation, normal T expressed and secreted*
RESPIRE	=	*Randomised Exposure Study of Pollution Indoors and Respiratory Effects*
ROI	=	*Reactive Oxygen Intermediates*
RPMI	=	*Roswell Park Memorial Institute*
RR	=	*Relative Risk;*
RSV	=	*Respiratory syncytial virus*
SLPI	=	*Secretory Leukocyte Protease Inhibitor*
TB	=	*Tuberculosis*
TCR	=	*T-cell surface antigen receptor*
TIFF	=	*Tagged Image File Format*
TIP	=	*Total inhalable particulates*
TLR	=	*Toll-like receptors*
TNF	=	*Tumour Necrosis Factor*
TSI	=	*Trust Science Innovation ®*
TWA	=	*Time Weighted Average*
UCB	=	*University of California Berkeley*
WHO	=	*World Health Organisation*

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 What is Biomass Fuel?

Biomass fuel (BMF) refers to burned plant or animal material; wood, charcoal, dung and crop residues account for more than one-half of household energy used in most developing countries and for as much as 95% in lower income countries.[1] It is also referred to as solid fuel, although this term also encompasses coal. Around 2.4 billion people, use BMF as their primary source of household energy and a further 0.6 billion people use coal.[1;2] 700 million people in Africa use BMF to provide energy for cooking, heating and lighting.

1.1.1 Poverty and Indoor Air Pollution from Biomass Fuel Use

The evolution of mankind has been intimately linked to the use of fire. Evidence of hominids using fire exists from half a million years ago and it is likely that early man gained a survival advantage by using fire to cook food, heat living spaces and to keep insects and animals away.[3] As man built shelters the pollution associated with the burning of biomass fuel was brought indoors into the living area.

The use of solid fuels lower down the energy ladder or pyramid (Figure 1.1) is invariably associated with poverty in countries, in communities within a country, and in households within a community. If the world map of BMF use is superimposed on that of socio-economic development there is near perfect fit. For this reason health effect studies of indoor air pollution are often confounded by other socio-economic factors (Figure 1.2) which are powerful determinants of both disease and BMF use.[4] These factors can be difficult to control for in health effect studies.

Appropriate measures of poverty vary by location. There has been work investigating how best to measure poverty [5] and work done in Malawi

has demonstrated that that a proxy measure of poverty based on a detailed living standards measurement survey (such as the Malawi Integrated Household Survey) and qualitative indicators can be applied to draw up socio-economic profiles of individuals.[6] The model developed by Nhlema Simwaka et al for assessing socio-economic status was developed based on a model consisting of six variables, two of which were related to biomass fuel use (cooking with firewood and using electricity for lighting).

There is good correlation between the main fuel used and socioeconomic factors, however most households use several fuels in different settings. The four factors that appear to be most relevant in a household's choice of fuel type are: (a) cost of fuel, stove type and accessibility to fuels; (b) technical characteristics of stoves and cooking practices; (c) cultural preferences; and lastly, if at all, (d) the potential health impacts. [7]

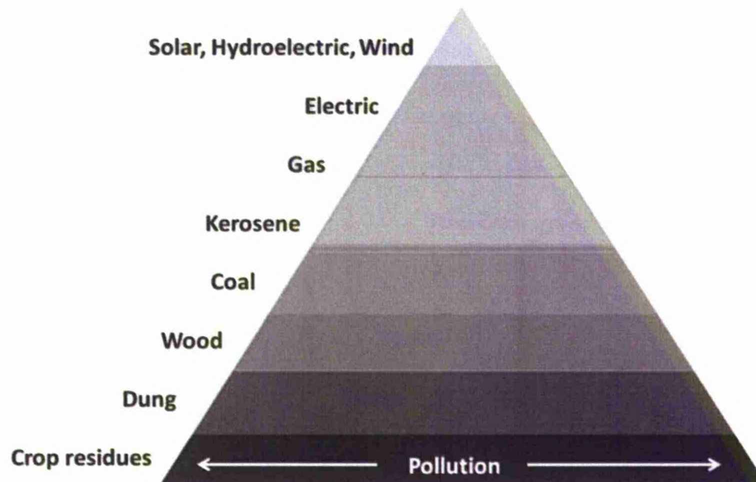


Figure 1.1: The Energy Pyramid. *The peak represents the cleanest, most efficient energy sources of energy; efficiency gradually decreases towards the bottom. The width of each section also represents the amount of pollution produced. Fuels located at the bottom of the pyramid tend to be cheaper and used in poorer households.*

There are age, gender and socioeconomic differences in levels of exposure to BMF smoke and the consequent health effects.[8] An increasing body of evidence exists on the health effects of household air pollution from BMF, as the results of the meta-analyses in Table 1.2 demonstrate.

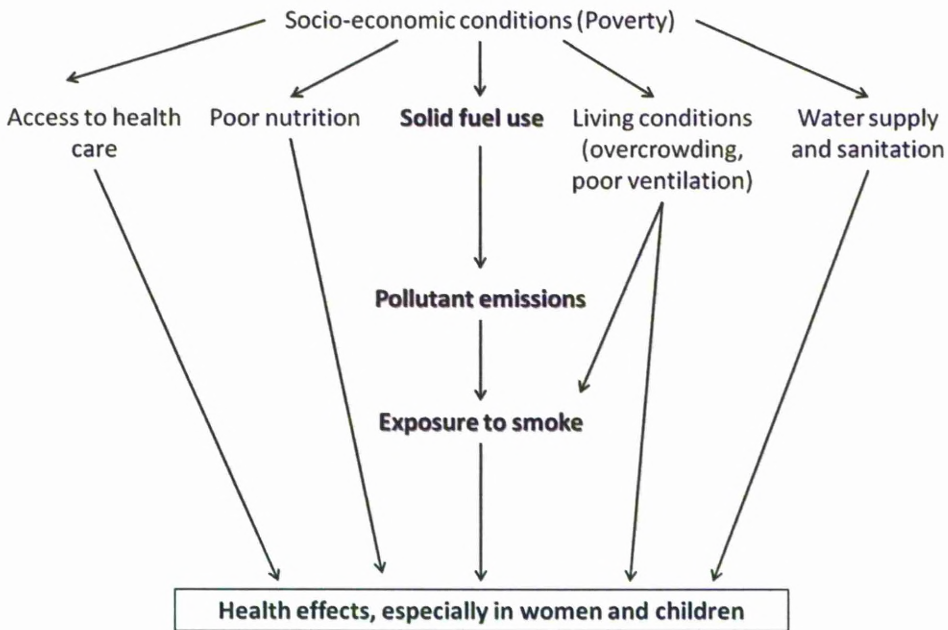


Figure 1.2: Solid fuel use and other influences on ill health. *One of the mechanisms that link poverty to disease is through the inhalation of solid fuel smoke in the home. Figure adapted from Perez-Padilla et al 2010*

1.1.2 The Toxic Products of Biomass Smoke

Biomass fuel is a toxic mix of compounds and elements.[9] Inefficient burning of BMF on an open fire or traditional stove generates large amounts of particulate matter as well as carbon monoxide, hydrocarbons, oxygenated organics, free radicals and chlorinated organics (Table 1.1).[9;10] The particulate matter (PM) component of this smoke is classified according to its size, with inhalable material <10μm in

aerodynamic diameter referred to as PM_{10}; **Total inhalable dust** approximates to the fraction of airborne material which enters the nose and mouth during breathing and is therefore available for deposition in the respiratory tract. **Respirable dust** approximates to that fraction which penetrates to the gas exchange region of the lung. The 24 hour mean PM levels set in the WHO guidelines for air quality are $50\mu g/m^3$ for PM_{10} and $25\mu g/m^3$ for $PM_{2.5}$, but in many parts of the developing world the peak indoor concentration of PM_{10} often exceeds $20{,}000\mu g/m^3$. [11-13]

Smoke particles consist of a carbonaceous core, associated with a wide variety of other substances. Carbon monoxide (CO), as well as carbon dioxide, have both been reported as having a toxic effect, although CO also acts as a surrogate for other pollutants.[14-17] PM itself is known to cause epithelial damage by size alone, leading to an inflammatory response related to the diameter of the inhaled particle; the 'ultrafine' fraction of inhaled smoke particles appear to cause the most effect; they are inhaled to the most distal part of the lung epithelium. [18]

The transition metal content of BMF smoke is likely to be an important cause of toxicity. The levels of transition metals vary depending on the location and the type of household fuel used. A biological hierarchy seems to exist in terms of tissue damage, with low valence transition metals being key to PM bioreactivity.[19] In India, where individuals burn dung cake, PM contains much higher levels of transition metals, as well as arsenic which has been shown to have a biological effect. [20]

There are many organic compounds that are more difficult to routinely measure (listed in Table 1.1). Benzene, naphthalene, polyaromatic hydrocarbons (PAH), methyl chlorides etc are all emitted at different concentrations when wood is burnt are likely to have a toxic effect at the respiratory tract mucosa. [9]

Finally, BMF smoke also contains large amounts of endotoxin.[21] The health effects of exposure to endotoxin may be considerable, particularly because exposure is sustained and occurs from birth. It is possible that inhaled endotoxin, being pro-inflammatory, may be one of the contributory factors that result in lung damage and the health effects observed.

Table 1.1: Chemical Composition of Wood Smoke and the potential health effects. *Data from the US EPA, UK HPA and references 9 and 10*

Species	**Grams per Kilogram of Wood**	**Potential health effects**
Carbon monoxide	80 to 370	Tissue hypoxia, cellular death, damages to the central nervous system, death, and reproductive toxicity – low birth weight.
Methane	14 to 25	Asphyxia (high exposures)
Volatile organic compounds (C2–C7)	7 to 27	Carcinogenic, impaired lung function, asthma, eye irritant, fatigue, ozone can damage plant life.
Aldehydes	0.6 to 5.4	Carcinogenic in high doses (animal studies), nasal irritation pulmonary inflammation, bronchoconstriction.
Substituted furans	0.15 to 1.7	Limited inhalational data – potential P450 damage
Benzene	0.6 to 4.0	Neurotoxicity, immunosuppressant and carcinogenic at high doses. Limited data for low dose chronic exposures
Alkyl benzenes e.g. Toluene	1 to 6 0.15 to 1.0	Limited data but studies suggest higher prevalence of Cardiovascular disease
Acetic acid	1.8 to 2.4	Ocular irritation
Formic acid	0.06 to 0.08	Cough, dermatitis, conjunctivitis
Nitrogen oxides (NO, NO_2)	0.2 to 0.9	Upper airway irritation. May cause impaired lung function and increased respiratory infections in young children. Exposure to high NO_2 levels can contribute to the development of acute or chronic bronchitis
Sulphur dioxide	0.16 to 0.24	Bronchoconstriction. Can worsen COPD and aggravate existing heart disease.
Methyl chloride	0.01 to 0.04	At high levels effects the CNS, crosses the placental barrier, and lowered fetal body weights have been noted. Probable human carcinogen.
Naphthalene	0.24 to 1.6	At high doses can cause haemolytic anaemia, hepatotoxicity, CNS effects, cataracts, possible human carcinogen.
Oxygenated monoaromatics	1 to 7	Limited data found
Total particle mass	7 to 30	Pulmonary inflammation; see text.
Particulate organic carbon	2 to 20	Respiratory and cardiovascular disease dependent on particulate size
Oxygenated PAHs Varied PAHs *Benzo[a]pyrene* *Dibenz[a,h]anthracene*	0.15 to 1 3×10^{-4} to 5×10^{-3} 2×10^{-4} to 2 3 10^{-3}	Respiratory and cardiovascular disease
Particulate elemental carbon	0.3 to 5	Respiratory and cardiovascular disease dependent on particulate size
Normal alkanes (C24–C30)	1×10^{-3} to 6×10^{-3}	Limited data found
Cyclic di- and triterpenoids *Isopimaric acid* *Lupenone* *Friedelin*	 0.02 to 0.10 2×10^{-3} to 8×10^{-3} 4×10^{-6} to 2×10^{-5}	Limited data found
Chlorinated dioxins	1×10^{-5} to 4×10^{-5}	Probable carcinogen, immunosuppressive and reproductive effects.
Particulate acidity	7×10^{-3} to 7×10^{-2}	Respiratory and cardiovascular effects

1.2 Evidence for Health Effects of Particulate Exposure

1.2.1 Epidemiological Studies

An epidemiological association between inhaled smoke and pulmonary ill health was reported after the Vesuvius eruption of AD45 in *Historia Naturalis* by Pliny the Elder.[22] There is a large body of evidence on the health effects of outdoor air pollution, especially PM pollution derived from diesel in urban settings; this has been reviewed elsewhere. [23-25]

The same amount of evidence does not exist for household air pollution from BMF. This discrepancy in terms of research is inversely proportional to where exposure occurs (Figure 1.3).

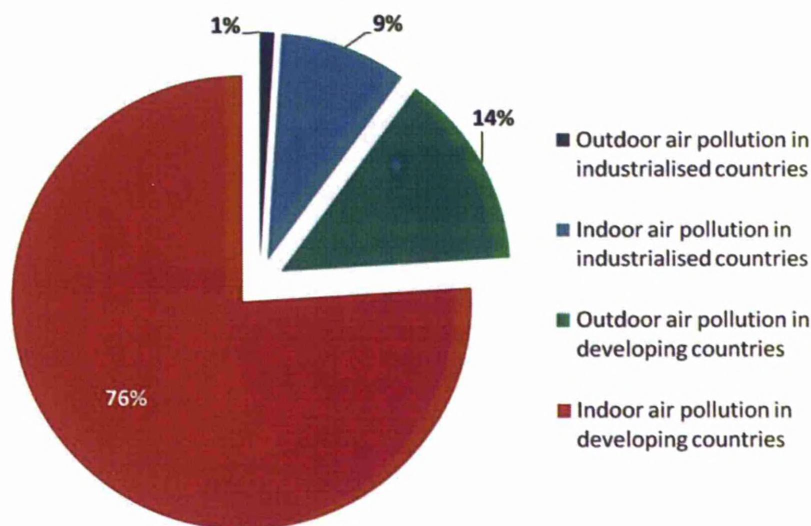


Figure 1.3: Total global exposure to particulate matter. [26]

Total global human exposure to PM, in terms of the number of people, exposure intensity and time spent exposed in various microenvironments varies greatly in different parts of the world. In industrialised countries approximately 1% of the global PM exposure sustained by human populations occurs in outdoor environments, with a further 9% occurring indoors. In the developing world 14% of global PM exposure occurs outdoors, while 76% of all human exposure to PM occurs in indoor

environments. As a result of this the vast majority of deaths that occur as a result of exposure to biomass fuels occurs in the developing world (figure1.4); indoor air pollution from biomass fuels is ranked 10th as a global risk factor for healthy years lost due to disease. [27]

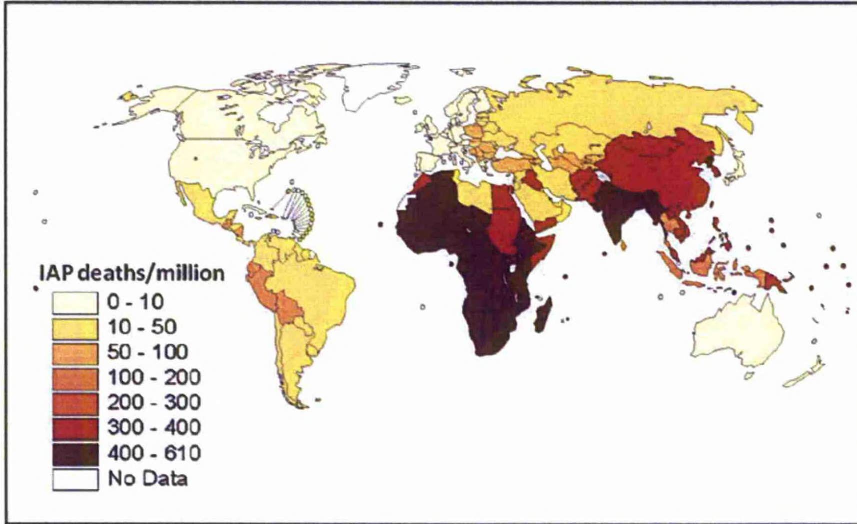


Figure 1.4: Deaths attributed to indoor air smoke from solid fuels. *The majority of deaths from indoor air pollution (IAP) occur in the southern hemisphere. As a risk factor for total healthy life years lost, IAP is ranked 10th, responsible for 2.4% of all deaths globally; Malaria is ranked 9th. Figure is taken from WHO World Health Report and is available at http://www.who.int/heli/risks/indoorair/en/iapmap.pdf.*

Modern epidemiological data have associated PM inhalation with adverse health outcomes; in industrialised countries because air quality is monitored closely it has enabled hospital admissions, as well as mortality, to be associated with outdoor air pollution levels.[23;28;29] Exacerbations of inflammatory lung conditions, lung infections and all-cause mortality are increased when particulate exposed populations are compared to controls.[28] These data continue to inform government recommendations on air quality.

Effects from short-term exposure, time series studies

The 1952 London smog is a dramatic example of the impact of air pollution on mortality and other health effects.[30] During this episode the daily levels of particles, increased 10-fold compared to the normal levels during this era (300-500μg/m^3). These air pollution episodes lead to regulations; the Clean Air Act in the UK and the subsequent air quality improvement over the last 30-40 years in industrialised countries.[31] The effect of air pollution control was clearly observed in Dublin in the 1990s when coal sales were banned and residential heating with coal was stopped.[32] 116 fewer respiratory deaths and 243 fewer cardiovascular deaths were seen per year in Dublin after the ban.

Time-series studies performed in the USA and Europe have demonstrated that days with higher concentrations of PM air pollution are associated with an increased rate of hospital admissions and death due to lung and cardiovascular disease. For example, Zanobetti et al demonstrated this in 10 European cities. [33]

Effects from long-term exposure, cohort studies

Dockery et al (1993) studied approximately 8000 persons in six cities and Pope et al (1995) studied about 550,000 persons in 151 city areas. [28;34] Both studies showed a significant association between mortality and particle concentrations ($PM_{2.5}$). The increased mortality was most pronounced among persons with pre-existing respiratory and cardiovascular disease.

These seminal studies have been followed up: Laden et al (2006) found an increase of 16% in overall mortality associated with each 10μg/m^3 $PM_{2.5}$ as an overall mean during the period.[35] Pope et al in 2002 updated their study and demonstrated a clear and significant association between mortality and $PM_{2.5}$ concentration.[36] There was a considerable reduction of about a third in $PM_{2.5}$ concentration in the period from 1979-83 to

1999-2000 in all areas. In 1979-83, a difference in the annual mean of $10\mu g/m^3$ $PM_{2.5}$ between the areas was associated with a difference in annual mortality of 4% while in the period of 1999-2000, a difference of $10\mu g/m^3$ $PM_{2.5}$ was associated with a difference in annual mortality of 6%. [36]

Jerret et al (2005) studied 23,000 persons in the Los Angeles area for the period 1982-2000. The population was a subset of the Pope et al study, however the exposure could be assessed more accurately as the exposure was determined using model extrapolations of PM-data from 23 measurement stations. After controlling for covariates, a $10\mu g/m^3$ increase in the annual $PM_{2.5}$ level was associated with an increase in mortality of 17%. The corresponding increase in ischaemic heart disease was 38% and for lung cancer 46%. This study found an association in relation to mortality that was three times higher than that of Pope et al in 2002. The increase was judged to be due to a more accurate exposure assessment than in the prior studies.[37] Further studies from the US and Europe support an even higher increased risk for cardiovascular mortality. [38;39]

1.2.2 Experimental studies

Human studies

There are few studies done with experimental exposure of human to BMF smoke. Barregard et al exposed volunteers to wood smoke PM ($PM_{2.5}$ concentrations of $240\text{-}280\mu g/m^3$) in a chamber. The experiment was performed in order to examine whether short-term exposure to wood smoke affects markers of inflammation, blood haemostasis, and lipid peroxidation in healthy humans. [40;41]

The authors concluded that wood smoke particles, at levels that can be found in smoky indoor environments lead to inflammation, coagulation, and possibly lipid peroxidation. All of these processes are involved in

the mechanisms whereby particulate air pollution affects cardiovascular morbidity and mortality.[40] They also concluded that wood smoke exposure lead to signs of increased oxidative stress in the respiratory tract, especially in the lower airways. [41]

Animal Studies

Animal and *in vitro* studies are discussed in more detail in Chapters 6 and 7 but in general toxicological studies of air pollutants tend to be short-term experimental studies often analysing early events instead of waiting for final disease, such as the development of emphysema or impaired respiratory system development. [42]

Two approaches have normally been taken in animal studies: 1) Placing suspensions of the test substances in the nose or the trachea, or 2) Inhalation of aerosols. Rats have been mainly used in these type of health effect studies and they tend to be performed in order to test the mechanisms of pollutant induced lung and airway injury or as models for infective processes and the functioning of the immune system.[43-45] Rats in particular appear to be susceptible to chronic inflammation, fibrous tissue development, and cancer from insoluble, non-cytotoxic particles, via a process believed to involve the overwhelming of normal particle removal mechanisms (particle overload).

Anatomical and physiological differences make comparison between the effects in rodents and those in human difficult, and these differences can result in significantly different concentrations in sensitive regions of the respiratory tract and the lungs of animals, compared to similar regions in humans. Additionally, experimental animals used in toxicological studies tend to be genetically very similar, whereas human populations are more heterogeneous. Consequently extrapolation of results from experimental animals to humans must also take strain and species differences into account.

Issues around dose and concentrations of particles at the tissue level are critical factors in determining toxicity. Also, many toxicological effects are related to exposures that occur over time, especially in those situations where exposure occurs over longer periods. However, certain effects, for example susceptibility to infection, may be more related to peak exposures.

In vitro Studies

The effect of air pollution can also be studied in isolated lungs from animals, sections of lungs removed from humans during surgery, cultures of various anatomical structures of the respiratory tract from animals or human cell lines and in, cultures of various cell types lining the respiratory system, and subcellular fractions of tissues and cells from animals or humans. These type of experiments tend to focus on cellular responses and biochemical systems rather than whole animals.

An example of this type of study is recent work carried out using the human cell line A549. *In vitro* work has demonstrated the adverse effect of wood smoke particulates on DNA, caused by oxidative stress. Small particle size, high levels of PAH and low levels of water-soluble metals from wood smoke leads to high levels of free radicals which in turn leads to DNA damage as well as inflammatory and oxidative stress response gene expression in cultured human cells.[46] This study supports previous data from mouse macrophage work that showed that wood smoke can cause lipid peroxidation, activate nuclear transcription factor and enhance the release of TNF-α, indicating that the free radicals generated by wood smoke are able to cause DNA and cellular damage and may potentially act as a fibrogenic agent. [47]

Like animal studies, *in vitro* studies have been useful in order to characterise the mode of action and mechanisms by which air pollutants cause damage, and they can also be used to assess differences between

different types of smoke or chemical constituents within the smoke. However, the complex physiological and pathological interactions that occur in the intact organism when animals or humans are exposed to air pollutants via inhalation mean that *in vitro* studies in isolation cannot be used in order to fully characterise the hazards associated with smoke exposure. That said to date there is little work characterising the *in vitro* effect of wood smoke and so these type of mechanism studies remain important in order to strengthen the foundation of biological plausibility of health effects associated with BMF smoke exposure.

Intervention Studies

Improved cookstoves are the major class of technical intervention for lowering household pollution exposures from solid cooking fuels. Other potential interventions include kitchen ventilation improvements, educational strategies and introduction of cleaner burning fuels.

To date there is little data on the effects of alternative cook stoves or other measures taken to reduce air pollution on improving health. Searching the MEDLINE database and the ClinicalTrials.gov website reveals four trials that have either taken place, are ongoing or are yet to start recruiting. They are located in:

- **San Lorenzo, Guatemala**. Title: The randomised exposure study of pollution indoors and respiratory effects (RESPIRE) using a plancha stove. This trial has been completed and is under review at the Lancet.
- **Michoacán, Mexico**. The use of a patsari stove has published effects of the stove on PM exposures and the respiratory health of women. This trial was not registered on ClinicalTrials.gov
- **Kathmandu, Nepal**. Title: Cookstove replacement for prevention of ALRI and low birth weight in Nepal. This trial started recruiting in August 2009 and is due to be completed in September 2013.

- **Kintampo, Ghana.** Title: Intervening to improve infant health in Ghana. This trial has will start recruiting September 2011 and is not due to report until 2016.

The RESPIRE study has yet to publish on the primary outcome measure (pneumonia in children) or the secondary outcome measure (severe (hypoxaemic) and RSV pneumonia), however there are currently nine publications associated with this trial.

Work from RESPIRE has demonstrated that indoor carbon monoxide levels were significantly lower among the intervention group compared to the non-intervention group.[48] A significant reduction in wheeze and other respiratory symptoms reported by the women who used the improved stove has been shown, although no significant effect on lung function has been reported as yet.[49] Women's perception of their health was improved, especially with regard to non-respiratory symptoms such as eye discomfort and headache.[50;51] There has also been data that imply that blood pressure is lowered by the use of improved stoves. [52]

The Patsari intervention trial in Mexico has demonstrated a significant reduction in the level of CO and PM in the intervention arm and has also shown an improvement in the symptoms and lung function of exposed women.[53;54] Despite adherence to the intervention being low (only 50% of women continue to use the stove) women who did use the stove most of the time, compared with those using the open fire, had significantly lower risk of respiratory symptoms as well as less eye discomfort, headache, and back pain. Importantly the use of the Patsari stove was associated with a lower FEV_1 decline (31 ml) compared with the open fire use (62 ml) over 1 year follow-up after adjusting for confounders. [53]

This is an area of research that requires more trials in order to answer questions not only about health effects but stove adoption. Data from Nepal and Ghana will be extremely important in informing the debate

around which interventions and how much resource to invest in such programs.

Health Effect	Meta-analysis RR (95% CI) * [1]
Strong evidence †	
Acute lower respiratory infection (ALRI) in children <5 years of age in developing countries	2.3 (1.9–2.7) 1.78 (1.45–2.18) [55]
Chronic obstructive pulmonary disease (COPD)in women >30 years of age, mainly homemakers residing in rural areas of developing countries	3.2 (2.3–4.8) 2.14 (1.78–2.58) [56]
Lung cancer (coal smoke exposure) in women >30 years of age	1.9 (1.1–3.5)
Moderate evidence ‡	
COPD in men >30 years of age	1.8 (1.0–3.2)
Lung cancer (coal-smoke exposure) in men >30 years of age	1.5 (1.0–2.5)
Lung cancer (biomass smoke exposure) in women >30 years of age	1.5 (1.0–2.1)
Asthma in children aged 5–14 years	1.6 (1.0–2.5)
Asthma, >15 years of age	1.2 (1.0–1.5)
Tuberculosis, >15 years of age	1.5 (1.0–2.4)
Low birth weight and perinatal mortality	1.38 (1.25-1.52) [57]
Insufficient evidence §	
Upper airway cancer Cardiovascular diseases	

Table 1.2: Diseases associated with solid fuel use [4]

** Meta-analysis results from reference Smith et al, unless otherwise stated.* [1]

† Strong evidence: Some 15–20 observational studies for each condition, from developing countries. Evidence is consistent (significantly elevated risk in most, although not in all, studies); the effects are sizable, plausible, and supported by evidence from outdoor air pollution and smoking. [1]

‡ Small number of studies, not all consistent (especially for asthma, which may reflect variations in definitions and condition by age), but supported by studies of outdoor air pollution, smoking, and laboratory animals. [1]

§ Insufficient for quantification based on available evidence. [1]

1.3 Health Effects of Biomass Fuel Smoke Exposure

The adverse health effects of indoor air pollution are often exacerbated by lack of ventilation in homes using BMF and by poor design of stoves that do not have flues or hoods to take smoke out of the living area.

Table 1.2 shows the diseases associated with BMF smoke exposure, and is divided according to the strength of evidence associated with each observed health effect.

As outlined in the previous section, the focus of research has been primarily on the health effects of outdoor air pollution in industrialised countries.[58] Despite this, there is an increasing body of evidence of the health effects of IAP.

For clarity the next section has been has been divided into four sub-sections: Respiratory and non-respiratory disease in adults and respiratory and non-respiratory disease in children.

1.3.1 Respiratory Illness in Adults

Women bear the brunt of the disease burden associated with BMF, primarily because it is women living in rural areas who are exposed to high levels of BMF smoke. For example in Nepal, the average PM_{10} level in kitchens using BMF was three times higher than in those using cleaner fuels such as kerosene, liquefied petroleum gas (LPG) and biogas, and 94% of the respondents were disadvantaged women.[59] Table 1.2 gives the meta-analysis results of the diseases associated with solid fuel use.

Interstitial Lung Disease

BMF smoke is associated with an interstitial lung disease referred to as 'hut lung' – a form of pneumoconiosis in rural women from developing countries; originally described as 'Transkei silicosis' because it was thought to be due to silica particles.[60;61] However, it is the contribution

of BMF smoke in the pathogenesis of chronic obstructive pulmonary disease (COPD) that causes a greater global burden of disease.

Chronic Obstructive Pulmonary Disease (COPD)

BMF smoke is strongly associated with COPD in non-smoking women living in rural areas.[1;62;63] In women from rural Turkey it is estimated that the fraction of COPD attributed to exposure to biomass smoke, after adjusting for possible confounding factors, is 23.1%.[64] A recent meta-analysis estimated the odds ratio of developing COPD to be between 2.29 and 2.96 for solid fuel exposure, although no data from Africa were included.[56] Cigarette smoking rates remain relatively low in developing countries compared with Europe and the USA.[65;66] However, in Mexico, women exposed to household BMF smoke develop COPD with clinical characteristics, quality of life and increased mortality similar in degree to that of tobacco smokers. [13;67]

Some of the most compelling data on the effect that household air pollution has on the development of COPD comes from China. A retrospective cohort study of 20453 people in Xuanwei province, demonstrated that the prevalence of COPD decreased markedly in men and women who lived in households with unvented coal stoves when ventilation was improved by the installation of a chimney.[68] Compared with people who did not have chimneys, the relative risk ratio of developing COPD was 0.58 (95% confidence interval 0.49 to 0.70) in men and 0.75 (0.62 to 0.92) in women.

There are few data available specific to Africa, however the burden of obstructive lung disease (BOLD) project assessed the prevalence of COPD in 12 cities and the only city in Africa where the work was done, Cape Town, had the highest prevalence of COPD in the world, in both males and females.[69] There are likely to be a number of contributing factors to the high prevalence of COPD observed, but the contribution

from household air pollution from biomass fuel smoke is likely to be significant. [70]

Tuberculosis

Evidence is emerging that the incidence of TB is increased amongst BMF exposed women. Studies from Mexico, Nepal and India have implied a causal role of current BMF smoke exposure and the development of TB.[71-74] Although these finding have not been seen in all studies, overall the evidence supports the hypothesis that exposure to respirable pollutants from combustion of BMF increases the risk of TB infection and disease.[75] It is known that BMF smoke impairs alveolar macrophage function,[43;76;77] and alveolar macrophages are not only the target of *Mycobacterium tuberculosis* infection but also contribute an important early defence mechanism against bacteria. Therefore, it seems intuitive that BMF smoke also leads to an increased incidence of TB. However, along with definitive intervention trials, more epidemiological and laboratory data are needed to support this hypothesis.

Lung Cancer

Data from China imply that household coal smoke is a significant risk factor for the development of lung cancer.[78;79] In studies from India and Mexico, data for non-smoking women exposed to BMF smoke for a number of years suggests that long-term exposure to smoke from cooking may contribute to the development of adenocarcinoma of the lung.[80;81] The International Agency for Research on Cancer (IARC) has termed biomass smoke a 'probable carcinogen' (Group 2a) and coal (used as household fuel) was termed carcinogenic to humans (Group 1). [82]

1.3.2 Non Respiratory Illness in Adults

Cardiovascular Disease

Particulate air pollution is statistically and mechanistically linked to increased cardiovascular disease.[83] Long-term prospective cohort studies show an association between levels of air pollution consisting of fine particulate matter ($PM_{2.5}$) and an elevated risk of death from all causes and from cardiovascular disease.[28;34] More recent data have shown that non-fatal ischaemic events are also associated with an increase in fine particulate concentrations in the community. [38]

There is a paucity of data on the association between cardiovascular disease and BMF, but it is known that particulate air pollution leads to rapid and significant increases in fibrinogen, plasma viscosity, platelet activation and release of endothelins, a family of potent vasoconstrictor molecules.[83] Biomass smoke in Guatemalan women, taking part in the RESPIRE trial, has been shown to increase diastolic blood pressure and data from Nicaragua have also implied that there is an increase in blood pressure that is associated with increased CO concentrations, although these increases were not significant.[52;84] Together with data from industrialised countries and the evidence from these two studies, as well as *in vitro* work makes, it highly likely that BMF smoke represents a considerable risk to cardiovascular health. If the risks from outdoor air pollution are translated to BMF use, the number of premature deaths globally will be even larger - approaching 4% of the total global burden of disease. [85]

Cataracts

Epidemiological studies from Nepal and India have associated indoor cooking using BMF with cataracts or blindness. [86;87] Smoke induces oxidative stress and depletes plasma ascorbate, carotenoids and glutathione, which provide antioxidant protection against cataract

formation. The prevalence of cataracts is high in developing countries, [88] nevertheless in a large, 89000 household, Indian national survey, women in homes using BMF had an adjusted odds ratio of 1.3 for blindness compared to women in homes using non-BMF, even after correction for a wide range of potentially confounding socioeconomic factors.

1.3.3 Respiratory Illness in Children

Acute Lower Respiratory Tract Infections (ALRI)

The risk of pneumonia in young children is increased by exposure to unprocessed solid fuels by a factor of 1.8 to 2.3 compared with those living in households using cleaner fuels or suffering less exposure to smoke.[55] In children under 5 years, the mortality attributable to ALRI is estimated to be over 2 million deaths per year. [89-91] The first report of indoor cooking smoke associated with childhood pneumonia and bronchiolitis was in Nigeria; however not until the 1980s was this followed by reports from other areas.[92-97] One carefully conducted cohort study in rural Kenya found that the amount of pollution to which a child is exposed directly correlates with the risk of developing pneumonia.[12] To date the work of Ezzati et al is the only study that has explored the exposure-response curve of BMF smoke and health outcomes. There have currently been two intervention trials that have used alternative cook stoves to improve respiratory health (in women and children), both in Central America, however the main findings from the RESPIRE study from Guatemala have not been published yet. [48;53]

Lung Development

The effect of air pollution on chidrens lung development has been recently reviewed by Grigg and gaps in the global evidence have been highlighted. [98] It has been demonstrated that outdoor air pollution has chronic adverse effects on lung development in US children aged 10-18 years, which leads to clinically significant deficits in attained forced

expiratory volume in 1 second (FEV_1) as children reach adulthood.[99] Kulkarni et al (2006) demonstrated that carbon particles, similar to those found in ambient air and a biomarker of exposure to air pollution, were present in the airway macrophages of exposed children, and an increased level of carbon in their macrophages correlated with decreased lung function. [100] It is likely that the effect of IAP also leads to deterioration in lung function when children are exposed to high levels of IAP from BMF, and data suggest that symptoms of wheeze are more frequent amongst households that use an open fire compared with a stove with a chimney.[101;102] A review of data from China has shown reductions in children's FEV_1, forced vital capacity and peak flow associated with household coal use.[103]

Work done in India, China and Mongolia has demonstrated that higher pollution levels is associated with impaired lung function.[104-107] In India, Padhi et al showed that exposure to cooking smoke from biomass combustion is significantly associated with decline in lung function (as well as asthma). In China, He et al have shown that school children living in the most polluted districts had worse lung function and Dashdendev et al demonstrated that 'normal' FEV_1 was actually 40% higher in rural Mongolian children than in urban children, suggesting that the FEV_1 of apparently healthy children living in urbanized societies may in fact not be normal, but may instead reflect the deleterious effects of air pollution in cities, as indicated by increased levels of both environmental and exhaled CO.

Despite this increasing evidence there as yet remains no longitudinal data on BMF exposure and children's lung function done in Africa (or elsewhere in the world).

1.3.4 Non-Respiratory Illness in Children

Low Birth Weight

Evidence exists that implicates exposure to BMF smoke to adverse effects on different birth outcomes.[108] It has been established since the 1970's that maternal tobacco smoking is associated with low birth weight and recently there are several published studies that associate low birth weight (LBW), intrauterine growth retardation and perinatal mortality with air pollution.[109-112] Figure 1.5 demonstrates the potential mechanism that BMF smoke exposure may lead to low birth weight and other poor health outcomes.

An association between birth weight and type of fuel used has been identified. The babies of mothers using open wood fires were on average 63g lighter compared with babies born to mothers using cleaner fuels; [113] the use of an open fire produced average levels of PM_{10} of $1000mg/m^3$. A similar (slightly larger) effect has also been reported in Zimbabwe. [114] Pope et al (2010) recently systematically reviewed the results of five low birth weight studies and three stillbirth studies. They reported an increased risk of percentage LBW has an odds ratio of 1.38.[57] Evidence from second hand tobacco smoke, ambient air pollution, and animal studies as well as biological plausibility (illustrated in Figure 1.5) substantiate these associations.

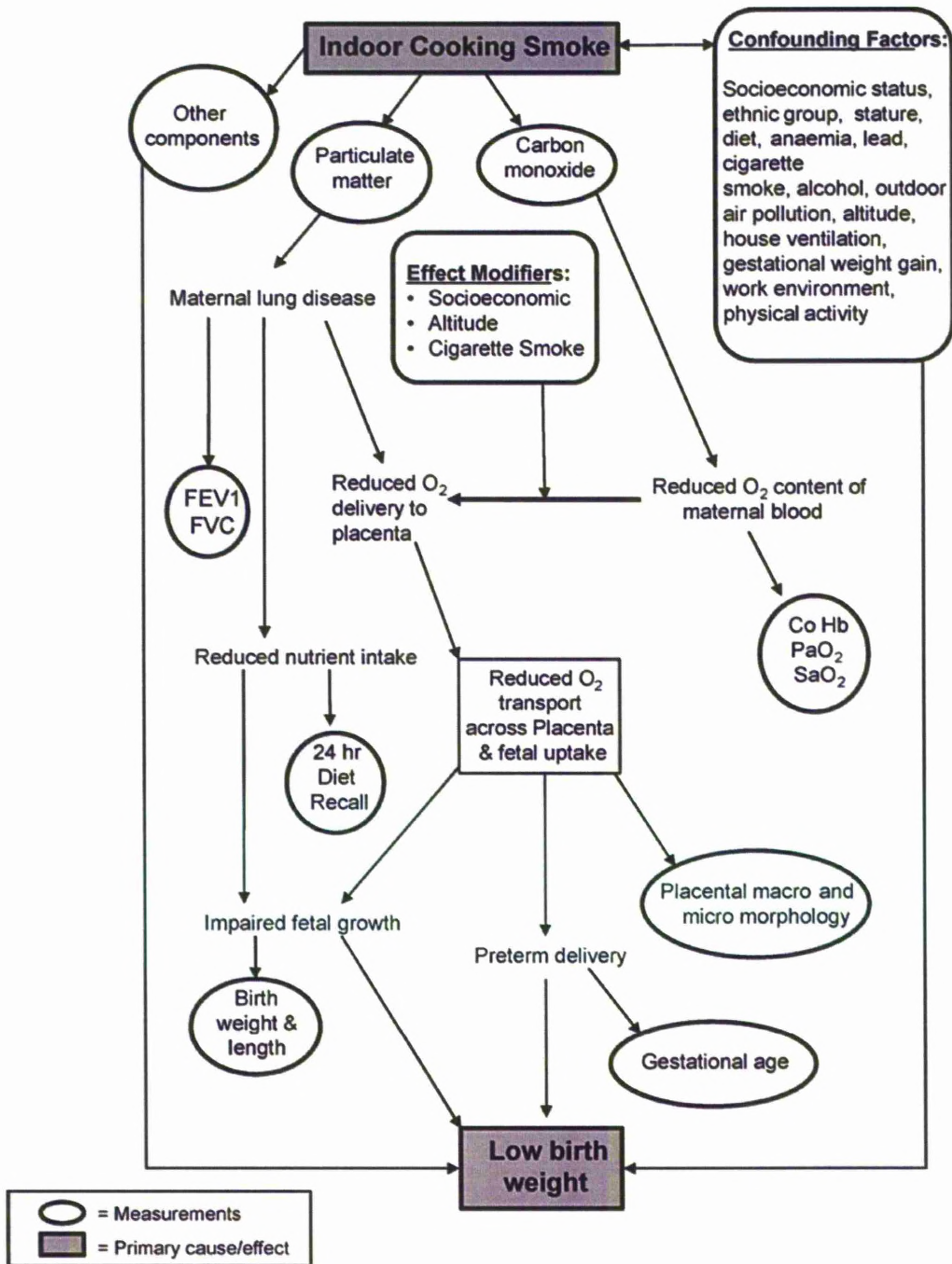


Figure 1.5 Pathways relating smoke exposure and childhood health: *Potential mechanisms for the effect of indoor cooking smoke on fetal growth Reproduced with permission of J.D. Haas.*

Nutritional deficiency

Mishra et al (2006) studied nearly 30000 children, taking into account potential confounders such as exposure to tobacco smoke, recent episodes of illness, maternal education and nutrition, and other factors. They showed an association between exposure to BMF smoke in young children and chronic nutritional deficiencies including anaemia and stunted growth. [115]

Despite their analysis, the possibility of residual confounding still exists: the mix of fuels at different locations; BMF exposed children are more likely to live in a rural area and to be in households of a lower standard of living and to live in lower-quality housing; and mothers are less likely to have received iron supplementation during pregnancy. Although Mishra et al use multiple logistic regression models in an attempt to control for these factors, the extent to which confounding can be controlled depends on the accurate measurement of the full set of confounding factors and their inclusion in the specified model.[115] They relied on national survey data, which inherently limits confounder assessment. For example, there were no data on intensity of hookworm or other helminth infections, which are important causes of anaemia and malnutrition in this and other regions of the world. These findings do however demonstrate another set of health outcomes from BMF smoke exposure that requires more thorough investigation.

1.4 HIV Disease

In parts of the world where HIV infection is common, BMF is the main household energy source. Both BMF use and HIV are associated with an increase in the incidence of pneumonia.[55;116-118] They are likely to represent the two of the most important risk factors for pneumonia in African adults, and may indeed interact.

1.4.1 Epidemiology of HIV in Malawi

Malawi is facing a severe HIV and AIDS epidemic with an estimated 12% of the adult population aged between 15 and 49 living with the virus. [119;120]

The past 8 years have seen major advances in access to antiretroviral therapy (ART) in Malawi and much of Africa. From 2002 onward, there has been an international drive to scale up antiretroviral treatment, most notably with the establishment of the Global Fund to Fight AIDS, Tuberculosis, and Malaria, the "3 by 5" Initiative of the World Health Organization (WHO) as well as U.S. President's Emergency Plan for AIDS Relief (PEPFAR). It is estimated that 2·7–3·1 million patients had started ART in this region in sub-Saharan Africa by 2008. [121]

1.4.2 The Prognosis of HIV infected individuals in Africa

The role out of ART does give significant cause for optimism. However the prognosis of HIV infected individuals who manage to get established onto ART depends on several factors.

May et al (2010) analysed the prognosis of patients with HIV-1 infection starting ART in sub-Saharan African countries (including Malawi).[120] They showed that in the African setting, several factors detrimentally affect prognosis. These factors included male gender, anaemia, total lymphocyte count, and body mass index.

The observed higher mortality in men compared to women is potentially because women are younger and start treatment with less-advanced disease than men, perhaps attributable to differences in health-seeking behaviour and women's more timely access to ART through antenatal care.

Anaemia in patients with HIV-1 infection is associated with higher rates of disease progression and death, independent of CD4 cell count and other prognostic factors, as is seen in Europe and the USA. [122;123] Anaemia in HIV-1 infection might be a manifestation of anaemia of chronic disease, infections of the bone marrow, or myelosuppressive drugs; in a study in Malawi most non-pregnant adults admitted to hospital with severe anaemia had HIV-1 infection.[124] Total lymphocyte count is prognostic and is explained by its correlation with CD4 cell count.

Bodyweight was also independent of CD4 cell count as a prognostic measure; bodyweight changes reflect changes in the rate of viral replication and bodyweight is affected by severe opportunistic infections or malignant diseases. [125;126]

1.5 Pneumonia in Africa

Pneumonia is the dominant global cause of death in children; killing more children than AIDS, malaria, and measles combined.[127] It is estimated that the condition causes 2 million premature deaths every year accounting for 19% of all under-5 deaths worldwide.[128] *Streptococcus pneumoniae* is responsible for at least 50% of these deaths in children.[129] It has now been established that pneumonia in children has the strongest association with IAP from biomass fuel smoke. [55;130]

In adults, in industrialised countries it tends to be the elderly population who are most affected by pneumonia – the "old man's friend". However, in countries affected by the HIV epidemic, pneumonia is the leading cause of death, with *S. pneumoniae* being the major pathogen in all patient groups; the young, old and the immuno-deficient.[131-136] There is currently only data from a few outdoor air pollution studies on the effects on pneumonia incidence but exposure to air pollution appears to be a significant risk factor. Despite this in populations where exposure to BMF smoke is ubiquitous and pneumonia is the main cause of adult hospital admissions and deaths, there are few published data examining the association between BMF smoke exposure and adult pneumonia. [137-139]

1.5.1 Aetiology and Prognosis of Pneumonia in African Adults

Acute adult pneumonia in sub-Saharan Africa is a frequent cause of admission in hospital-based series; in Malawi it is the leading cause of medical admissions.[138;140;141] Despite this there is little descriptive epidemiology of acute pneumonia in sub-Saharan Africa; to date the only descriptive study is from Scott et al in 2000.[141] In this study of 281 hospitalised Kenyan adults (52% of patients were HIV positive); *Streptococcus pneumoniae* was documented in almost half of the patients although the true proportion was probably greater than two thirds with acute pneumonia.

Aetiology and outcome between HIV positive and HIV negative patients is very similar (Table 1.3) and the appropriate first-line therapy in most of Africa remains high-dose benzyl penicillin. Failure to respond to this is likely to be due to other bacterial pathogens (*H influenzae*, S*almonellae*, and *Staph aureus*), intermediate-resistant pneumococci, or tuberculosis.

Mycoplasma, Coxiella, Chlamydia, or Legionella are not common causes of pneumonia in Africa. Cryptococcosis, Nocardiosis, and *Pneumocystis jiroveci* pneumonia (PCP) have all been documented in HIV-positive African patients with undiagnosed respiratory disease, however most studies of African HIV associated respiratory disease include patients with a longer duration of illness and even in these cases, PCP is rare; the most common diagnosis is pulmonary tuberculosis. [142-145]

Tuberculosis can present as acute pneumonia. This may occur either because *M. tuberculosis* itself is the cause of acute pneumonia but also if the history is abbreviated by the patient or if there is co-infection with a bacterial respiratory pathogen. The data from Kenya showed positive sputum cultures for *M. tuberculosis* in 9% of all pneumonia patients and in 7% of those in whom a conventional respiratory pathogen was isolated.[141] Many TB culture-positive patients temporarily improve on benzyl penicillin and only half were smear positive. In the absence of sputum culture or a follow-up visit the diagnosis of tuberculosis can easily be missed.

Pathogens		HIV Positive (%)	HIV Negative (%)
Gram Positive Bacteria	*Streptococcus pneumoniae*	64 (44)	65 (49)
	Staphylococcus aureus	0 (0)	4 (3·0)
Gram Negative Bacteria	*Haemophilus influenzae*	6 (4·1)	4 (3·0)
	Salmonella spp	6 (4·1)	0 (0)
	Other	3 (2·0)	1 (0·7)
Mycobacteria	*Mycobacterium tuberculosis*	19 (13)	7* (5·2)
	Atypical mycobacteria	6 (4·1)	5 (3·7)
Other Pathogens	Nocardia spp	0 (0)	1 (0·7)
	Mycoplasma pneumoniae	4 (2·7)	3 (2·2)
	Viral pneumonia	11 (7·5)	5 (3·7)
Number of patients for whom aetiology was unknown		49 (33)	50 (37)

Table 1.3: Distribution of pathogens observed in HIV-positive and HIV-negative patients. *Acute pneumonia in hospitalised Kenyan adults was caused predominantly by bacteria; Streptococcus pneumoniae was documented 46% cases, although the true proportion is probably greater than two thirds. (NB: * = statistically significant difference (p=0·04) between HIV positive and HIV negative rates of M. tuberculosis). Data from Scott et al (2004).* [141]

The mortality of hospital-treated pneumonia in African series is 5–23%, similar to that in more developed countries.[146-154] An important difference however is in the age distribution. The median age at death in the Kenyan study was 33 years; in industrialized countries it is normally 65 years.[141;153] The mortality recorded may also be a substantial underestimate as patients often present very late to facilities that have limited diagnostic facilities. Pneumonia deaths that occur rapidly in hospital may not be differentiated from other fatal conditions.

1.5.2 Epidemiology of Streptococcus pneumoniae [155]

Streptococcus pneumoniae is the most common cause of pneumonia, sepsis and meningitis in Africa. It was described by Sir William Osler in the early 1900's as "the captain of all the men of death".[156] and it remains the most frequent cause of community-acquired pneumonia worldwide.

Streptococcus pneumoniae is a Gram-positive aerobic commensal bacterium and is a common inhabitant of the upper respiratory tract, existing alongside other co-resident microorganisms. There are currently 91 different serotypes known to be found in the flora of the nasopharynx. After colonisation with a new serotype other competing pneumococcal serotypes are eliminated and the new serotype persists for weeks or months (in adults and children respectively), usually without any clinical manifestation of disease. This carrier state has enabled the organism to persist in human populations, and induces some acquired B-cell-mediated immunity to re-infection. [157;158]

Epidemiological and genetic evidence demonstrate that pneumococcal clones adopt one of two possible survival strategies; either an invasive pneumococcal disease phenotype or a persistent colonisation phenotype with low risk of tissue invasion; these strategies account for the success of *S pneumoniae*. The invasive pneumococcal disease phenotype depends on its capacity for rapid disease induction and efficient person-to-person spread by coughing. The non-invasive phenotype on the other hand uses various immune evasion strategies, surface adhesins, and secretory defences such as IgA1 protease and inhibitors of antibacterial peptides to help with long-term carriage within the nasopharynx.[159] Persistent carriage of pneumococci in the upper airway enables low-level and long lasting transmission, thereby retaining non-invasive strains in human populations. Host defence deficiencies can alter this host–

pathogen interaction and allow strains of low virulence to invade the immunocompromised host. [159-161]

The pneumococcus is mainly transmitted by direct contact with contaminated respiratory secretions between household members, infants, and children, and isolation is not required for patients with infection. However epidemic spread of serotype 5 has been reported, both in the community and in day-care centres, military bases, jails, and men's shelters.[162-165] Colonisation by pneumococci is detectable in about 10% of healthy adults. 20–40% of healthy children are carriers, and more than 60% of infants and children in day-care settings can be carriers. [159;166]

Intrinsic and Extrinsic Influences on Pneumococcal Infection

The incidence of bacterial pneumonia and bacteraemia (i.e. of invasive pneumococcal disease) varies substantially by age, genetic background, socioeconomic status, immune status, and geographical location. Although numerous genetic differences have been suggested as contributors to immune dysfunction and increased risk of disease, these alone cannot explain the varying susceptibility. Risk factors common to sub-Saharan Africa, such as environmental and social factors, nutritional deprivation and the HIV epidemic, can all have detrimental effects on mucosal immunity. Also, the human mucosa is constantly exposed to both pathogenic microbes associated with severe disease and largely asymptomatic infections by commensal bacteria, helminths and other parasites.[167;168] Aside from HIV infection other risk factors include sickle cell anaemia and asplenia, alcoholism, diabetes mellitus, age greater than 65 years, underlying lung disease, severe liver disease, recent influenza infection and other immuno-deficiencies (e.g. immunoglobulin and complement deficiencies).

Socio-economic status determines living conditions, access to nutritious food, access to good sanitation as well as the choice of fuel used for cooking and heating. Work in The Gambia has demonstrated that living in close proximity to others is linked to carriage of the same disease-causing pneumococcal serotype by siblings within the same household. [169] In the context of direct low-level mucosal immune activation by the pneumococcus and frequent high exposure to smoke from BMF, there is potential for further enhancement of person-to-person spread through the generation of respiratory droplets. In this setting, crowding, poor ventilation and increased exposure to BMF smoke have all been implicated as risk factors for pneumonia. [55]

Consideration is now given to the normal pulmonary mucosal defence and the potential effects that BMF smoke has on this mileu.

1.6 Pulmonary Defence Mechanisms

The lung is constantly challenged by inhaled particles and microbial pathogens. The first line of defence is the cough reflex and the mucus covered ciliated epithelium.[170] The next line of defence consists of innate responses that are non specific host defence responses to foreign particles occurring predominantly at the epithelial surface.[171;172] Mediators of the innate response include soluble factors and host cells, including alveolar macrophages and epithelial cells. Finally humans mount acquired responses to invading pathogens. These are specific humoral (antibody) or opsono-phagocytic responses regulated by the cellular immune system.

The entire immune system must respond rapidly to remove inflammatory stimuli because the respiratory tract is a fragile tissue with a delicate structure that is designed for gas exchange and excessive inflammation impairs this function.[173] Disruption or subversion of the respiratory tract epithelial barrier leads to increased invasion by mucosal pathogens and a high frequency of life-threatening bacterial disease. It is likely that a process of epithelial barrier dysfunction and immune dysregulation at the mucosal surfaces leads to the much higher rates of pneumonia seen in resource-limited countries. [174]

1.6.1 Airway Clearance: The Mucociliary Escalator and Cough

Each day approximately 8000 litres of air are drawn through the nose. This is where large particles are deposited. Particulate material greater than 0.5μm in diameter are deposited in the lining fluid (mucous) of the trachea and the bronchi.[175] Clearance of this debris, particles and secretions is by mucociliary action. The lower airways are ciliated and lined by epithelium that contain goblet cells and submucosal glands. These glands produce enough mucus to cover underlying cilia which constantly sweep the mucus towards the throat where it is swallowed.[176]

Cough can be either a voluntary action or a reflex respiratory response to inhaled PM, irritants, high or low temperatures and humidity. Most commonly cough is a symptom of infection but in developed nations it is also associated with cigarette smoking. In Malawi, chronic cough is often associated with tuberculosis infection.[177] The incidence of cough as a symptom was common among Malawians with HIV and pneumococcal disease, but the duration of cough was not predictive among hospital admissions. [116]

1.6.2 Innate Immune Factors

A range of soluble innate immune mediators meets organisms and particles that pass through the mucociliary blanket. Some of these factors are produced at low levels and others are induced by specific activation.

Lysozyme

Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, was first discovered by Alexander Fleming in 1921. It is a bactericidal protein capable of lysing the carbohydrate polymers (peptidoglycans) that comprise the external membrane of bacteria. It is present in lung lining fluid, tears and saliva as well as in cytoplasmic granules of neutrophils. [178;179]

Lactoferrin and Secretory Leukocyte Protease Inhibitor (SLPI)

These are airway defence proteins produced by serous cells in a number of locations in the respiratory tract. Lactoferrin is a multifunctional protein of the transferrin family that is capable of killing and agglutinating bacteria. It recognises bacteria on the basis of carbohydrate motifs – it binds to the lipopolysaccharide of bacterial cell walls, and the oxidized iron part of the lactoferrin oxidizes bacteria via formation of peroxides. This makes the membrane more permeable and results in cell breakdown (lysis).[180] It also stimulates super oxide production by neutrophils. [179]

SLPI is one of the dominant proteins present in nasal epithelial lining fluid and other nasal secretions. Diseases such as emphysema and cystic fibrosis are characterized by increased levels of neutrophil elastase. SLPI is one of the major defences against the destruction of pulmonary tissues and epithelial tissues by neutrophil elastase. SLPI is considered to be the predominant elastase inhibitor in secretions. Increased levels of SLPI in plasma may be indicative of pneumonia. [181]

α and β Defensins

These molecules show broad antimicrobial activity against Gram-positive bacteria, mycobacteria, fungi and some viruses.[182] They act by inducing permeabilisation and are up regulated in the lung in response to the inflammatory cytokine interleukin-1 (IL-1). [183]

Cathelicidin

The cathelicidins are a family of antimicrobial peptides that are found in the phagolysosomes of polymorphonuclear leukocytes and macrophages. [184] Members of the cathelicidin family are characterized by a highly conserved region (cathelin domain) and a highly variable cathelicidin peptide domain. Cathelicidins were originally found in neutrophils but have since been found in many other cells including epithelial cells and macrophages after activation by bacteria, viruses, and fungi. [185]

Collectins

The collectins are a family of proteins that bind to carbohydrates on the surface of pathogens. This triggers the alternate complement cascade. They also have direct effects on the activation of immune cells including macrophages and lymphocytes. Key members of this family include surfactant proteins A and D (SP-A and SP-D) and mannan-binding lectin (MBL). [186]

Complement

The complement system consists of over 25 proteins found in the blood. It is a biochemical cascade that helps the ability of antibodies to clear pathogens. Complement proteins are important components of the innate and the acquired immune defence and are found within lung secretions. [187;188] Patients with complement deficiencies (e.g. Sickle Cell Disease) experience recurrent infections with capsulate organisms including *Haemophilus influenza* and *Streptococcus pneumoniae*. [189;190]

Toll-Like Receptors

Host cells are also capable of mounting a cellular innate response; Toll-like receptors (TLRs) are of particular interest.[191] TLRs are primitive receptors on host cells that respond to well-conserved structures of microorganisms such as lipopolysacharides and carbohydrates or forms of bacterial DNA. The TLR on the surface of alveolar macrophages has been shown to mediate innate responses to *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* in human alveolar macrophages. The inflammatory response of alveolar macrophages to an intracellular product of *S. pneumoniae* called pneumolysin has been shown to be dependent on TLR4. [192;193]

1.6.3 Cellular Components of Immune System

Macrophages, dendritic cells and lymphocytes act together to orchestrate the rapid uptake and removal of foreign material from the lung.

Alveolar Macrophages

Alveolar macrophages (AMs) are mononuclear phagocytes that are produced in the bone marrow and reach the lungs via the bloodstream. [194] Based on their location, macrophages found in the lung are classified into 4 different types; i) the alveolar macrophage; ii) the interstitial macrophage; iii) the intravascular macrophage and iv) dendritic cells.[195] AMs occupy a unique site as they are the only macrophages in the body that are exposed to air. They are located within the alveolar surfactant film, which is produced by type II alveolar epithelial cells and is composed of phospholipids and proteins.[196] Figure 1.6 (taken from Russell et al) demonstrates two different macrophage phenotypes and the different way that they process antigen. [197]

The AM is considered a key cellular component in pulmonary innate immunity. They are the predominant phagocyte of the alveolar space and are found at a density of approximately one per alveolus.[198] They represent the first line of defence against any inhaled constituents of the air that reach the alveolar space.[195] Approximately 90% of cells obtained from healthy individuals by bronchoalveolar lavage (BAL) are AMs.

AMs are a major component of inflammatory and immunologic reactions in the lung; they respond to environmental factors through multiple ligands present on their surface as well as their ability to secrete cytokines. AMs are responsible for the phagocytosis and clearance of most microparticles and microorganisms in the distal airways and the alveolar spaces.

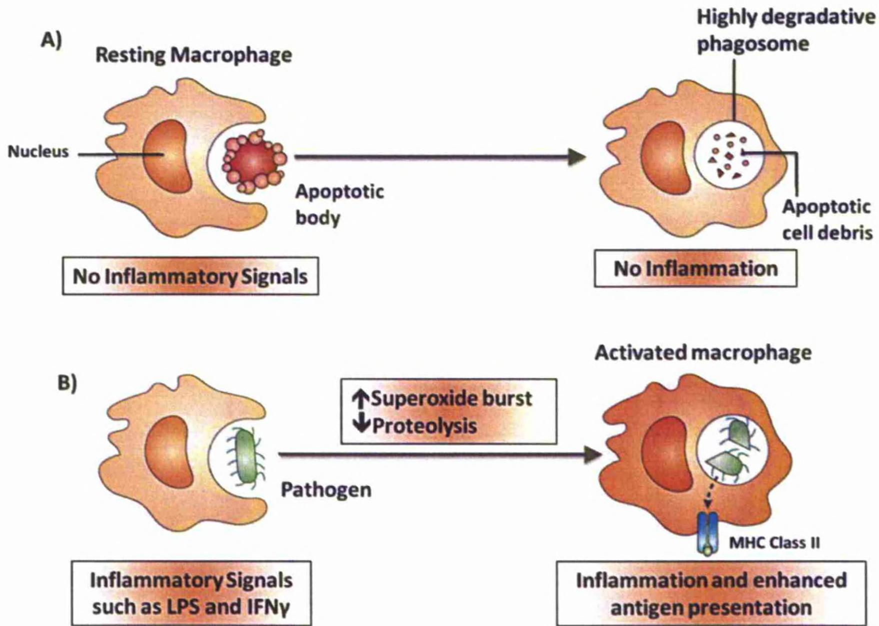


Figure 1.6: The phenotypic differences between resting and activated macrophages. *These differences are reflected in the physiology of their phagosomal compartments. The functions carried out by macrophages are modulated by their degree of stimulation by exogenous mediators, such as microorganism-derived Toll-like receptor (TLR) agonists, or cytokines and chemokines. A) Resting macrophages function in the absence of any inflammatory stimuli and their primary role is to remove cellular debris such as apoptotic cells from the body. The phagosomal compartment of the resting macrophage is highly degradative for efficient processing of this material. B) By contrast, macrophages activated by exposure to TLR agonists, such as lipopolysaccharide (LPS), or cytokines, such as interferon-γ (IFNγ), show alterations in several aspects of phagosome physiology. The superoxide burst in activated macrophages relies more heavily on the production of reactive oxygen and nitrogen intermediates.*

AM ingest small numbers of particles, bacteria or apoptotic cells and release oxidative agents and proteases onto this material in the form of phagolysosomes.[188] Some bacteria (e.g *Legionella spp* and *Mycobacteria spp*) can frustrate phagolysomal processing, either by resistance to the phagosome contents or by escape into the cytosol.[199] Following digestion in the phagosome, macrophages present antigen to lymphocytes either locally or in the regional lymph node.[200] The precise mechanism by which antigen are transported to the regional lymph nodes is poorly understood in humans but is likely to involve dendritic cells and other pulmonary macrophages (interstitial macrophages).

AM play a central role in the inflammatory process; they secrete chemotaxins that attract polymorphonuclear leukocytes to the airways and within the phagolysosome they release potent bactericidal agents (oxygen radicals and proteolytic enzymes) in response to infection. [76;201] AM protect the lung from bacterial or viral infections by at least three mechanisms: production of interferon (an antiviral agent), production of inflammatory cytokines that recruit and activate lung phagocytes, and production of antimicrobial reactive oxidant species. [174;202;203]

Overwhelmed macrophages undergo apoptosis and are removed by the mucociliary escalator and cough reflex.[204] Apoptosis is a preferable response compared to necrosis as it reduces the release of inflammatory mediators that might otherwise damage the respiratory epithelium. [205]

An important aspect of AM function that is often overlooked is the anti-inflammatory cytokine production.[206] AMs have a predominantly immunosuppressive influence on the alveolar surface that is altered in favour of phagocytosis and inflammation in the presence of bacteria or viruses.

Lymphocytes

In normal individuals, lymphocytes account for approximately 10% of cells harvested from BAL fluid. In addition, lymphoid aggregates and follicles containing mainly B-Lymphocytes are found in contact with the visceral surface of the epithelial layer – known as bronchus-associated lymphoid tissue (BALT).[207] The lymphocytes harvested from BAL are approximately 99% T-Lymphocytes. These cells distinguish self from foreign antigen presented by macrophages and B cells to T-cell surface antigen receptors (TCR). Different subpopulations of T-Lymphocytes defined by surface markers have different functions. CD4 expressing cells are helper T cells with a predominant role in regulating antibody responses and activating macrophages. CD8 expressing lymphocytes are cytotoxic cells and produce a cytokine profile that activates macrophages and Natural Killer (NK) cells to an effective anti-viral response. [208]

Neutrophils and Monocytes

Neutrophils and monocytes make up about 1-2% of lung lavage from healthy individuals but the vast majority of cells in acutely pneumonic lung.[209] A large number of neutrophils are sequestered by the fluid dynamics and small capillaries of the pulmonary circulation and are so effectively on "stand by" near the air space.[210] Monocytes are immature macrophages that circulate like lymphocytes and neutrophils and form an important immune reserve, rapidly recruited when necessary. Monocyte emigration follows the neutrophils, starting at 6 hours and peaking at 12-18hours.

1.6.4 Cellular Functions of Innate Immune Function

Oxidative Burst

Reactive oxygen intermediates (ROIs) are key components of the innate defense system.[211] Oxidative burst (sometimes called respiratory burst) is the rapid release of reactive oxygen species. AMs and neutrophils produce O_2 metabolites, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), in association with phagocytosis.[212] AMs can be activated to produce ROIs by many substances and they can also be activated or primed by cytokines (IFN-γ, platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF and leukotriene B4 (LTB4)) to enhance secretion of ROIs.[212-215] ROIs play an important role in both the intracellular and extracellular defence mechanisms directed against micro-organisms and this capacity to produce ROIs is a prerequisite of the non-specific anti-infectious defence system.

Proteolysis

Proteolysis refers to the degradation of proteins by cellular enzymes called proteases. Proteases released by alveolar macrophages are of major importance in the tissue damage that is associated with chronic inflammatory lung disease.[216;217] These enzymes can be classified according to the optimal pH in which they are active. Activated leucocytes secrete proteolytic enzymes with activity at acid and neutral pH and both types of proteases have been implicated in causing lung tissue damage. [218-221]

The mechanisms by which proteolytic functions are involved in defence against infection are complex. The phagolysosomal milieu of activated macrophages (Figure 1.6) is actually less proteolytically active than that of resting macrophages, the implication is that this may maximize the antigen sampling and presenting capacity of activated macrophages.

1.6.5 Acquired Immunity

The acquired or adaptive immune system is made up of highly specialised cells and processes that avert or eliminate pathogenic challenges. The adaptive immune response enables an organism to recognize and remember specific pathogens and to intensify host responses each time the pathogen is encountered.

This aspect of the immune system is highly adaptable because of somatic hypermutation; a mechanism that allows a small number of genes to generate an enormous number of different antigen receptors, which are then uniquely expressed on each individual lymphocyte. Gene rearrangement leads to a permanent change in the DNA of each cell so all of the progeny of that cell will have genes encoding the same specific receptor; Memory B and T lymphocytes therefore enable immunity to be long lived.

Immunoglobulin

Immunoglobulins, either secreted by plasma cells or attached to the surface of B lymphocytes, are found at all levels of the respiratory tract.[187;222] Immunoglobulin in the respiratory tract agglutinates bacteria, activates complement and acts as an opsonin for phagocytosis by resident AMs or neutrophils migrating into the parenchyma in response to inflammation.[223] IgA is the predominant immunoglobulin of the upper respiratory tract and IgG predominates in the parenchyma. Respiratory epithelium facilitates the secretion of IgA.[224] IgG is thought to be produced locally by pulmonary plasma cells that have matured in regional lymph nodes and migrated back to the tissue where antigen was first presented.[225] In acute inflammation, the epithelium becomes increasingly permeable and so plasma proteins, including albumin and immunoglobulin, leak in to the respiratory tract.[226] In patients with IgG deficiency, recurrent respiratory tract infections are a major problem.[227]

Immunoglobulin facilitates phagocytosis by binding to immunoglobulin receptors on macrophages and neutrophils.

1.6.6 Cytokines and Chemokines

Cytokines are peptides produced by metabolically active cells of the immune system. In the face of overwhelming infection, macrophages and lymphocytes generate pro-inflammatory cytokines to recruit polymorphonuclear cells and monocytes to the lung. Cytokines have the ability to activate other cells and include proteins such as interleukins, interferons, tumour necrosis factor and chemokines. Their effects include chemotaxis, degranulation, protein production, cell division, and activation, cytoskeletal rearrangement and immunomodulation. Cytokines usually act over short distances and bind to target cell receptors; e.g. RANTES (Regulated on activation, normal T expressed and secreted) is a cytokine that is a member of the interleukin-8 family of cytokines. It is a protein that is a selective attractant for memory T lymphocytes and monocytes.

Two key inflammatory cytokines that are involved in the pulmonary and systemic inflammatory responses are interleukin 6 (IL-6) and interleukin 8 (IL-8).

Interleukin 6 (IL-6)

Macrophages as well as T cells secrete IL-6. It stimulates an immune response to the inhalation of particles or as a response to tissue damage (e.g. burns) that subsequently leads to inflammation.[228-230] In mice, it has also been shown to be required for resistance against *Streptococcus pneumoniae*.[231]

Alveolar macrophages are able to secrete IL-6 in response to specific microbial molecules, referred to as pathogen associated molecular patterns (PAMPs). PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition

receptors (PRR), including Toll-like receptors (TLRs). This in turn can induce intracellular signalling cascades that give rise to inflammatory cytokine production.

Interleukin 8 (IL-8)

Macrophages as well as other cell types, such as bronchial epithelial cells, produce IL-8; it can be secreted by any cell with TLRs, which are involved in the innate immune response.

Macrophages are the first cells to encounter and phagocytose PM, which in turn leads to the secretion of IL-8. Two of IL-8's key functions are to recruit neutrophils and to promote oxidative burst within the neutrophil.[173;232] In individuals who produce excess IL-8 the severity of respiratory syncytial virus bronchiolitis appears to be worse. [233]

1.6.7 Lung Inflammation and Pulmonary Infection

Lung inflammation increases susceptibility to bacterial respiratory infection (Figure 1.7B). Inflammation of the respiratory mucosa, caused by viral upper respiratory tract infections is associated with increased bacterial adherence and secondary bacterial infection of the pulmonary epithelium.[234] This effect has been modelled *in vitro* when inflamed alveolar epithelium showed up-regulated epithelial receptors, particularly platelet activating factor receptors (PAFr).[235;236] It has also been shown that PAFr deficient mice have decreased susceptibility to pneumococcal pneumonia due to decreased bacterial binding. Inflammation increases susceptibility to bacterial infection by facilitating bacterial adherence and secondary viral infection.[235;237] In summary, *in vitro* inflamed epithelium shows up regulated epithelial receptors (PAFr) and this receptor specifically binds surface of *S. pneumoniae* and therefore facilitates invasive bacterial disease.

1.6.8 Host Responses against Streptococcus pneumoniae Colonisation [238]

Colonisation of the nasopharynx occurs before infection of the lower respiratory tract, but this is normally asymptomatic and not usually followed by disease.[157] The local host immune response in the upper airway is critical to prevent invasive disease. A vigorous local host immune response to *S. pneumoniae* involving phagocytes (neutrophils and macrophages), B cells (antibodies against pneumococcal polysaccharides and proteins) and T cells rapidly eliminates colonisation. By contrast a poor mucosal immune response results in protracted colonisation. Innate and adaptive immunity both play an important role in host defence responses against the pneumococcus. [157]

Innate Immune Response during Colonisation

Innate responses outlined in Chapter 1.6.2 are important in assisting in the removal of the organism from the upper airway. C-reactive protein (CRP) also appears to play an important role in the defence against colonisation. CRP is an acute-phase protein which is mainly found in serum and is elevated during inflammation. It is also found in the nasopharynx of healthy individuals as well as being elevated during inflammation.[239;240] CRP has several functions in relation to cell-surface phosphorylcholine-expressing bacteria (e.g. S. *pneumoniae* and *H. influenzae*), which includes activation of complement by the classical pathway, enhancing opsonisation and inhibition of the attachment of bacteria to epithelial cells.[241] These functions help in clearance of colonising pneumococci from the upper respiratory tract. The role of complement in colonisation is not fully elucidated yet but it is known that complement plays a role in protection against pneumococcal infection through the promotion of opsonophagocytosis. [242;243]

The innate response also includes cellular responses from both neutrophils and macrophages. In murine models it has been

demonstrated that colonisation of the upper respiratory tract by pneumococcus triggers an acute inflammatory response characterised by a robust influx of neutrophils into the lumen of the paranasal spaces and release of cytokines (TNF-α) and chemokines (IL-8).[244-246] This acute inflammatory response is ineffective at controlling initial mucosal colonisation, but it enhances the adaptive immune response and subsequent bacterial clearance. [247]

Adaptive Immune Response during Colonisation

Secretory IgA antibodies and serum IgG are produced in response to colonisation of the nasopharynx.[248] However, it is unclear whether these antibodies are protective against colonisation. Many serotypes of *S. pneumoniae* secrete a zinc metalloprotease which can inactivate IgA1 (a subclass of IgA). Also the cleaved IgA1 fragment may assist in translocation of the opsonised bacteria across the host respiratory epithelium.[249] Raised concentrations of serotype-specific antibodies against pneumococcal polysaccharides and antibodies against pneumococcal proteins in serum and saliva have been correlated with increased protection against carriage.[158;249;250] These antibodies opsonise pneumococci, making it easier for phagocytes to recognise, ingest and clear bacteria from the respiratory tract.[251] This has long been thought to be the primary mechanism for protection against pneumococcal colonisation.

In addition to antibody-mediated immunity, recent data suggest that other mechanisms of protection against pneumococcal carriage are also required. The observation that pneumococcal pneumonia is greatly increased in patients with HIV infection indicates the protective role of CD4+ T cells in resistance to pneumococci. Data from murine studies imply that the course of experimental colonisation is not affected in mice that are unable to produce pneumococcal specific antibody.[252] Secondly, the adaptive immune response is improved in the presence of a pore-

forming cytotoxin called pneumolysin and neutrophils. The relationship between pneumolysin and neutrophils promotes delivery and release of pneumococcal-specific antigens to the nasal associated lymphoid tissues, a process that is impaired in either neutrophil- or pneumolysin-deficient conditions which in turn is associated with prolonged nasopharyngeal colonisation.[247;252] Lastly, mice lacking the ability to induce a cell-mediated immunity owing to the absence of appropriate molecules to present antigens to CD4+ T cells (MHC-II knockout mice) show prolonged carriage, suggesting an important role for CD4+ T cells rather than antibody mediated immunity. [253]

Recent data suggest that immunity to pneumococcal colonisation is mediated by a specific subset of CD4+ T cells (Th17) which produce IL-17A.[254-257] Again data from mice suggest that IL-17A-mediated protection against pneumococcal colonisation results in recruitment of neutrophils into the upper airway lumen to clear bacterium.[255;257] However, it is still not clear whether Th17 cells are involved in immunity against human pneumococcal colonisation.

1.6.9 Host Responses against Pneumococcal Pneumonia

As outlined previously respiratory epithelial cells not only provide a mucociliary blanket to continually remove potential pathogens from the lower airways, but they also actively respond to the presence of pathogens. The respiratory epithelium releases an assortment of mediators such as cytokines, chemokines, and antimicrobial peptides (e.g. lysozyme defensins, and cathelicidins), contributing to the innate immunity against *Streptococcus pneumoniae.*

Once that colonising pneumococci in the human nasopharynx are aspirated into the distal airways and alveolar air spaces they interact with pulmonary defence mechanisms and will be either cleared or will cause disease. Excessive replication of the bacteria in the alveoli initiates infiltration of immune cells which, if not properly regulated, impairs gas exchange and leads to the clinical syndrome of pneumonia.

Bacterial clearance in the lung is a highly regulated process; an excessive response might potentially lead to tissue damage, whereas a weak response leads to exponential growth of the pathogens. The primary host immune defence against small numbers of pneumococci during early infection is phagocytosis by alveolar macrophages, which is enhanced through opsonisation by immunoglobulin and complement; a process called opsonophagocytosis.[245;251] Host defence mechanisms against pneumococci during later infection are different; they involve multiple immune cells and a combination of innate and adaptive immunity.

Early Infection in the Lung

Alveolar macrophages are the first phagocytic defence in the lungs and can phagocytise and kill low numbers of pneumococci.[251;258] This helps to clear the bacteria without recruitment of inflammatory cells such as neutrophils, and hence maintaining a low inflammatory state in the

lung.[259] When sizeable numbers of pneumococci are introduced into the lower airways, neutrophils are recruited and they become the main phagocytic cells in the acutely inflamed lung. AMs are then relegated to removing apoptotic neutrophils.[201] They also go through apoptosis during pneumococcal pneumonia; macrophage apoptosis helps with killing of phagocytised *S pneumoniae* and keeps pneumococcal invasion into the bloodstream to a minimum. [260]

It is still not clear whether antigen presentation takes in the lung, in the draining lymph nodes, or in both, and whether alveolar macrophages are part of this antigen presentation process. There are *in vitro* data that imply that alveolar macrophages are able to present antigens to T cells, although less effectively than other antigen-presenting cells (APCs).[261] AM's might induce antigen-specific unresponsiveness in CD4+ T cells as a result of antigen recognition in the absence of co-stimulation. [261]

Late Infection in the Lung

When the alveolar bacterial load rises above a critical threshold, alveolar macrophages cease to perform effective opsonophagocytosis and produce an increased pro-inflammatory cytokine response dominated by TNF-α and IL-8.[259] The presence of TNF-α is not a prerequisite for pulmonary anti-pneumococcal responses because successful clearance of the bacteria can occur independent of this cytokine, but it is beneficial during systemic infection.[262] Inflamed epithelial cells enhance neutrophil recruitment into the lung by secretion of IL-8, both as a direct result of pneumococcal binding to epithelial receptors and in response to macrophage proinflammatory signalling. [263]

When a proinflammatory signal (TNF-α and/or IL-8) is produced in the alveolus, there is up regulation of adherence molecules on endothelial cells, which bind to their receptors on neutrophils. Neutrophils then become the major immune cell population responsible for pneumococcal

clearance in the lung. Gross pathological inspection of the lung in pneumococcal pneumonia lead to the term 'red hepatisation'; this occurs when the alveolar spaces are packed with neutrophils, red cells, and fibrin i.e. neutrophils predominate within cellular infiltrates.

The conventional notion of neutrophil migration with selectin-mediated rolling and β2-integrin-mediated tight adhesion to the endothelium does not apply to pneumococcal pneumonia.[264] β2-integrin-deficient mice show normal neutrophil trafficking into lung tissue after infection with S. *pneumoniae*.[265] Another host-derived, soluble adhesion molecule known as galectin-3 has been implicated as a major neutrophil recruitment signal in pneumococcal pneumonia.[266] Galectin-3-/- mice inoculated with *S. pneumoniae* have accelerated lung infection, with early-disseminated disease.[267] In addition galectin-3 increases neutrophil phagocytosis and exerts bacteriostatic effects on *S. pneumoniae*.

T cells are also recruited in high numbers to the lung in late infection – the peak of T cell infiltration in the lung during intranasal pneumococcal infection in mice *in vivo* coincided with the phase when bacterial growth ceased.[268] The recruited T cells are predominantly of the effector memory phenotype and potentially secrete interferon gamma IFN-γ to activate alveolar macrophages. It has been shown that T cells expressing the gamma delta receptor (γδ T cells) act as regulators of alveolar macrophages and pulmonary dendritic cells during the resolution of pneumococcus-mediated lung inflammation. [269]

Cytotoxicity mediated by γδ T cells helps to restore mononuclear phagocyte numbers to homeostatic levels, and hence prevents excessive inflammation in the lung.[269] After clearance of pneumococci from the lungs, neutrophils, some macrophages and T cells undergo rapid apoptosis. Macrophage apoptosis leads to reduced TNF-α expression, which subsequently leads to reduced neutrophil recruitment and enhanced neutrophil apoptosis.[260] Dead cells are then removed by

phagocytosis, efferocytosis (clearance of apoptotic cells by phagocytes) and the normal function of the mucociliary escalator, whereas the surviving T cells remain in the alveoli as resident effector memory cells. The actual mechanisms of the role played by T cells are still not clear.

This process of mucosal responses are critical in regulating carriage and defence against infection *S. pneumoniae* and the interaction of this whole process with biomass fuel smoke has yet to be examined in any great detail.

1.6.10 Effect of HIV on Immune Function

The literature covering this area is large and so focus here has been on the limited number of studies that have been carried out that are relevant to the African population.

Innate factors in the lung lining fluid from Malawian volunteers have been investigated in order to determine their relation with HIV infection. No significant difference was found in the level of lactoferrin, lysozyme or SLPI in comparison between HIV negative and positive adults, with or without a history of pneumococcal disease.[270] HIV infected adults were found to have increased RANTES (and beta-chemokine) in lung fluid. This pro-inflammatory response is thought to be an appropriate response to the challenge posed by the HIV infection itself. [270]

HIV infects and affects **alveolar macrophages.**[271] This leads to altered function of receptor expression, activation, cytokine production, accessory cell function, phagocytosis and apoptosis of the macrophages themselves. Phagocytosis and killing of *Cryptococcus neoformans* and *Pneumocystis jiroveci* may be impaired in alveolar macrophages from HIV infected individuals but no impairent of *Staphylococcus aureus* or *S. pneumoniae* has been observed.[272-275] Some differences in alveolar macrophage function may therefore be due to the prevailing cytokine milieu in the alveolus and so these findings need to be repeated in the

light of different lymphocyte populations and cytokine measurements in the lung fluid.

Gordon et al (2001) have also reported that alveolar macrophages from HIV-infected adults do not exhibit impaired pneumococcal phagocytosis. They suggested that alternative mechanisms of susceptibility to pneumococcal pneumonia exist in HIV positive individuals. [272]

In studies of HIV infected patients from the USA, work has suggested that **IgG levels** in lung fluid might be low in patients with HIV.[276] However, Gordon et al (2003) investigated the levels of pulmonary immunoglobulins both in serum and the lung fluid of Malawian adults and demonstrated in individuals with HIV infection, increased levels of IgG, IgM and IgA were found in plasma as well as in lung fluid. The lung fluid levels were not significantly altered by correction (using albumin levels) for the effect of plasma leakage. In addition, pneumococcal polysaccharide specific IgG was particularly increased in the lung following recent pneumococcal disease both in HIV infected adults as well as control patients but the increase was significantly greater in HIV infected adults. This increase in total and specific IgG was thought to be due to the increased production of IL-6 in HIV infected adults; this cytokine being responsible for driving B cell antibody production. [277]

Cytokine responses from alveolar macrophages also appear to differ in HIV infected individuals. Alveolar macrophages from HIV positive individuals produce significantly more IL-1β and IL-6 level in response to *S. pneumoniae* compared to HIV negative individuals. However IL-8 release was significantly lower in HIV-infected than in non-HIV-infected which potentially may result in impaired neutrophil recruitment, and therefore increased susceptibility to pneumococcal infection in HIV-infected subjects. [278]

More recent work has suggested that BAL antigen-specific CD4(+) T cells responses against important viral and bacterial respiratory pathogens are impaired in HIV-infected adults. A higher frequency of antigen-specific CD4(+) T cells against influenza virus, *S pneumoniae* and *M. tuberculosis* was observed in BAL compared to blood in HIV-uninfected adults. Influenza virus and *M tuberculosis* specific CD4(+) T cell responses in BAL were impaired in HIV-infected individuals i.e. Proportions of total antigen-specific CD4(+) T cells and of polyfunctional IFN-γ and TNF-α-secreting cells were lower in HIV-infected individuals than in HIV-uninfected adults. These findings may contribute to the susceptibility of HIV-infected adults to lower respiratory tract infections such as pneumonia and tuberculosis. [279]

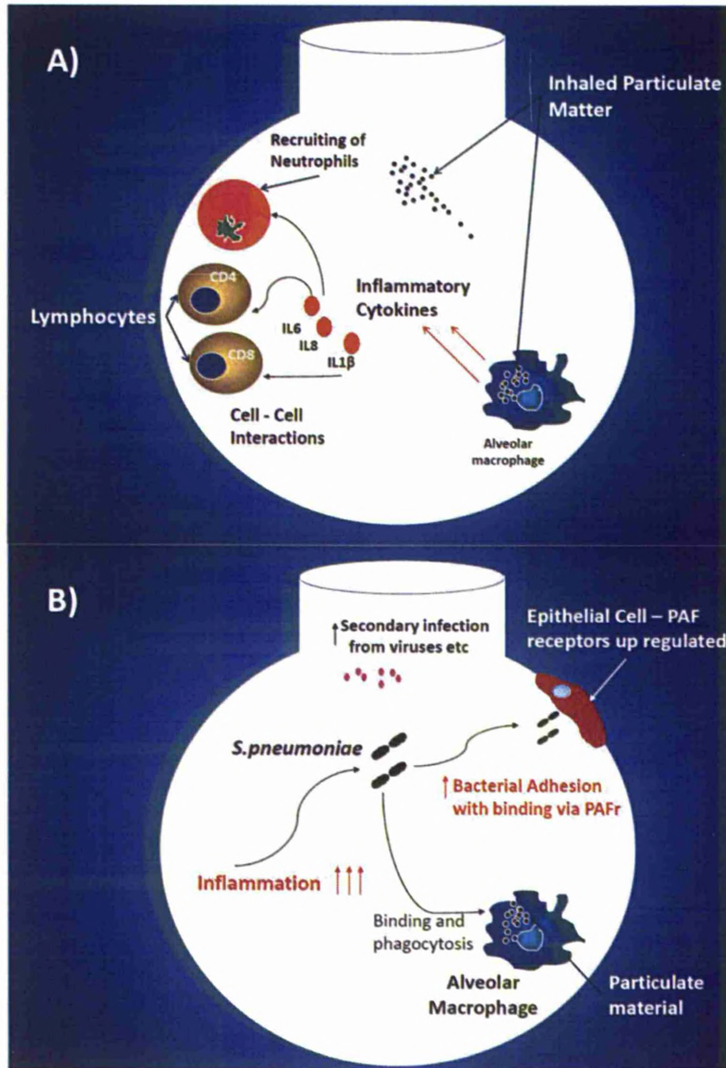


Figure 1.7: Schematic representation of the Alveolus Space. *A) Inhaled PM is phagocytosed by resident alveolar macrophages resulting in an increase in cytokine production and recruitment of other cells. B) The increase in inflammation leads to up regulation of Platelet Activating Factor (PAF) receptor on the surface of epithelial cells, which in turn facilitates S pneumoniae to cross the epithelial lining of the alveolar space.*

1.7 The Effect of PM on Pulmonary Defence Mechanisms

BMF smoke may cause damage to the respiratory tract mucosa by a number of mechanisms. These are summarised in Table 1.4.

After exposure to PM, alveolar macrophages mediate the inflammatory response in the lung (illustrated in Figure 1.7A).

1.7.1 Inhaled Particulate Matter and Lung Inflammation

African households are exposed to levels of carbon particulate matter that exceed those experienced by forest fire fighters.[280] It is likely that the smoke and PM from biomass fuel impair pulmonary macrophage, dendritic cell (DC) and T-cell functions, resulting in epithelial inflammation and apoptosis characterized by cytokine release (tumour necrosis factor-alpha (TNFα), interleukin-1 (IL-1), IL-6, and IL-8), activation of cyclo-oxygenase and lipoxygenase (producing leukotrine B4 and prostaglandin E2), and increased reactive oxygen species activity.[281-283]

PM leads to lung inflammation by mechanisms related to the particle size, the transition metal content and endotoxin associated with the inhaled PM.[284-288] The pro-inflammatory effect of inhaled particulates on human AMs is characterized by increased cytokine production, particularly IL-6 and IL-8, Tumour necrosis factor-alpha (TNF-α), IL-1β and granulocyte macrophage colony-stimulating factor (GM-CSF).[289;290] This inflammatory response is modified by the AMs location, being amplified by epithelial cells and bystander neutrophils that respond to IL-8 secretion.[291;292]

The route by which PM is internalised by alveolar macrophages is important as particles taken up by specific scavenger receptors scavenger receptor e.g. MARCO (macrophage receptor with collagenous structure) do not result in inflammation, but uptake of pollutant particles by any other receptors results in an inflammatory response.[293-295] This route-

dependency of the inflammatory response implies a mechanism by which the alveolus might be protected from the effect of low dose chronic exposure to particulates but susceptible to the inflammatory effect of higher doses.

In healthy young subjects exposed to air pollutants from forest fires acute smoke exposure leads to an increased circulating neutrophil count. This is a bone marrow effect mediated by increased IL-8 production derived from alveolar macrophages.[290] A similar inflammatory response has been observed in Canadian fire-fighters. [280]

The pathogenesis of COPD caused by BMF smoke has yet to be examined but it is probable that it will be similar to cigarette smoke induced lung damage.[13;296] Data from individuals from the USA suggest a novel link between wood smoke exposure and gene promoter methylation that synergistically increases the risk for reduced lung function in cigarette smokers.[297] The chronic inflammatory response to the effects of BMF smoke in the lung, are however only just beginning to be described.

1.7.2 Inhaled Particulate Matter and Susceptibility to infection

Chapter 1.2.2 outlined the data that are available on the effect of wood smoke from human studies. The majority of animal and *in vitro* studies have looked at the effect of PM, derived from outdoor air and diesel, primarily on factors involved in cardiovascular disease, but there are data on the effect that PM has on susceptibility to infection.

Although a direct link between biomass fuel smoke, lung immune dysfunction and susceptibility to infection has yet to be established, it is probable that this is common in resource poor settings.

Carriage

There are limited data on the effects that BMF smoke has on the colonisation and invasion of pathogenic organisms. A recent systematic review found that second hand smoke exposure may be associated with invasive meningococcal disease but there was insufficient epidemiologic evidence to show an association between second hand smoke exposure and invasive *Haemophilus influenzae* disease or *Streptococcus pneumoniae*.[298] More studies are required to be able clarify the effect that smoke exposure has on this aspect of infectious disease pathogenesis.

Mucocilary Clearance

There are no data on the effect BMF has on mucociliary clearance however clearance is substantially impaired by cigarette smoke due to both increased viscosity and volume of mucus produced by smokers as well as damage to cilia caused by increased levels of proteases in the mucus.[299] Cigarette smoking is the biggest risk factor for pneumonia amongst immune-competent adults in the USA, although recent data also imply outdoor air pollution is also associated.[131;137] It is highly likely that BMF will make adults more susceptible to pneumonia in countries such as Malawi, but no data on the impact on adults are currently available.

Innate Responses to to Particulates and Susceptibility to Infection

The effect of inhaled particles is characterized by increased cytokine production, especially IL-6 and IL-8. It is likely that these inflammatory cytokines are involved in the interaction between PM and alveolar macrophages that lead to the observed increased susceptibility to infection (Figure 1.7).

Zhou et al (2007) have demonstrated in murine macrophages *in vitro* that particulates derived from outdoor air pollution cause an increase in the

binding of *S. pneumoniae*, but internalization of the organism is reduced. [43] The rate of killing of internalized bacteria is similar, but particulates lead to a reduction in the absolute number of bacteria killed by macrophages, mainly due to a decrease in internalization. Zhou et al proposed that phagocytic internalization was a potential specific target for the toxic effects of air pollution particles on alveolar macrophages.

Recently Phipps et al have explored the mechanisms by which cigarette smoke exposure impairs host defence against *S. pneumoniae*.[300] After exposure to either room air or cigarette smoke for 5 weeks mice were challenged with *S. pneumoniae* in order to assess the effect on AM phagocytosis of *S. pneumoniae in vitro*. Cigarette smoke-exposed mice had a greater bacterial burden and a more pronounced clinical appearance of illness as well as increased inflammatory cytokines.

Sigaud et al (2007) have shown in a murine model of pneumonia, that IFN-γ priming, administered in order to simulate viral infections, and exposure to particulates derived from outdoor air pollution enhance lung inflammation, evidenced by increased neutrophil recruitment in to the lung and an elevated expression of pro-inflammatory cytokine mRNAs.[301] Both IFN-γ priming and particulate exposure lead to impaired pulmonary bacterial clearance, as well as increased oxidant production and reduced bacterial uptake by alveolar macrophages and neutrophils. It was suggested that priming and particulate exposure lead to an inflamed alveolar milieu where oxidant stress causes loss of anti-bacterial functions in AM and recruited neutrophils.

Paradoxically, also using a murine model of *S. pneumoniae* pneumonia Tellabati et al (2010) have shown that ultrafine carbon black (UF-CB) instilled intranasally leads to a reduction in morbidity and mortality after infection with pneumococci.[44] They concluded that acute high level loading of AM with UF-CB does not increase the susceptibility of mice to pneumococcal infection *in vivo*. The mechanism by which UF-CB

protects mice against pneumococcal is unclear. The authors have suggested that increased anti-pneumococcal function in carbon-laden AM is unlikely, since loading of AM with UF-CB *in vitro* has been shown to significantly impair pneumococcal killing.[302] They have speculated that because increased urinary 8-oxodG (a marker of oxidative stress) was shown in UF-CB-exposed mice, suggestive of increased pulmonary oxidative stress, partial protection against pneumonia in UF-CB exposed mice was due to oxidative stress stimulating the recruitment of neutrophils into the airway.

It is possible that the findings of Tellabati et al may be related to the relatively acute exposure, as opposed to chronic exposures that occur in biomass exposed individuals or alternatively to the high dose of pneumococci given is not comparable the *in vivo* situation of carriage and chronic exposures to smoke. Zhou et al (2007) also outlined that adsorbed compounds, such as metals, endotoxin or other volatile compounds associated with PM from BMF smoke may have a very different effect to the carbon alone used by Tellabati et al. [43]

Table 1.4: Potential effect of biomass fuel smoke on different parameters of the immune respone. (overleaf)

This is a summary table of a literature review of the potential immune dysfunction that is attributable to BMF smoke exposure.

Part of Immune Function Affected	Potential Effect of Biomass Fuel Smoke	References
Increased carriage of micro-organisms	Evidence from direct smoking and biological mechanisms, indicates that SHS exposure may be associated with invasive meningococcal disease although data for pneumococcal disease is less clear.	**Lee (2010)**[298] *PLoS one*
Mucociliary clearance	There are no data on the effect BMF has on mucociliary clearance however clearance is substantially impaired by cigarette smoke due to both increased viscosity and volume of mucus produced by smokers as well as damage to cilia caused by increased levels of proteases in the mucus.	**Tegner (1985)**[299] *Eur J Respir Dis Supp*
Innate Immunity	MARCO has been identified as playing a dominant role in the human AM defence against inhaled particles and pathogens. The effect that BMF smoke has on this and other receptors is not yet clear.	**Arredouani (2005)**[295] *J. Immunol*
	Pulmonary inflammation secondary to microbial components of PM mediated through TLR 2 and 4	**Becker (2002)**[287] *Am J Phy L Cell Mol Phy*
Increased inflammation and reactive oxygen species	Enhanced release of TNF and cell toxicity by increased oxidative stress	**Imrich (2007)**[281] *Toxicol. Appl. Pharm*
	Proinflammatory effects by modulating intracellular calcium concentrations, activation of transcription factors, and cytokine production through a ROS-mediated mechanism	**Brown (2004)**[282] *Am J Phy L Cell Mol Phy*
	Pulmonary and systemic inflammation; increased oxidative stress within the lung	**Swiston (2008)**[280] *ERJ* **vanEeden (2001)**[290] *Am.J.Resp.Crit Care Med*
	Burning dung smoke produces highly redox active fine particulates.	**Mudway (2005)**[285] *Part Fibre.Toxicol.*
Alveolar macrophages	Biological components of PM induce inflammatory response in macrophages	**Alexis (2006)**[288] *J.Allergy Clin.Immunol*
	In murine macrophages *in vitro* particulates derived from outdoor air pollution cause an increase in binding of *S. pneumoniae*, but internalization of the organism is reduced.	**Zhou (2007)**[43] *Am J Phy L Cell Mol Phy*
	The bacterial burden in cigarette smoke-exposed mice ss increased. Increased cytokines IL-1beta, IL-6, IL-10, and TNF-alpha were observed. Cigarette smoke exposed murine AMs showed reduced complement-mediated phagocytosis of *S. pneumoniae*.	**Phipps (2010)**[300] *Infect.Immun.*
	In mice, IFN-γ priming and exposure to PM derived from outdoor air pollution enhance lung inflammation, evidenced by increased neutrophil recruitment in to the lung and an increase in expression of pro-inflammatory cytokine. Both IFN-γ priming and PM exposure lead to impaired bacterial clearance, increased oxidant production and reduced bacterial uptake by AMs and neutrophils.	**Sigaud (2007)**[301] *Toxicol Appl Pharmacol*
Epithelial Cell interactions	Interaction between AMs and epithelial cells during PM exposure induce a systemic inflammatory response	**Fujii (2002)**[291] *Am J Phys L Cell Mol Phy*
	Interactions between neutrophils and lung epithelial cells can amplify inflammatory responses to PM	**Ning (2004)**[292] *Am J Phy L Cell Mol Phy*
Adaptive Immunity	PM exposed mice have increased airway hyper-responsiveness & increased numbers of BAL lymphocytes, eosinophils, neutrophils, & mucus-containing cells. These effects were associated with increase in IL-17A, IL-17F, & TH2 cell (IL-13, IL-5) cytokine levels in lung cells, as well as reductions in the suppressive cytokine IL-10. PM exposure potentially increases the pathophysiological features of asthma via activation of lymphocyte-dependent pathways.	**Saunders (2010)**[303] *Env Health Perspect*
	Smoke exposure seems to be specific to the IFN-gamma-producing CD8+ cells (in adenoids)	**Marseglia (2009)**[304] *J.Interferon Cytokine Res*
Immunoglobulin	Effect is unclear. PM component of diesel exhaust appears to enhance humoral immune responses (specific IgE and IgG) in a rat model. Little other data.	**Siegel 2004**[305] *J Toxicol Env Health*
Interaction with DNA	Wood smoke exposure interacts in a multiplicative manner with gene (p16 or GATA4) promoter methylation increasing the risk of impaired lung function.	**Sood (2011)**[297] *Am.J.Resp.Crit Care Med*

1.8 Conceptual Framework

The background to the problem of biomass fuel exposure, its relationship with socio-economic stautus, its effect on health (in adults and children) and the mechanisms by which it may lead to an increased incidence of pneumonia have been outlined in the preceding sections of this chapter.

This research project was originally designed to assess the biological effects of BMF smoke exposure on pulmonary defence mechanisms against infection and so this has been discussed in detail in Chapter One. However, after a visit by the Directors of the Wellcome Trust to MLW (after one year of the project/funding) the project was critiqued and a strategic decision was made to re-focus on defining the nature of the problem (i.e air pollution levels) and the potential health effects (i.e. lung function) as well as continuing with some aspects of macrophage function work. This lead to a re-defining of the *project hypothesis* and *thesis aims*.

1.8.1 Project Hypothesis

The general hypotheses of this project are that: (1) Indoor air pollution levels in Malawian homes are high; (2) BMF smoke impairs lung function and (3) Particulate matter (PM) impairs the function of alveolar macrophages (AM).

1.8.2 Thesis Aims

The aims of this thesis are to:

I. Assess the **levels of indoor air pollution** (IAP) from Biomass Fuels (BMF) in a population that is exposed in Malawi.

II. Investigate whether there was a **difference in lung function** between individuals who burnt different types of biomass fuel.

III. Investigate if there is an association between reported smoke **exposure and the amount of particulate material (PM) observed** in alveolar macrophages (AM).

IV. Model the *in vivo* situation in the laboratory by **challenging macrophages with PM *in vitro*** and assessing the effect that this has on pulmonary defence mechanisms

V. To assess in the laboratory **the function of BMF exposed smoke. alveolar macrophages** obtained from individuals

CHAPTER 2: GENERAL MATERIALS AND METHODS

2A: PARTICIPANTS

2.1 Study setting

2.1.1 Malawi

Malawi is located in Southern Africa and is bordered by Mozambique to the south, west and east, Tanzania in the northeast and Zambia in the northwest (Figure 2.1). Lilongwe is the capital city and is located in the central region. The biggest commercial city is Blantyre, located in the southern region. Population density is shown in Figure 2.2.

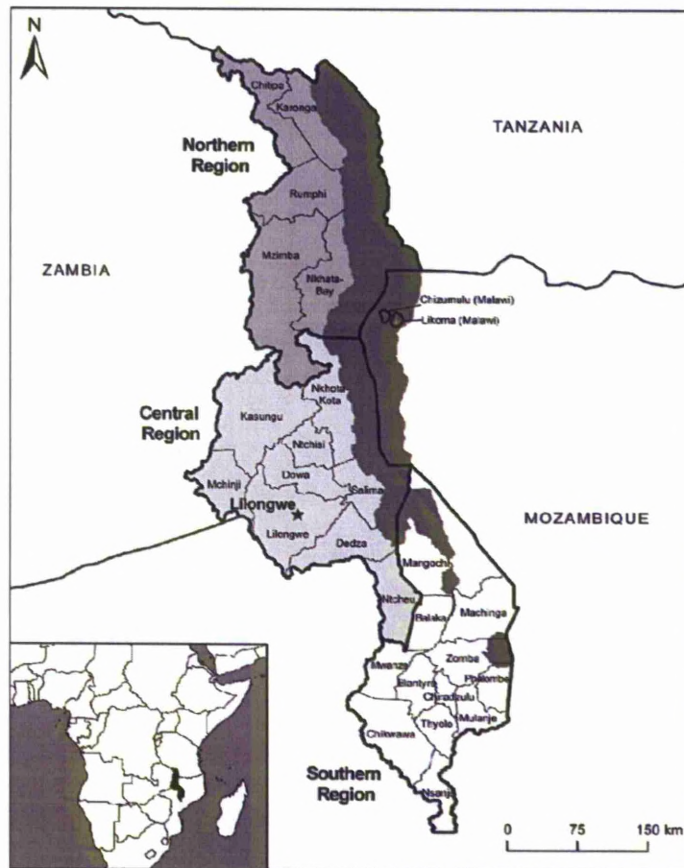


Figure 2.1: Map of Malawi and location in Southern Africa (inset).

Malawi has a population of approximately 13 million people. There are at least 9 different ethnic groups including the Chewa (making up 33% of the population), Yao, Lomwe, Nyanja, Tumbuka, Ngoni, Sena, Tonga and Nkhonde. English and Chichewa (language of the Chewa) are the main languages spoken. [306]

Malawi is one of the poorest countries in the world. The Gross Domestic Product (GDP) per capita is approximately $350 USD per annum. Infant mortality in Malawi is 109/1000.[307] The economy is agriculturally based, contributing more than one-third to GDP. Tobacco accounts for at least half of all exports. Like many other countries in this region of Africa, 95% of Malawians use biomass fuel as their main source of domestic energy. [308;309]

2.1.2 The Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW)

MLW is based at the College of Medicine in the University of Malawi in Blantyre; it is a Wellcome Trust Major Overseas Project. The programme aims to conduct medical research of relevance in Malawi; to provide opportunities for clinical and science graduates from Malawi and abroad to gain additional experience of research by collaborating in or conducting projects; to seek further training opportunities for Malawi graduates on site and in centres of excellence elsewhere; to assist in the establishment of a vigorous research capacity within the College of Medicine, with a strong local base and international collaborative links.

The research strategy at MLW has been formulated to accommodate new scientific innovations and new ideas while continuing to support the Programme's areas of excellence and build on previous achievements. MLW focuses on six major multidisciplinary research themes namely: Malaria & Brain Diseases, Therapeutics in the Tropics, Severe Bacterial Infection, Mucosal & Vaccine Immunity and Health in the Population.

The MLW laboratories are situated next to the Queen Elizabeth Central Hospital (QECH). However, there is an increasing community based aspect to the work of MLW, based in Health Centres in the peri-urban and rural areas around Blantyre.

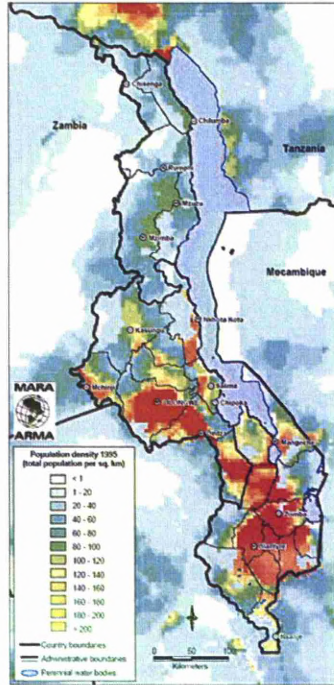


Figure 2.2: Population Density in Malawi

2.1.3 Liverpool School of Tropical Medicine (LSTM)

Founded in 1898 by Sir Alfred Lewis Jones, a Liverpool ship-owner, the LSTM was the first of its kind in the world. As this thesis was being started the Centre for Tropical and Infectious Diseases (CTID) was being built. CTID is a £23 million state of the art facility which puts LSTM at the forefront of infectious disease research.

LSTM holds a research portfolio worth £159 million. It has links with MLW as well as the Royal Liverpool University Hospital which is based within a short walking distance from the laboratories at LSTM.

2.2 Ethics

All aspects of this study were given ethical approval by the College of Medicine Research Ethics Committee (P. 05/06/460), University of Malawi, the Liverpool School of Tropical Medicine Research Ethics Committee (06.45) and Sefton Local Research and Ethics Committee (06/Q1501/192) - see Appendix 1.

All participants read the patient information sheets and signed a consent form. In the rural community the project was explained in village meetings before any work was commenced.

2.2.1 Compensation for Participation in Studies

Participants were given financial compensation for their time and inconvenience for taking part in the research activities in Malawi and the UK.

In Malawi individuals who had air sampling devices placed in their homes were given 500 Malawi Kwacha (MWK); in 2008-2009 there was approximately 250 MWK to £1. For spirometry, which was performed at a location away from the participant's home, 200MWK was given. For attendance at a pre-bronchoscopy out patients appointment 200MWK was given and the equivalent of approximately 50kg of Maize flour was given (1500MWK) for participation in research bronchoscopy, regardless as to whether the procedure was successful or not.

Since 2001 more than 1000 research bronchoscopies have taken place at MLW, without any significant complication. An audit of participants in 2004 suggested that the main incentive to participation in research bronchoscopy was in order to access to healthcare and not for financial compensation. [310]

In Liverpool individuals were given £10 for attendance at a pre-bronchoscopy out patients and £70 for a research bronchoscopy.

2.3 Recruitment of Study Participants

2.3.1 Adults in Malawi

Air Sampling and Spirometry

These two studies were carried out between March and August 2008. The sampling strategies for each study are outlined in Chapter 3.3.1 and 4.3.2 respectively.

The area where the work was planned to be carried out was visited by a field worker in order to explain the project and what was involved with the each study.

Details on air sampling device placement and use as well as spirometry technique and method are outlined in Chapters 3 and 4 respectively.

Bronchoscopy

Advertisements were placed in and around QECH. Interested individuals made contact with the Clinical Investigation Unit, based on one of the hospital wards. Patients were given more information and asked to attend for a clinical assessment. After this second visit they were given a third appointment to attend for bronchoscopy. In this way patients were given three opportunities to not attend or express concern at undergoing a bronchoscopy and lavage (BAL); i.e. a three stage consent procedure was undertaken.

Participants were only included if they were:

- Between 18 and 60 years old
- Willing to have an HIV test
- Not pregnant
- In good health (assessed clinically by history and physical exam)

Participants were excluded from the study if they were:

- Unwell in any way (febrile, cough, wheeze etc)

- Being treated for any chronic medical conditions (e.g. Diabetes or uncontrolled hypertension)
- Anaemic (<8g/dl)
- Taking anti-retroviral therapy or immunosuppressive medication

Only individuals who read the patient information leaflet and signed the consent form were admitted into the study.

After physical examination and history, only patients that were fit enough were invited to take part in the study.

2.3.2 Adults in Liverpool

Bronchoscopy

Research bronchoscopy and BAL had not been established in Liverpool prior to this research project being commenced, although there are several other centres in the United Kingdom where it is performed.

Advertisements for participants aged 18 to 60 were placed in and around the Campus at Liverpool University (e.g. Libraries, Students Union buildings etc) as well as in the Royal Liverpool University Hospital and LSTM. Participants made initial contact with a member of the study team by email or phone and the same three stage consent process that had been established at MLW Blantyre, was performed. The same inclusion and exclusion criteria were used.

Monocyte Derived Macrophages (MDM)

Specimens of whole buffy coat blood were obtained from the UK Blood transfusion service. Buffy coat refers to a blood component prepared by centrifugation of a unit of whole blood. It contains a considerable proportion of the leucocytes (and platelets). MDM were isolated each week at LSTM (outlined in section 2.8.1).

2B: PROCEDURES AND SAMPLES

2.4 Air Sampling Devices and Techniques.

This is described in more detail in Chapter 3.3.4.

Briefly, four different devices were used to measure IAP in Malawian homes:

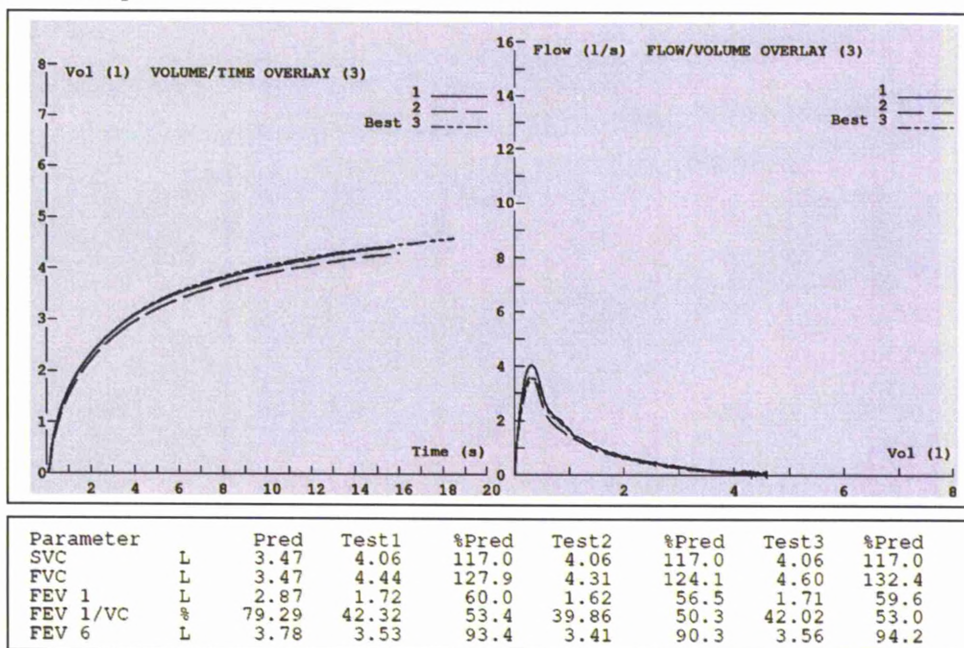
- A gravimetric sample (the internationally agreed bench mark for air quality) was obtained using a **Apex pump** and either a Cyclone head for respirable dust or a IOM sampling head for total inhalable dust
- Two photometric devices to measure PM concentration. These gave a time / exposure pattern. Photometric devices use diffraction of light to measure PM concentrations.
  The **University of California, Berkeley** (UCB) device is based on a modified smoke-alarm and uses the voltage changes generated by particles passing across a photometric chamber to express airborne concentrations of fine particulate matter and the **TSI SidePak AM510** which are laser photometers that measure particles by refraction of a laser beam and active (pump) sampling of the air.
- A **HOBO® Carbon monoxide** device was used to measure indoor CO levels.

This equipment was placed in participant's homes or on their person for 24 hours.

2.5 Spirometry [311]

Spirometry is the measurement of the volume and flow of air that can be inhaled and exhaled from the lungs. It is performed for diagnostic purposes, monitoring lung function, disability/impairment evaluations, epidemiological surveys and clinical research. Spirometry is used in the clinical diagnosis and assessment of asthma, pulmonary fibrosis, cystic fibrosis, and COPD.

Spirometry is reproducible, responsive, discriminates different outcomes and has physiologically valid known performance standards. It is also relatively easy to perform, can be portable and the devices are relatively cheap.



Parameter		Pred	Test1	%Pred	Test2	%Pred	Test3	%Pred
SVC	L	3.47	4.06	117.0	4.06	117.0	4.06	117.0
FVC	L	3.47	4.44	127.9	4.31	124.1	4.60	132.4
FEV 1	L	2.87	1.72	60.0	1.62	56.5	1.71	59.6
FEV 1/VC	%	79.29	42.32	53.4	39.86	50.3	42.02	53.0
FEV 6	L	3.78	3.53	93.4	3.41	90.3	3.56	94.2

Figure 2.3: An example of a participant's spirometry traces. *The FEV_1 was 1.72L and the FVC was 4.60L. The FEV_1 was 60% of predicted, based on the participant's anthropomorphic data. The long expiratory time, concave appearance to the flow-volume loop and the FEV_1/FVC ratio <0.7 are all suggestive of moderate (GOLD Stage 2) COPD.*

Normal spirometric values are predicted by age, gender, height and ethnicity. Key variables obtained by spirometry that are used in the diagnosis of COPD are the Forced Expiratory Volume in one second (FEV_1) and the Forced Vital Capacity (FVC) which is the maximal amount of air a person can exhale with forced effort (Figure 2.3).

2.5.1 Quality control

Daily calibration of the instrument (Vitalograph ® 2120) was performed; the volume was checked using a 3L calibration syringe. The most common cause of inconsistent readings was poor patient technique caused by one of the following; sub-optimal inspiration, sub-maximal expiratory effort, delay in forced expiration, shortened expiratory time or an air leak around the mouthpiece. Subjects were observed and encouraged throughout the procedure and the operator had a good understanding about technique and what was an acceptable result; clear instruction was given to the participant.

2.5.2 Acceptability criteria

ATS/ERS guidelines define an acceptable test as one which fulfils the following criteria:

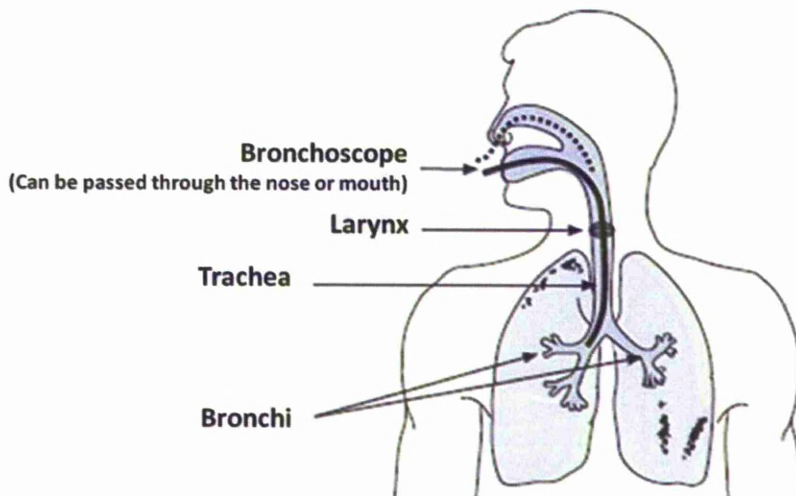
- Fast start with extrapolated volume of no more than 5% of FVC or 0.150 L, whichever is greater.
- After a good start the blow must continue for at least 6 second and the Volume-Time curve must reach a plateau for at least 1 second.
- The manoeuvre must also free from artefact such as cough during one second of blow, leak, glottis closure, early termination, and variable effort.

The blows were repeated until 3 reproducible and acceptable manoeuvres were achieved. The two highest FVC and FEV_1 value of the three must did not differ by more than 0.150 L.

2.6 Bronchoscopy and Bronchoalveolar Lavage

Bronchoscopy was carried out using a standard protocol. [278] Topical lignocaine and xylocaine were applied to the nasal and pharyngeal mucosa in semi-recumbent participants and a fibre-optic bronchoscope (Olympus, UK) was passed to the level of the sub-segmental bronchus of the right middle lobe (Figure 2.4). A 200 ml (warmed normal saline) lavage of the right middle lobe was performed and the BAL fluid was collected into sterile 50ml centrifuge tubes on ice and delivered to the laboratory within 30mins for processing.

Figure 2.4: Bronchoscopy Being Performed to the Right Lung



2.7 Measuring Socio-Economic Status

Questionnaires were asked of all participants (see Appendix 5). From this data an assessment of socio-economic status was made.

From Question 42: Do you own any of the following? Car, phone, bike, radio etc a binary score was obtained.

These scores together with answers to the questions 33 to 43 were then used into a socio-economic living index (SLI). This was a sum of all of the following:

- House Density (number of rooms/number of residents in the home)	0 point ≤ 1 1 point = > 1
- Roof Material	1 point = Grass 2 points = Corrugated metal 3 points = Tiles
- Window Material	0 point = No glass 1 point = Glass
- Sum of assets	0 point = Zero items 1 point = > 1 item
- Type of water supply	1 point = Piped water 0 points = Not piped

Table 2.1: Scoring system used for socio-economic status

The SLI was calculated using the above table. If the SLI points were:

≥ 5 points = High SLI

> 2 and ≤ 4 points = Medium SLI

≤ 2 points = Low SLI

These variables were used in the statistical analysis in Chapter 4.

2C: LABORATORY TECHNIQUES

2.8 Cell Culture

2.8.1 Human Peripheral Blood Monocyte Isolation and Culture

Buffy coat (supplied by the National Blood Service) was gently overlaid onto Lymphoprep™ (Axis-Shield) (2:1 dilution) and centrifuged at 2000 rpm for 25 minutes. The flocular band at the interface (Figure 2.5) was recovered without disturbing the red blood cell layer underneath and PBS added (1:2 dilution). This was centrifuged at 1500rpm for 8 minutes. The supernatant was discarded and the pellet re-suspended in PBS by roughly flicking the Falcon tube. The cell suspension was centrifuged at 1000rpm for 5 minutes, the PBS discarded and the pellet re-suspended in 10ml complete media.

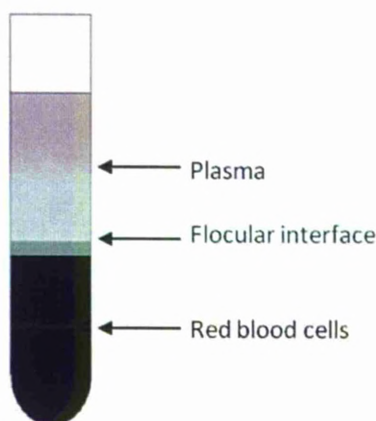


Figure 2.5: The position of the flocular band after centrifugation of buffy coats overlaid on Lymphoprep™

An equal volume of cell suspension was added to 0.4% Trypan Blue. 35µl was pipetted beneath a cover slip that had been placed on the surface of the Improved Neubauer haemocytometer which was used to count the cells. This number was needed to determine the volume of cell suspension that should be added to a specific volume of complete media

to enable an appropriate number of wells to be seeded at $1.5x10^6$ cells per well. 1 ml of this final cell suspension was added to each of 8 wells on a 24-well plate.

Plates were incubated for 4 hours at 37°C, 5% CO_2, after which time the media was aspirated, the cells washed twice; once with 1 ml pre-warmed PBS, once with 1ml pre-warmed RPMI 1640 and finally 1ml complete media was added to each well and the plates returned to the incubator at 37°C and 5% CO_2. The media was changed every third day until the cells were ready to use at between 10 (estimated minimum time for adhered monocytes to develop into macrophages) and 17 days old. No loss of macrophage function or significant change in appearance was observed between these days.

2.8.2 Human Alveolar Macrophage Isolation and Culture

First, 5ml of unprocessed BAL fluid was stored in a cryovial at -80°C. The remaining BAL fluid was filtered through gauze and centrifuged at 800g for 10mins. The supernatant was stored at -80°C and the cell pellet was washed twice with RPMI and then re-suspended at 1×10^6 cells/ml in RPMI 1640 medium (GibcoBRL, Paisley, Scotland) supplemented with 10% fetal calf serum and antibiotics (penicillin concentration of 40U/ml, Streptomycin concentration of 40µg/ml and Neomycin to achieve a final concentration of 80µg/ml).

Depending on the assay to be performed either 1ml or 3ml of the cell solution was pipetted in to a 24 well or 6 well plate respectively.

Greater than 95% viability of adherent cells was demonstrated using Trypan blue; these cells have been demonstrated previously to be predominantly (> 98%) macrophages. [272]

2.9 Particulate matter challenge of cells in vitro

In order to establish image analysis protocols, fine carbon black (FCB) was used. Fine carbon black (purchased from H. Haeffner & Co Ltd, Chepstow, UK, as Huber 990 kindly donated by Dr R Duffin, University of Edinburgh) is a powder with a surface area of $7.92m^2/g$. [312] It was used as a surrogate for field derived biomass fuel samples i.e. before wood or charcoal smoke had been obtained from field studies. This work was carried out at Liverpool School of Tropical Medicine.

In order to treat MDM or HAM in culture a stock solution of PM was made. Using a microbalance, FCB was weighed and added to a known volume of phosphate buffered saline. Sonication of the solution was performed to prevent agglutination of PM and serial dilutions of the stock solution were then added to MDM in a 24 well plate, at known concentrations. MDM were treated with different doses of fine carbon for different times.

The stock solution of PM was stored at 4°C and re-sonicated before further dilutions and treatment of ceels in culture. Non-agglutination was prevented by using glassware as opposed to plastic and was confirmed both by visual inspection of the dilutions made as well as inspection of the cells under an inverted microscope after PM had been added to them.

2.10 Cell Staining Techniques and Preparation of BAL Cytospin

2.10.1 Cytospin preparation

In order to obtain an even dispersal of cells on a microscope slide that allows identification of the cell and particulate matter within it, cells derived from BAL were spun on to the slide using a cytocentrifuge.

250μl (1 x 10 ^{6} cells/ml) cell suspension of undiluted cell suspension was diluted with PBS (1:2 dilution). Microscope slides were cleaned with 70% ethanol placed in the cytocentrifuge. Each cytocentrifuge cup reservoir was loaded with ≤75 μl of cell suspension and spun at 800g for 6 min. The cytospin spot on the microscope slide was air dried.

2.10.2 Staining of Cellular Material

Different cell staining protocols were carried out to ascertain which stain gave the most accurate representation of PM within the macrophage under light microscopy and allowed image analysis software to be able to interpret and analyse the image.

Romanowsky stains and Fluorescein Diacetate (FDA) were all trialled in preliminary cell staining and culture work.

The Romanowsky stains - Giemsa, Hemacolor® (Merck), Fields and Leishman stains were used to ascertain which gave the most accurate images of MDM containing PM. After several trials Giemsa staining gave the best resolution of PM, cell and cytoplasm. An iterative process, that adjusted the staining protocol to accommodate the strengths and weaknesses of image analysis software, resulted in a final staining protocol that only used Fields B (Red) (for cell culture) and Hemacolor® (for cytospins) as identification of the nucleus was not required to ascertain PM load in the cell (see Figure 2.9 and 2.11)

Fluorescein Diacetate (FDA) and Propidium Iodide (PI) Staining

Fluorescein Diacetate (FDA) is deacetylated by intracellular cytoplasmic esterases to yield green fluorescein and accumulates only inside viable cells. Propidium iodide (PI) is used as a DNA stain to evaluate cell viability and to visualise the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. PI is commonly used for identifying dead cells in a population and as a counter stain in multicolour fluorescent techniques. Therefore FDA/PI staining can be used to distinctly visualise viable and non-viable cells under fluorescent microscopy.

Under fluorescent light viable cells appear white, with PM appearing black. 10µl FDA working solution (and 50µl propidium iodide) was added to MDM and incubated for 10 minutes at room temperature.

Fields B and Hemacolor® Staining

<u>For cells in culture plates:</u>

300ul/well of 2% paraformaldehyde was added to MDM for 6 minutes, after the culture medium had been removed. The cells were air dried for at least 10 minutes and using a pipette 300µl of methanol was added for a further 10 minutes. Each well (one at a time to ensure consistent staining) was stained with 400µl Fields B for 9 seconds while gently agitating the plate. The stain was aspirated and the well washed three times with tap water. Once all wells had been stained, the plate was inverted and left to dry at room temperature.

<u>For cells on cytospin microscope slides:</u>

Microscope slides were fixed by dipping them 10 times in Hemacolor® methanol and then tapping them hard on a paper towel to remove excess methanol. Each slide was then dipped in Hemacolor® Red 10 times then tapped hard again on a paper towel to remove the excess stain as much as possible. Slides are stood in the horizontal position so that excess

stain can run off and allowed to air dry. A cover slip was placed on top using DPX (Distyrene, a Plasticizer, and Xylene) mountant as this improved the quality of the image as well as creating a permanent record of the BAL cytospin.

2.10.3 Rationale for staining technique

It was decided that FDA staining of live MDM was not the correct staining method to be used in order to improve the image acquisition and analysis SOP. FDA staining of MDM did have a number of advantages:

(i) There were no artefacts from fixation and killing cells.
(ii) Staining procedure was very straight forward
(iii) There was less variability between samples and was therefore more robust (no washing or de-staining techniques)
(iv) Less labour intensive
(v) Clearly distinguished between cells, cytoplasm, nucleus and PM
(vi) Cells would not be required to grow on a cover slip

However, staining with Hemacolor® and Fields B staining (red only) proved to be more appropriate for the following reasons:

(i) The staining method proved to be quick and uniform and there was little variation different users on different days (Figure 2.12).
(ii) A permanent record of the cells was created.
(iii) This permanent record (cover slip or 24 well plate) was of very limited infection risk (which in terms of handling potentially HIV infected material was felt to be more appropriate)
(iv) When taking images of the cells it did not require inverted fluorescent microscopy: This is not widely available (secondary to cost) and each image obtained using fluorescence requires a significant exposure time, making high through put more difficult.

2.11 Image Acquisition, Analysis and Calculation of PM load in Monocyte Derived Macrophages and Human Alveolar Macrophages

Particulate loading of lung macrophages provides a direct measure of inhaled pollutants derived from biomass smoke. The following section details the testing of:

a. Two methods of enumerating the area of the cell containing PM (Paint Shop Pro and Scion Image Grabber versus Image SXM)
b. Two methods of analysing the cells (including the cell nucleus versus not including the nucleus)

2.11.1 Image Acquisition of MDM and HAM after phagocytosis of PM in vitro

In Chapters 6 and 7, the cells were fixed, in either a well plate on a cytospin spot and stained. They were examined using a Leica DMRB microscope and images were acquired with a DFC 300 camera and Leica Firecam image acquisition software.

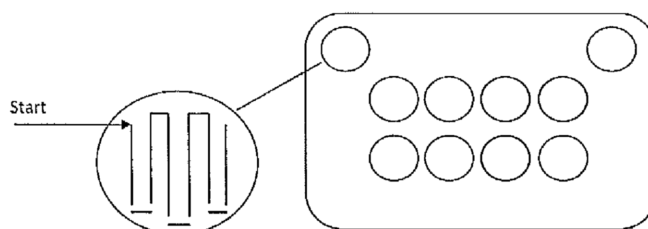


Figure 2.6: Image acquisition pattern in 24 well plate and/or cytospin dot. *(Left hand image represents one well of 24 well plate (or a cytospin dot); right hand image represents a 24 well plate with 10 wells being used) The microscope was moved across the well or cytospin spot in the pattern shown on the left hand image. An image was taken at each new microscope field to ensure that the set of images of cells taken were systematic.*

The acquisition of images required the camera configuration to remain as constant as possible whilst obtaining the best quality image. Images were captured at the highest resolution. The exposure, gain (used to amplify the image signal), gamma (which adjusts the contrast range) and colour settings of the camera were modified very slightly to optimise the quality of the image taken. The images were saved as TIFF's in a specified folder.

2.11.2 Image Analysis Software

Two methods of cell staining and image analysis are used in this thesis. Method One was originally described by Dr Neeta Kulkarni and is used in Chapter 5.[313] This method was very successful at identifying PM within the cell but is labour intensive and depends on subjective observations. Therefore one of the aims of this thesis was to develop a digital image analysis method to enable data to be collected from subjects in a highly reproducible and quick manner. Method Two is the result of a sequence of trials of cell staining, cell fixation and software changes in order to improve on Method One. Both are outlined below.

Method One – Scion Image

Images were viewed under oil immersion using a Leica DMRB microscope. They were then acquired using a Sony HR Trintron video camera. Each HAM image was processed using Paint Shop Pro software (Paint Shop Pro 8; Jasc Software, Eden Prairie, MN, USA) and then transferred to a Power Macintosh 7500/100 CPU. The original images had the appearance demonstrated in Figure 2.7. Method One image analysis is demonstrated in Figure 2.8.

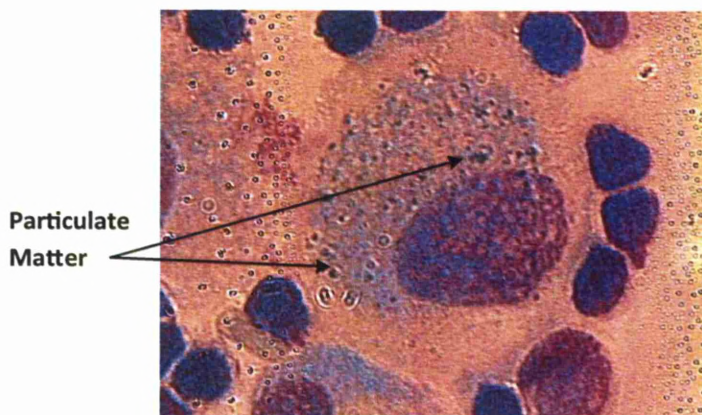
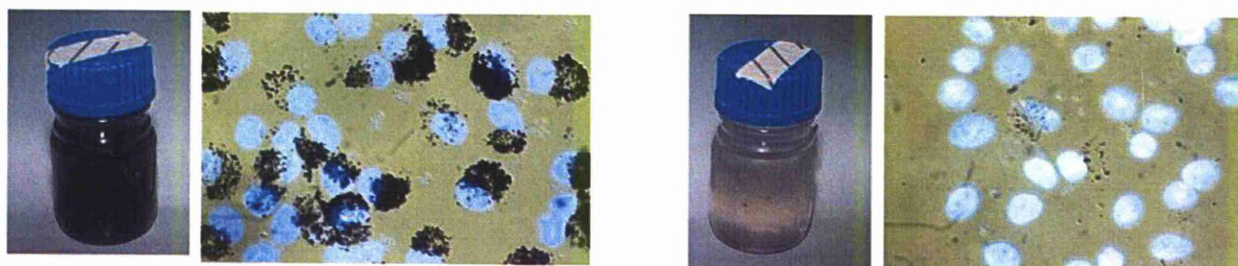


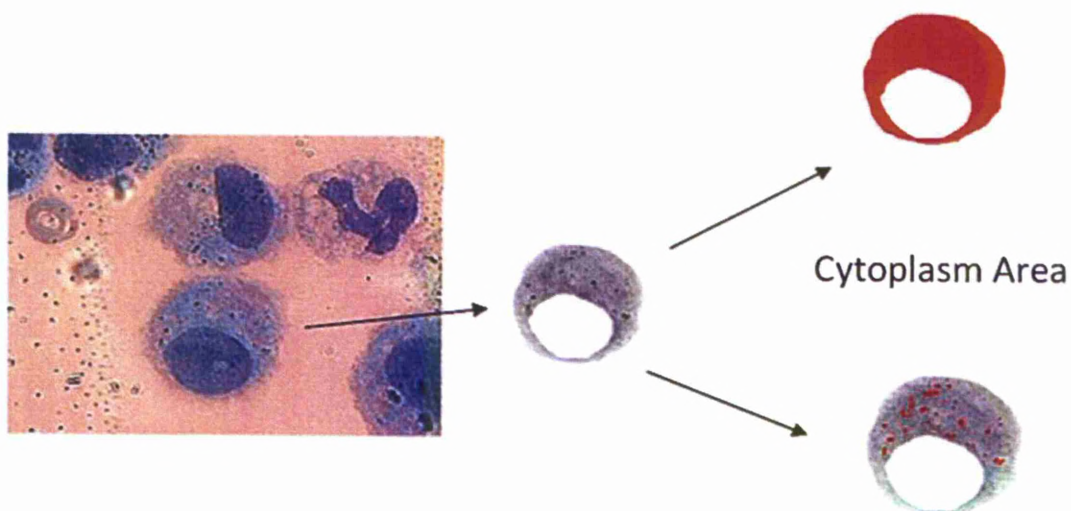
Figure 2.7 Alveolar macrophages containing Particulate Matter: *Obtained from a cytospin of a bronchoalveolar lavage specimen from adult in Malawi (and stained with Giemsa). Particulate Matter (PM) in the cytoplasm is indicated by the arrows.*

The freehand selection tool of Scion Image Grabber software (Scion Image, Frederick, MD, USA) was used to select and manually cut out each cell from the original image. The same freehand process was repeated in order to extract the nucleus from the whole cell. The image was then converted into a grey scale image and using the density slice, the slider was moved to maximum density in order to calculate the cytoplasmic area. The same process was done for particulate matter within the cytoplasm; by selecting the particles only using the density slider and then measuring the area. Because the image had been converted to greyscale the area selected was compared with the original colour image in order to adjust for artefact, which interfered with the interpretation of PM load within the cytoplasm.

To calculate total PM load per cell, the selected PM area was divided by the cytoplasm area.



(A)



(B)

Figure 2.8: Appearance of carbon in alveolar macrophages and digital image analysis to objectively quantify the PM load:

(A) Bottles of freshly obtained BAL taken from two different subjects on the same day, and AM obtained from the same samples viewed under standard transmitted light. The left hand sample is dark in colour due to PM within the AM that can be seen in the cytoplasmic compartment under transmitted light. The right hand sample is macroscopically cloudy and the AM cytospin preparation shows cells with pale coloured cytoplasm. Supernatant fluid after removal of the cell pellets from both samples was clear. In (B) a dissected cell image is shown. The ratio of the particle area (red dots in lower figure) to cytoplasmic area (red colour in upper figure) was calculated and described as the PM load.

Method Two – Development

Using different image analysis software programs (Image SXM, Image Pro Plus, Image J, Digital Pixel Imaging and Andor IQ analysis software; Appendices 2 and 3) Method One was developed further. This was done at LSTM; see section 2.9 for treatment of macrophages with FCB.

Image SXM is a public domain image analysis software program (http://www.liv.ac.uk/~sdb/MIASMA/) that is written, supervised and administered by Dr Steve Barrett (Department of Physics, University of Liverpool). With Dr Barrett a PM analysis application was developed within Image SXM to identify PM, cytoplasm, nucleus and cell boundary from digital images. Treating MDM with known concentrations of FCB assessed the reliability of this and other software. For each different concentration of FCB, the PM to cell area ratio was calculated for each image. The results of time and concentration experiments and cytokine responses to this exposure are reported in Chapter 6.

The digital image analysis method was optimised to enable a PM load score to be derived from alveolar macrophages in a describable, reproducible and quick manner. Image SXM was chosen above other software packages for the following reasons:

(i) It enabled large number of images to be analysed more quickly and efficiently; this gave the assay strong power and small standard deviations when interpreting PM load.

(ii) It was developed at the Department of Physics, University of Liverpool (Dr S Barrett) which enabled various iterations to be tested and improved locally.

(iii) Batches of images can be processed at one time.

(iv) A 'blink comparator' facility enabled rapid comparison of raw image and PM and Cell maps. See Figure 2.11 and Appendix 3.

(v) It only required a Mac Operating System to run the program and the software is free to download from the internet at http://www.liv.ac.uk/~sdb/ImageSXM/#what; there is minimal cost implication, enabling it to be used globally.

Method Two – Effect of Removing the Cell Nucleus from the Analysis

During the development of the image analysis SOP an assessment was made as to whether the nucleus should be included in the PM:Cell area ratio or not. Image SXM software was at first developed to exclude the nucleus as per Method One and demonstrated in Figure 2.9. However because methylene blue stains the nucleus a dark colour, in a large number of images the image analysis software often found it was difficult to distinguish nucleus from PM.

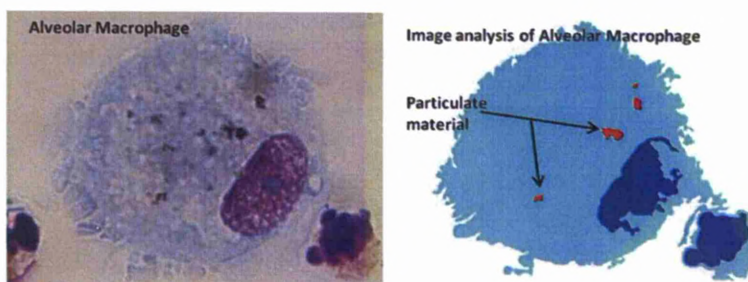


Figure 2.9: An alveolar macrophage and an Image SXM map. *In this example an attempt has been made by the Image SXM program to identify the nucleus.*

Figure 2.10: Analysis of cells using Image SXM – the effect of nuclear staining on PM load. *Different concentrations of FCB were used and images were taken. The images were then analysed by either 'excluding' the nucleus or including the nucleus in the PM load score. (A) Shows comparison of PM load from the same images analysed either with (red) and without (blue) the nucleus included. (B) is a set of images after 12.5µg/ml of FCB is added to MDM. The PM load for each analysed image where the nucleus is excluded is plotted against the same image where it is included. The PM area relative to the whole cell rather than cytoplasm (minus the nucleus) gave a scaling factor of 0.88.*

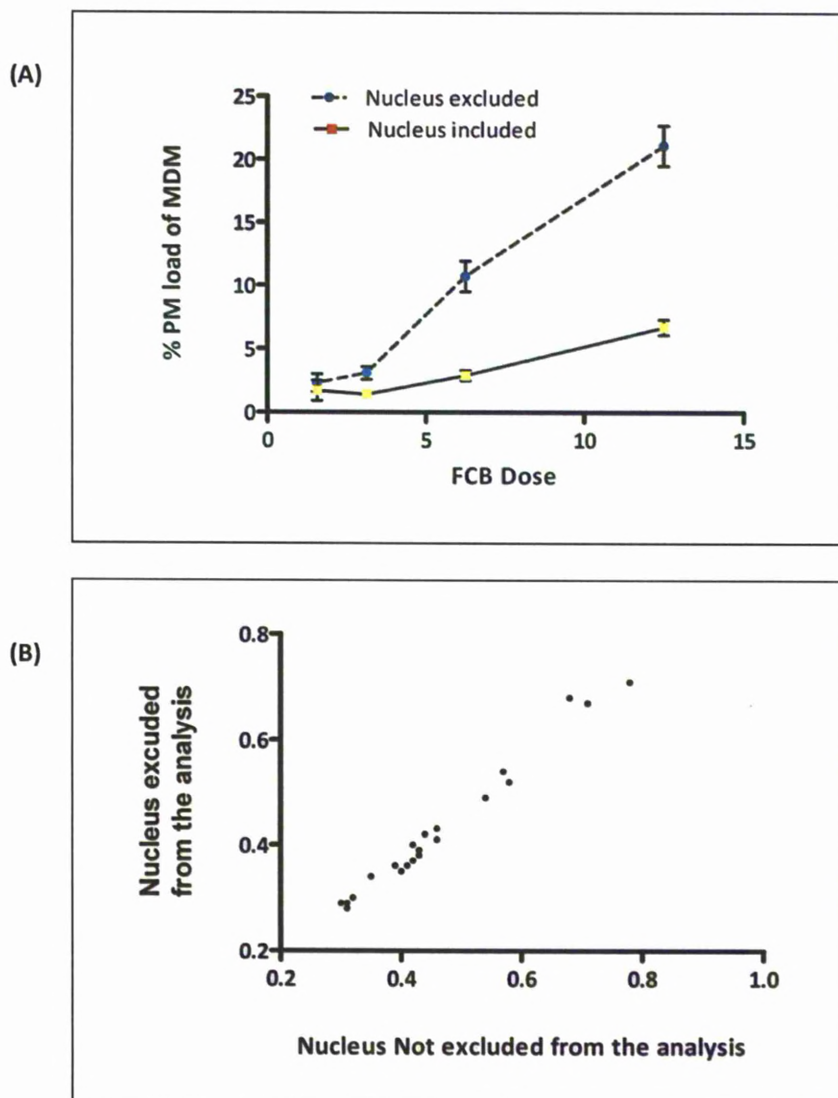


Figure 2.10A shows the results where attempts to identify the nucleus were compared to the results where no attempt to identify the nucleus. It demonstrates that when nuclear staining was used and the software program attempted to identify this staining (and therefore remove it from the final ratio) the PM load was lower. However when nuclear staining was used and the program did not attempt to identify this staining, the PM load was higher, reflecting what the program interpreted as PM but was actually nuclear material.

Figure 2.10B demonstrates that if the nucleus is stained and the image analysis software attempted to remove it, then the reported PM load of the cells was a scaling factor lower. Whilst this scaling factor was similar in different examples and on different days, often nuclear staining lead to a falsely elevated PM load as Image SXM was not able to successfully distinguish between PM and nuclear material.

In summary; when nuclear staining was used there was much greater staining variability. It also meant that the software had difficulty distinguishing the nucleus from PM within the cell. For these reasons the final staining protocol used either Hemacolor® (Red) or Fields B only (VWR International) i.e. no nuclear staining. By staining the cells with red only, a more homogeneous colour, within each cytospin spot or fixed cells in a 24 well plate, was achieved. This enabled Image SXM to interpret the image more effectively.

Figure 2.11 demonstrates the final method that was used at MLW. It can be seen that in this method there is no attempt to identify the nucleus.

Method Two – Image SXM

Image SXM can analyse acquired images in bulk. This analysis methodology is outlined in detail in Appendix 3.

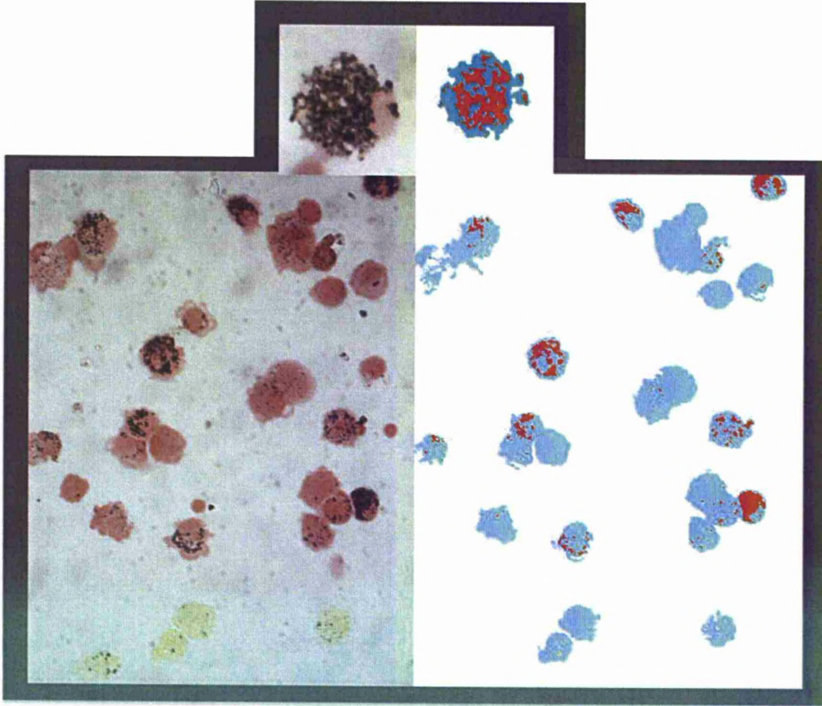


Figure 2.11: Alveolar macrophages containing particulate matter and Image SXM analysis. *Left hand image is of an AM (Hemocolor® Red) obtained from a cytospin of a BAL specimen from adult in Malawi. Computer software image analysis of left hand image produced right hand image – a map of PM in AM where cells are blue and PM red. Inset shows a cell and its map at a higher magnification.*

Image SXM requires a Mac operating system. The following section outlines the method used to calculate the PM load from cells images, using Image SXM software (more detail is in Appendix 3):

- The 'particulate matter analysis' option in the software was selected and the 'Fields B' stain was ticked.
- The input and output files locations were chosen.
- A 'PM load (% of cytoplasm that is PM) was calculated for each image
- A mean PM load was calculated for each individual by the software and the PM-G values (geometric mean) were used for the analysis.

Image SXM enables easy comparison of the original image with the image 'output map' that is generated by the software program (Figure 2.11; right hand image). The 'output map' shows cells and PM within the cytoplasm. This important feature of the software is referred to as the **blink comparator**.

Using the blink comparator different variables were set at beginning of the analysis (i.e. the upper and lower limits of the size of cells and PM threshold). This is done after examination of several different individuals cell images and the settings are then fixed for the whole analysis so that the examination of all cells is objective i.e. uses the same software settings. These settings can be reported, in order to allow comparison between different geographical locations if required and also allows the program to be used with different microscopes, under different magnification etc. (see appendix 3; pages 250-255)

2.11.3 Protocol Development in Malawi: Between day and individual variability

The reproducibility of cytospin image analysis from 10 participants was tested. 25 -30 images were taken by two separate researchers (DF and DM) to assess between day and between individual variability; the differences were analysed using Spearman's correlation coefficient.

There was no significant difference the results of the two observers. The linear R^2 value for the correlation between the two observers was 0.933, $p < 0.001$ (Figure 2.12).

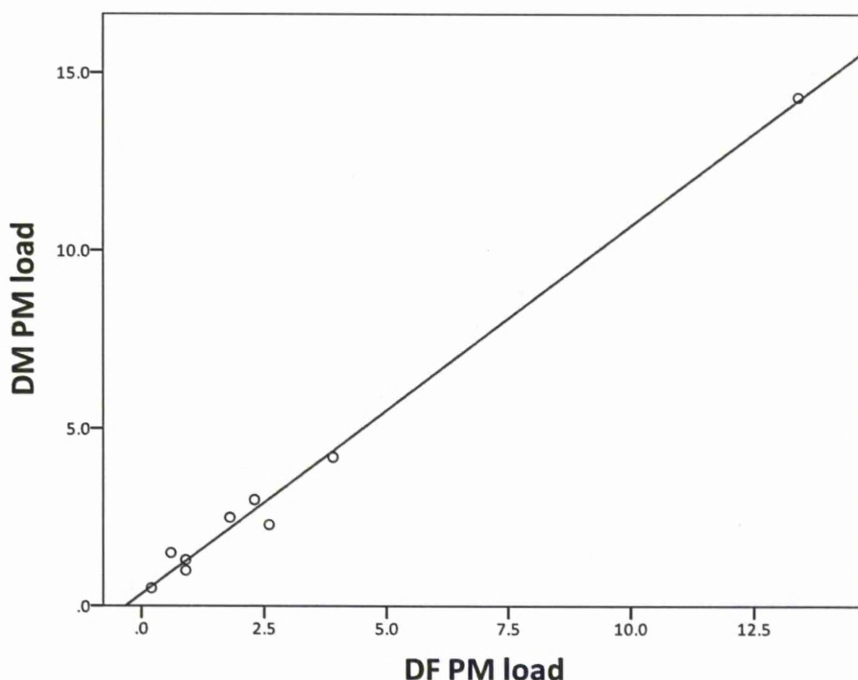


Figure 2.12: Scatter plot of PM load score obtained by two individuals (DF and DM). *PM load Values were obtained using Image SXM software, HAM were obtained from BAL of healthy volunteers and cytospins were stained with Hemacolor® Red only.*

2.12 Enzyme-linked immunosorbent assay (ELISA)

In this thesis, ELISA was used in experiments to detect and quantify the concentration of inflammatory cytokines (IL6 and IL8) in culture media from MDM and HAM experiments *in vitro*, in response to challenge with PM (Figure 2.13).

ELISA was carried out using standard kits. For the experiments described in Chapter 6, the concentrations of IL-6 and IL-8 were measured using a BD OptEIA™ Human ELISA set (BD Biosciences) according to manufacturer's recommendations. The ELISA was carried out in 96-well plates. These were coated overnight with capture antibody, blocked with FBS and loaded with serially diluted standards and supernatant samples for minimum 2-hour incubation.

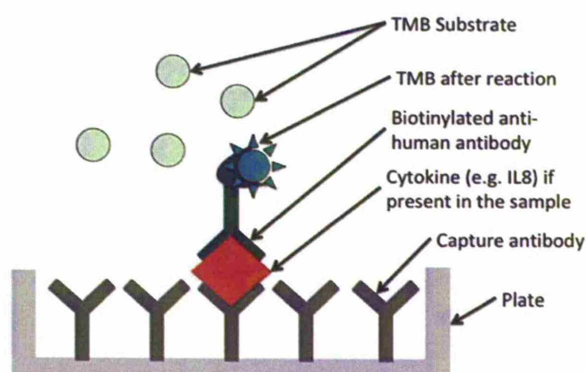


Figure 2.13: Enzyme-linked immunosorbent assay (ELISA). *In this thesis a "sandwich" ELISA technique was used. Antibody specific to the inflammatory cytokine was used to quantify the amount present in MDM cell culture supernatants challenged with PM*

A biotinylated anti-human IL-6 or IL-8 antibody was used for detection, followed by Tetramethylbenzidine (TMB) substrate. The reaction was stopped with sulphuric acid.

Each step of the protocol was followed by 3 washes with PBS using a Labsystems™ Wellwash 4 (Mk 2) plate-washer. The plates were read at 450nm within 30 minutes on a Dynex Opsys MR™ plate reader.

2.13 Reporter Bead Assay – using Flow cytometry

Flow cytometry allows the examination of the heterogeneity of the activity across the cell population at the level of each individual cell. (Figure 2.14) Analysis by flow cytometry can also be combined with immunofluorescence with antibodies against pathogens or cell surface markers.

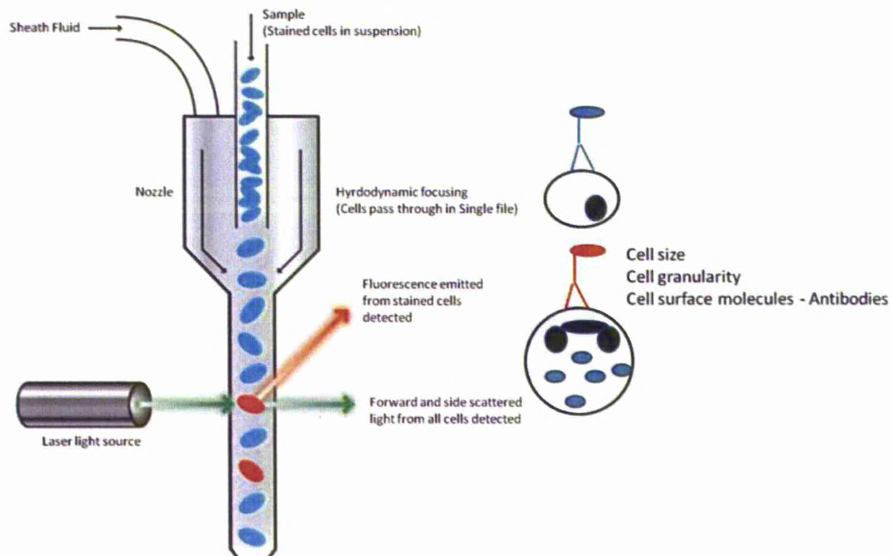


Figure 2.14: Flow Cytometry. *A beam of light (normally a laser light) of a single wavelength is directed onto a hydrodynamically focused stream of fluid. Detectors are aimed at where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors*

Macrophage phgosome activity was measured using a modification of an assay developed by Prof David Russell (Cornell University).[197] It enabled bulk protease activity and intra-phagosomal superoxide (oxidative) burst, as well as other potential macrophage functions, to be measured. Figure 2.15 demonstrates the assays. The assay results are presented in Chapter 7.

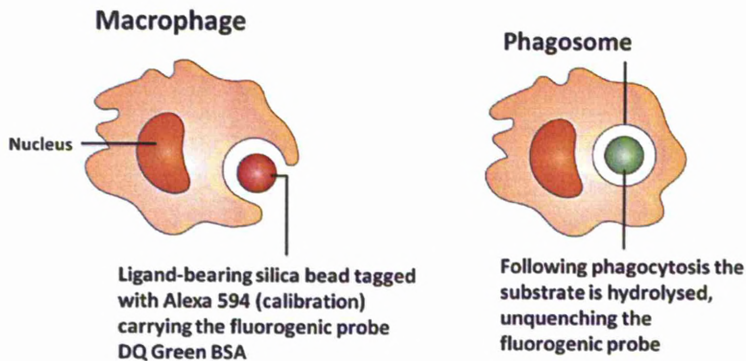


Figure 2.15: Reporter Bead Assay. *The assays use fluorogenic probes that are linked to silica beads and are coupled with an opsonizing molecule (IgG or mannosylated bovine serum albumin (BsA) to facilitate their uptake by macrophages). Results are expressed as a ratio of substrate fluorescence to calibration fluorescence. In the example illustrated experiments with beads coupled with the protease substrate DQ Green BsA, which consists of albumin combined with a self-quenching fluorochrome. The ensuing change in fluorescence generated through the release of fluorescent peptides can be measured by either flow cytometry or a spectrophotometer.*

Bulk proteinase activity was assessed using IgG beads that were coated with the generic substrate DQ Bodipy bovine serum albumin (BSA), which is BSA combined with a self-quenching Bodipy fluor, and a calibration fluor. Activity was measured by the increasing fluorescence

of the DQ Bodipy relative to the calibration fluor. When the DQ Bodipy flurochrome is excited at 490nm the emission wavelength is 520nm; when the Alexa 594 calibration fluorchrome is excited at 594nm the emission wavelength is at 620nm.

Superoxide burst activity was assessed using IgG beads coated with dichlorohydroxyfluorescein (DCDHF), which increases fluorescence upon oxidation, and Alexa 633 for calibration purposes. When dichlorohydroxyfluorescein is excited at 490nm the emission wavelength is at 520nm and when Alexa 633 calibration fluorchrome is excited at 635nm the emission wavelength is at 670nm.

The variable that was used in the analysis of this data was referred to as the **Activity Index.** This was calculated by the mean reporter fluor (FL1) / mean calibration fluor (FL4). The analysis was carried using FloJo ® Software (Tree Star, Inc. Oregan USA).

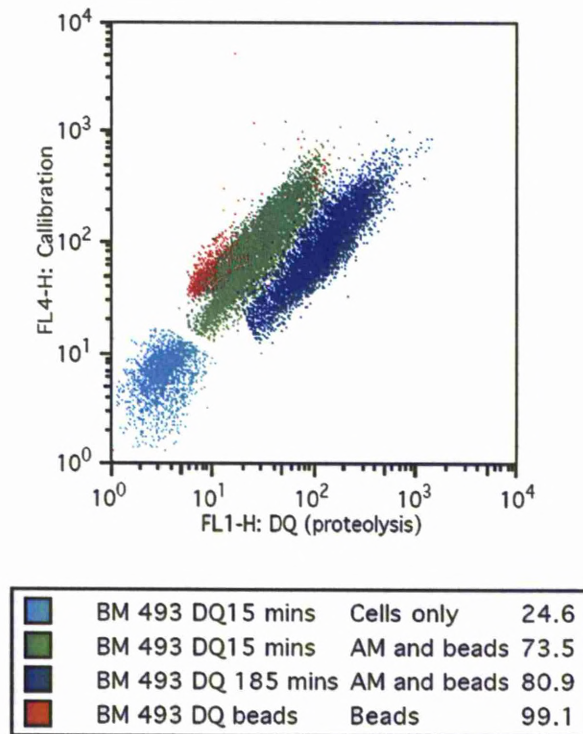


Figure 2.16: Flow Cytometry Plot from Reporter Bead Assay. *This assay is described in more detail in Chapter 7. Each individual dot (or event) essentially represents an Alveolar Macrophage. This graph represents one participants BAL. The four colours represent the beads alone (with no cells present (in red), the cells alone with no beads (turquoise) and cells with beads added at 15 (green) and 180 minutes (blue). The key shows the shift in the fluorescence signal (and therefore proteolysis) that occurs as the bead is metabolised in the macrophage phagosome.*

CHAPTER 3: INDOOR AIR POLLUTION LEVELS IN MALAWIAN HOMES

3.1 Aims

The aim of this work was to:

I. Describe and quantify the concentrations of indoor air pollution (respirable dust, total inhalable dust and carbon monoxide) from burning biomass fuel in Malawian homes

II. To assess transition metal and endotoxin content of biomass smoke from Malawi.

III. Compare four different types of air sampling device in order to assess their suitability for this environment and to help develop appropriate methodology for future exposure assessment work that may be useful in the evaluation of intervention programmes.

3.2 Introduction

In Malawi, it has been estimated that 95% of people use biomass as their main source of domestic energy.[1] These data were obtained using regional and national fuel use surveys.

The World Health Organization (WHO) has produced guidance on acceptable levels of fine particulate matter with an aerodynamic diameter of up to 2.5μm ($PM_{2.5}$) in inspired air, predominantly based on studies of environmental pollution and outdoor air. As outlined in Chapter 1.1.2 BMF smoke contains a large number of pollutants. The focus of this study is primarily PM because it is an air quality gold standard and is most commonly associated with health effects; PM is also the most practical to measure. In settings where there is incomplete combustion of biomass fuels carbon monoxide (CO) levels have been demonstrated to correlate well with airborne PM concentrations.[314;315]

CO is also practical to measure in domestic settings and was recorded during this study.

The recent WHO guidelines suggest that air quality should be maintained at not higher than a maximum 24h average concentration of $25\mu g/m^3$ $PM_{2.5}$ in order to avoid the detrimental health effects outlined in Chapter One.[316] Published studies of biomass burning in homes in Asia have measured average $PM_{2.5}$ concentrations over $2000\mu g/m^3$ and a small number of studies have reported similar levels in different parts of Africa. [11;12;317;318]

There has been no previous household description of biomass fuel use and exposure levels in Malawi, therefore the aim of this study was to quantify and describe the levels of exposure in Malawian homes.

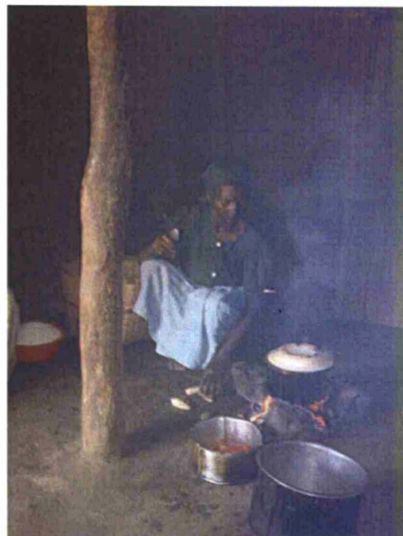


Figure 3.1: Woman cooking indoors in rural Chikwawa. *In the dry season it is more common to cook out doors or on the veranda (khondi), however in the wet season or the evenings it is often done indoors. The situation in the urban environment is similar although indoor cooking (and heating) is more frequent because of cooler average temperatures.*

3.3 Materials and Methods

3.3.1 Study population and sampling strategy

Urban and rural homes were sampled in Southern Malawi. Rural Chikwawa is approximately 30 km from and 1200 m lower altitude than urban Blantyre. The study was carried out during April 2008 (dry season).

Urban homes were randomly selected from a list of 360 Blantyre residents who had previously volunteered for studies at the Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW). Selected homes were then visited by a fieldworker, and the residents invited to participate.

Rural homes: Entomology studies based in Chikwawa had established contacts with the local health surveillance officers in the area. Contact was made with village elders through health surveillance officers and the project was explained to the community. Village homes were very homogeneous, similar in design and construction; the first home was chosen as closest in a random direction from the village elder's house and a snowball sampling strategy was then used to select the other homes.

3.3.2 Ethics

See Chapter 2.2

3.3.3 Questionnaire

Study participants completed an interviewer-administered questionnaire (See Appendix 5 for Case Report Form). This was used to obtain information about exposure to potential risk factors, smoking history, occupation and exposure to biomass fuels used in the home for cooking, heating and lighting, details of the house construction, demographic data and an assessment of socioeconomic status (described in Chapter 2.7)

3.3.4 Indoor Air Pollution Measurements

Air samplers (shown in Appendix 4) were located in the main room of the dwelling and were placed at a height of approximately 1 m from ground level and between 0.5 and 1.0 m from any cooking stove. If more than one device was placed in the same home, they were placed within 10 cm of each other; this did not lead to interference of the devices with each other. The main room of the building was defined as the main living area; a room was defined as a separate area divided by a wall that did not necessarily either go to the ceiling or have a door in the entrance to it. Cooking areas were only sampled if they were in the main living areas. Devices were placed in homes between 07:00hr and 15:00hr in order to capture at least two cooking periods. A second visit was made where possible to extend sampling time by changing batteries if needed.

It was not possible to place one of each type of device in all homes. This was because there were different numbers of each devices (5 Apex pumps, 7 HOBO CO monitors, 3 SidePaks and 6 UCB monitors). Therefore different combinations of each device were placed in the homes selected to have air sampling performed.

Table 3.1*: *Summary table of four air sampling devices used and their advantages and disadvantages

Name of device	*Pollutant measured*	*Strengths and weaknesses of device*
UCB (Photometric)	Respirable dust	Long battery life – can monitor for 48hr plus Quiet Limited accuracy at low exposure levels but can detect high peak exposures. Can plot time against exposure Can measures peaks and time above specific levels Cost ~$500 USD
HOBO	Carbon monoxide	CO is only a proxy for PM Long battery life – can monitor for 48hr plus Quiet Can plot time against exposure Can measures peaks and time above specific levels Cost ~$500 USD
SidePak (Photometric)	Respirable dust	Short battery life Noisy Very accurate PM exposures – essentially gold standard for occupational settings in industry. Can plot time against exposure Can measures peaks and time above specific levels Cost ~$3000 USD
Apex pump and cyclone / IOM filter head	Respirable dust (cyclone) Total inhalable (IOM)	Moderatly cheap but requires samples to be analysed away from the field. Gold standard air quality measure (for 24 hours) Short battery life Enables smoke sample to be collected for analysis Noisy Only produces one result – PM mass/time sampled Cost ~$600 USD + consumerbles

Gravimetric Methods

Airborne particulate mass was measured using standard gravimetric methods.[319] An apex pump (Casella CEL, Bedford, UK) was attached to either an Institute of Occupational Medicine (IOM) sampling head for total inhalable dust (PM<100μm) at a flow rate of 2.0 l/min or a Higgins-Dewell cyclone for respirable dust at a flow rate of 2.2 l/min. Flow rates were calibrated using a using a TSI flow meter. Both sampling heads contained a pre-weighed 25 mm glass fibre filter with a 0.7μm pore size.

After sampling, each filter was re-packed and sent back to the UK for re-weighing. Field blanks were used to correct the data for changes in filter weight associated with manipulation. The concentration of PM was calculated by dividing the change in mass of the filter by the volume of air sampled.

Photometric Methods (SidePak and UCB)

The TSI SidePak AM510 Personal Aerosol Monitor (TSI Incorporated, Shoreview, Minnesota, USA) is a laser photometer (particle size range 0.1–10μm) which measures aerosol concentrations in the range of 1–20000$\mu g/m^3$. The devices were zero calibrated each day during the study, using the zeroing facility on the device and the flow rate was set to 1.7 l min−1 using a TSI flow meter. Using a Dorr-Oliver cyclone, the device can be used to measure the respirable size fraction of collected PM. In this study, the SidePak was set to log the recorded concentration every minute. Raw data were corrected by applying a calibration factor of 0.295; this was derived from gravimetric data from similar combustion-derived air pollution sources. [320;321]

The gravimetric and SidePak data reported were for the respirable size fraction and so are not directly comparable with $PM_{2.5}$. However, particle size distribution of the combustion-derived biomass fuel smoke

is likely to be almost entirely less than 2.5μm in size so it was therefore assumed that respirable dust measurements closely approximated to $PM_{2.5}$ concentrations.

University of California, Berkeley (UCB) monitors were developed to measure IAP in the developing world and obtained for this study after personal communication with Professor Kirk Smith (2007) at UCB. The UCB monitor measures PM concentrations of a size fraction similar to respirable dust (0.1–10μm) using photoelectric methods; it records changes in concentrations each minute.[322] The UCB device was zeroed by placing it in a dust free environment (i.e. a sealed plastic bag) for 30mins prior to placing the device in the sampling location. Data were downloaded from each device using UCB monitor manager 2.2 software. The UCB is a passive device and therefore uses less battery power allowing it to run for several days, whereas the pumped SidePak instrument runs for approximately 10–15h.

Carbon Monoxide (CO) monitors

HOBO monitors (Tempcon Instrumentation, Arundel, West Sussex, UK) were used to measure CO concentrations every minute. Data were downloaded from each device using Boxcar 4.3 software.

Co-location of Air Samplers

In a number of settings, the photometric device (Sidepak) was located side by side with a UCB device. Devices were placed within 10cm of each other during the monitoring duration in order for comparison to be made. Samplers were located in the main room of the dwelling as described previously.

Personal versus Static Monitoring

In order to obtain an estimate of differences between static and personal monitoring, individuals who had had a static device in their home were

also invited to wear two devices for 24 hours on a separate day. Individuals wore 2 devices; one active sampler (SidePak or Apex pump) and a passive sampler (UCB monitor or HOBO)

Chemical Analysis for Transition Metal Content

Filters used in homes were analysed for their metal content using a modification of Occupational Safety and Health administration (OSHA) ID121 http://www.osha.gov/dts/sltc/methods/inorganic/id121/id121.html analysed by inductively coupled plasma/atomic emission spectrometry at the IOM, Edinburgh using standard methods and calibration standards prepared from UKAS-accredited reference material. The samples were digested in concentrated nitric acid on a hotplate at 15°C. Samples were then filtered and made up to 25 ml with distilled H_20. The samples were then run in duplicate and a mean of these results was reported.

Endotoxin Content Analysis

Endotoxin concentrations of samples were measured using a modification of the kinetic chromogenic Limulus amebocyte lysate (LAL) assay (Lonza, Inc., Walkersville, MD). [323] This work was carried out by Prof Peter Thorne's group at the University of Iowa, USA. Briefly, air sampling filters were extracted in sterile, pyrogen-free (pf) water containing 0.05% Tween20 for 1 hr at 22°C, with continuous shaking. Filter extracts were centrifuged 20 min at 600g. Twofold serial dilutions of endotoxin standards (Escherichia coli O111:B4) and fivefold serial dilutions of sample extracts were prepared using sterile, pf water in heat-treated borosilicate glass tubes. A thirteen-point standard curve was generated ranging from 0.025 to 100 EU/ml (R^2>0.995) with absorbance measured at 405 nm (SpectraMax 340, Molecular Devices, Inc., Sunnyvale, CA). Endotoxin determinations were based upon the maximum slope of the absorbance versus time plot for each well.

The arithmetic mean (14.4 EU/sample) of six filter field blanks was subtracted from each of the other Malawi filter results. The analytical limit of detection (LOD) was derived from using a value of three times the standard deviation (9.24 EU/filter) of the field blank measurements (Malawi filter analytical LOD 27.7 EU/filter). Where corrected filter values were less than the LOD (n=12) the filter was assigned a value of one-half of the LOD (13.9 EU/filter).

3.3.5 Statistical analysis

The clinical database (MS Excel, Microsoft Corp) was verified and then analysed using SPSS 15. Proportions were compared using the χ^2 test and air sampling results between rural and urban homes were compared using the non-parametric Mann Whitney U test. Multiple stepwise linear regression analysis was performed on potential determinants of exposure.

3.4 Results

3.4.1 Study Participants

The characteristics of the urban and rural populations are summarised in Table 3.1. Rural homes cooked exclusively with wood and urban homes used primarily charcoal. In the rural area participants were more likely to cook outside in the dry season while most urban participants cooked indoors. Cooking outside in many cases actually meant cooking on the veranda (khondi); when this was the case much of the smoke was observed entering the main house. During the sampling period 2 separate cooking locations were often used.

Rural homes in general consisted of one main room divided by partitions that did not go to the ceiling. Most homes, especially in the rural area, did not have a specific kitchen area. The median number of cooking periods captured was 2 (range 0-4); residents in general burnt biomass

fuel between 2 to 3 times per 24 hour period and there was no difference observed between rural and urban homes in this regard.

Rural homes were more likely to use simple tin lamps fuelled by kerosene (sometimes diesel), or a flaming torch (86%) compared to urban homes (61%) ($p<0.01$). The rural population had a statistically significantly ($p<0.001$) lower economic status when assessed using either a simple score of possessions each household owned or the standard of housing (windows and roofing).

Table 3.2*: *Details of homes compared by rural and urban location. *Data are from questionnaire and visiting homes and is based on the individual who answered the questionnaire.*

Characteristic of Household		Urban N=31 (%)	Rural N=31 (%)	P value
	Gender (Female - %)	21 (67.7)	26 (83.9)	< 0.0001
	Age (SD)	36.3 (11.1)	43.4 (16.7)	< 0.0001
Main type of cooking material:	*Wood*	6 (21)	31 (100)	< 0.0001
	Charcoal	21 (66)	-	
	Wood and Charcoal	2 (6)	-	
	Electricity	3 (9)	-	
Main type of lighting:	*Simple Kerosene lamp, candle and /or flaming torch*	19 (61.3)	27 (86.5)	< 0.0001
	Hurricane lamp alone	3 (9.7)	3 (9.7)	NS
	Electricity	9 (29.0)	1 (3.2)	< 0.01
Main cooking location in Dry season	*Inside*	16 (51.7)	2 (6.4)	< 0.01
	Separate building	2 (6.5)	9 (29.0)	
	Outside	12 (38.7)	18 (58.1)	
	In and outside	1(3.2)	1(3.2)	
	Separate building & Outside	0	1(3.2)	
Main cooking location in wet season	*Inside*	23 (74.2)	7 (22.6)	< 0.01
	Separate building	2 (6.5)	12 (38.7)	
	In and outside	6 (19.3)	12 (38.7)	
	Do they heat the home?	16 (51.5)	15 (48)	NS
	Mean no. of rooms (SD)	4.29 (1.4)	4.27 (2.4)	NS
	Mean no. of residents (SD)	5.03 (1.8)	4.35 (1.7)	< 0.001
	Mean room/residents (SD)	0.93 (0.3)	0.99 (0.5)	NS
	Mean sum of household assets (SD)	2.29 (1.5)	1.48 (1.1)	< 0.0001
	Number of homes keeping animals	16 (52)	27 (87)	< 0.0001
Roof material	*Grass*	1 (3.2)	21 (67.7)	< 0.0001
	Corrugated material	29 (93.5)	10 (32.3)	
	Tiled	1(3.2)	0	
Type of window	*Space Only*	7 (22.6)	26 (83.9)	< 0.0001
	Glass	24 (77.4)	5 (16.1)	

***Table 3.3*: Number and amount of hours that Air sampling devices were used, and Indoor Air Pollution Measurements.** ** = Probability values refer to mean cooking times (not total hours devices were used) and ▲ = All air pollution indices are presented in Table 3.3. NB: The units of PM used in this table are mg/m³ not µg/m³; 1mg = 1000µg.*

		Urban N=31	Rural N=31	P value
Total number of air sampling devices used (hours used)	*Gravimetric (Apex)* Total inhalable dust	5 (102)	6 (115)	NS*
	Respirable dust	15 (317)	14 (290)	
	Carbon Monoxide	30 (671)	28 (569)	<0.001*
	SidePak	13 (204)	13 (167)	NS*
	UCB	24 (533)	23 (466)	0.001*
Mean Time Weighted Average (TWA) values for each device (SD) ▲	*Gravimetric (mg/m³)* Total inhalable dust	0.185 (0.197)	0.268 (0.214)	0.285
	Respirable dust	0.204 (0.69)	0.811 (0.541)	0.04
	Carbon Monoxide (ppm)	63.50 (69.86)	16.31 (22.77)	<0.001
	SidePak (mg/m³)	0.07 (0.08)	0.18 (0.27)	0.343
	UCB (mg/m³)	0.15 (0.36)	0.25 (0.40)	0.191

3.4.2 Indoor air pollution measurements

A total of 62 homes (31 urban; 31 rural) were sampled (Table 3.2). High levels of particulate material were measured in both urban and rural samples. A detailed summary of all the air sampling devices is shown in Table 3.3.

Gravimetric air sampling results (Table 3.3, 3.4 and Figure 3.2)

Forty homes were sampled (20 rural / 20 urban) ranging from 8.73h to 25.7h in duration, mean 20.6h (SD 3.07h). 29 homes were sampled using respirable dust sampling heads (range 30-856$\mu g/m^3$; mean 226$\mu g/m^3$ (SD 206$\mu g/m^3$); 11 homes were sampled using total inhalable dust sampling heads (range 130-1860$\mu g/m^3$; mean 535 $\mu g/m^3$ (SD 499 $\mu g/m^3$). The total inhalable dust levels were significantly higher in the rural homes compared to those in the urban environment ($p<0.01$). The time weighted average (TWA) levels for respirable dust were comparable between rural and urban locations (Figure 3.2 and 3.3). Multiple linear regression analysis (see Appendix 6 page 275) showed that higher total inhalable dust levels were associated with cooking with wood ($p = 0.036$, Beta 0.635) and that higher respirable dust levels were associated with more polluting lighting sources ($p = 0.046$, Beta = 0.403).

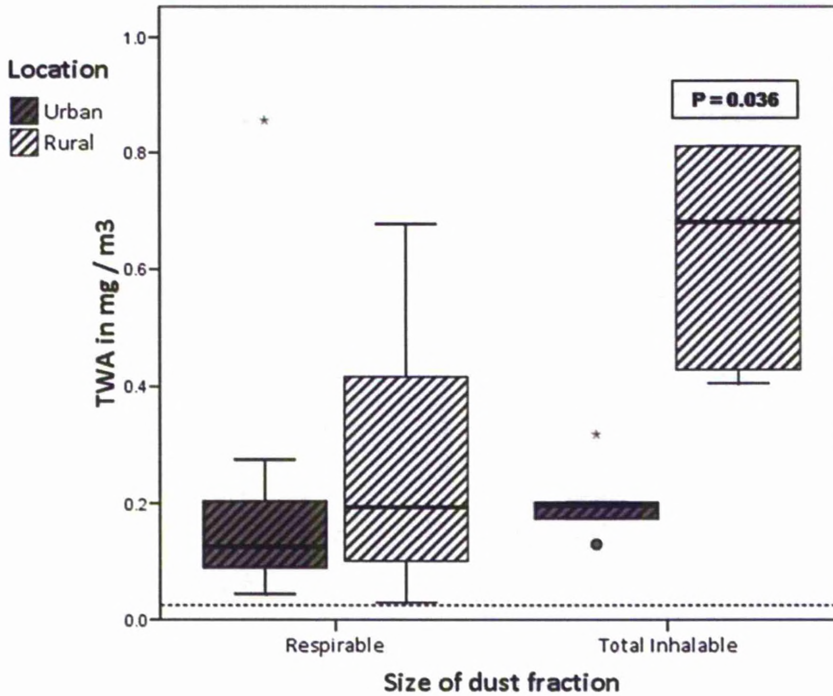


Figure 3.2: Total Weighted Average (TWA) concentrations of respirable and total inhalable particulate matter. *There was a significant difference between a rural and urban home in terms of total inhalable dust (p= 0.036) but not for respirable dust. The dotted line represents the WHO outdoor air quality level for* $PM_{2.5}$ *(25µg/m*^{3}*). It has been added to highlight that all the homes sampled were above this level, and 80% were over four times greater. An outlier home, with TWA of total inhalable dust of 1.86mg/m*^{3}*, has been removed for clarity. If this home was excluded from the analysis the statistical difference between the urban and rural population increases to p=0.008.*

Photometric Methods (SidePak and UCB) (Tables 3.3 and 3.4 and Figure 3.3)

Sidepak Aerosol Monitor: Twenty six homes were sampled (13 urban / 13 rural). The duration of sampling ranged from 3.4 to 24.6h, mean 14.3h (SD 4.98h). The mean time-weighted average respirable dust concentration across all homes was 120μg/m^3 (range 7-891μg/m^3, SD 199μg/m^3). There was no significant difference between the levels measured in the rural and urban environments (p=0.34). The median peak values for the rural and the urban environments was 950μg/m^3. The maximum value that was recordable using a SidePak was 5900μg/m^3; a typical home is represented in Figure 3.1 and Figure 4.3

UCB measurement: Forty seven homes (24 urban / 23 rural) were sampled. The duration of sampling was 17.4-25.1h, mean 21.3h (SD 2.10h). The mean TWA for all homes was 204μg/m^3, (range 1-1900μg/m^3, median 80μg/m^3 and SD 381μg/m^3). The TWA differences between the rural and urban environment were not significant. The duration when PM concentrations in homes exceeded 250μg/m^3 was higher in rural homes than in urban homes; 52% of rural houses spent more than an hour per day above the 250μg/m^3 concentration compared to only 17% of urban homes (p=0.112). The median peak values for the rural and the urban environments was 1400μg/m^3.

Carbon monoxide (CO) monitors

Fifty eight homes (30 urban / 28 rural) were sampled .The mean duration of sampling was 21.4h (SD 2.20h); range (17.4-26.8h). The mean carbon monoxide TWA for all homes was 4.08ppm (range 0.2-24.6; median 1.80ppm; SD 5.36).There was a significantly higher level of CO in urban homes compared to rural homes (6.14ppm vs. 1.87ppm; $p<0.001$). Urban homes also had significantly higher average peak values and time above 5ppm. Multiple linear regression analysis (with factors associated with

increased pollution levels included i.e. fuel type, where cooking was done, whether heating was used, lighting type) associated CO levels with the use of charcoal ($p<0.001$, Beta 0.539) – Appendix 6 page 279.

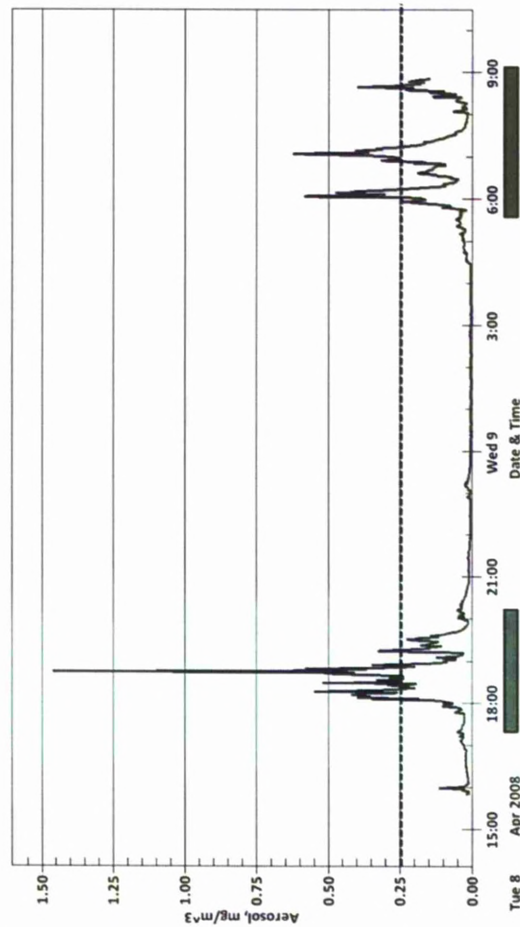


Figure 3.3: A plot of particulate matter against time, obtained using the photometric (SidePak) device. *Cooking periods are highlighted with the grey boxes on the x axis. In this particular home cooking took place on the veranda and the device was placed inside the main room of the home (approximately 1.5 metres from the fire). Peaks associated with cooking are seen and for over 1.5h of the day $PM_{2.5}$ levels are above $250\mu g/m^3$; a level deemed hazardous by the US EPA.*

Table 3.4: Summary data from all 4 air sampling devices compared by home location

	Urban homes (SD)		Rural homes (SD)		P value
Gravimetric (Apex) Data:					
Respirable dust: N	15		14		-
Mean number of cooking periods captured	2.21	(0.80)	1.93	(0.73)	0.324
Mean sampling time (hours)	21.1	(4.3)	20.7	(1.8)	0.217
Mean TWA (mg/m^3)	0.185	(0.197)	0.268	(0.214)	0.285
Total Inhalable dust: N	5		6		
Mean number of cooking periods captured	2	(0)	2.20	1.30	0.91
Mean sampling time (hours)	20.4	(3.2)	19.1	(1.1)	0.584
Mean TWA (mg/m^3)	0.204	(0.69)	0.811	(0.541)	0.04
Sidepak Data:					
N	13		13		-
Mean sampling time (hours)	15.70	(5.96)	12.86	(3.72)	0.161
Mean number of cooking periods captured	1.62	(0.87)	1.46	(0.52)	0.87
Mean TWA (mg/m^3)	0.07	(0.08)	0.18	(0.27)	0.343
Mean peak values (mg/m^3)	1.79	(2.20)	2.37	(2.65)	0.898
Mean time spent over $0.065 mg/m^3$ (hours)	2.20	(2.94)	1.85	(1.65)	0.980
Mean time spent over $0.25 mg/m^3$ (hours)	1.03	(1.76)	0.98	(1.36)	0.979
UCB Data:					
N	24		23		-
Mean sampling time (hours)	22.21	(2.06)	20.24	(1.66)	0.001
Mean number of cooking periods captured	2.27	(0.72)	1.96	(0.56)	0.101
Mean TWA (mg/m^3)	0.15	(0.36)	0.25	(0.40)	0.191
Mean peak values (mg/m^3)	4.04	(9.45)	7.94	(14.00)	0.156
Mean time spent over $0.065 mg/m^3$ (hours)	9.45	(10.53)	7.61	(8.51)	0.826
Mean time spent over $0.25 mg/m^3$ (hours)	1.55	(4.68)	2.61	(5.16)	0.112
Carbon Monoxide Data:					
N	30		28		-
Mean sampling time (hours)	22.35	(2.20)	20.32	(1.69)	<0.001
Mean number of cooking periods captured	2.23	(0.72)	2	(0.49)	0.069
Mean CO TWA (ppm)	6.14	(6.48)	1.87	(2.42)	<0.001
Mean CO peak values (ppm)	63.50	(69.86)	16.31	(22.77)	<0.001
Time spent above 5 ppm (hours)	4.14	(4.08)	1.63	(2.70)	<0.001

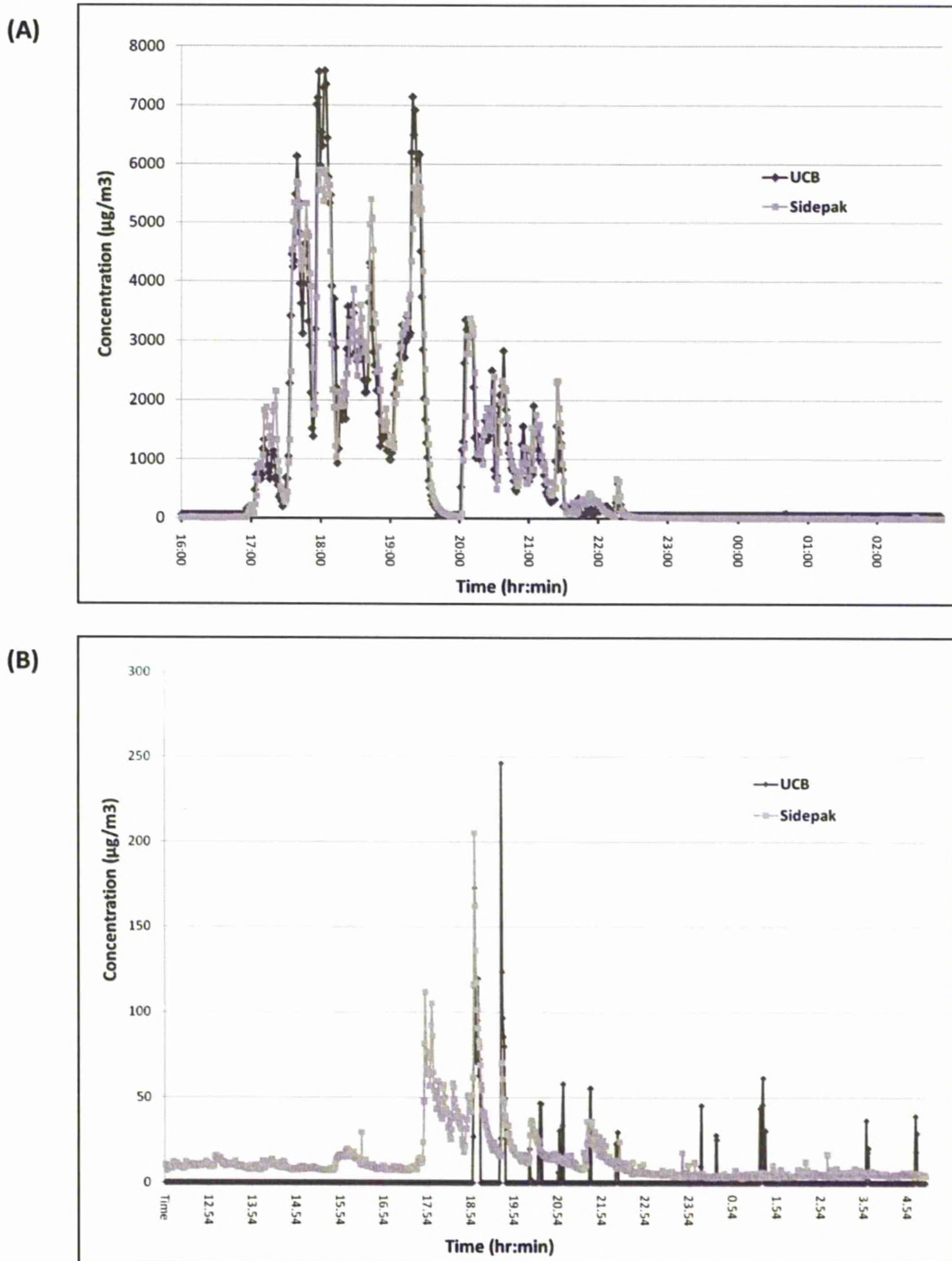
Co-location of devices (Figure 3.4)

25 homes had both a Sidepak and a UCB located together. TWA levels for both devices were not strongly correlated ($R^2 = 0.331$)

In homes where smoke levels were over 250µg/m^3 the UCB and the SidePak were strongly correlated ($R^2 = 0.772$). However, when a sub-group analysis was done on those homes where the SidePak recorded $PM_{2.5}$ levels over 250µg/m^3 for over 15min (N=12) and homes where the SidePak recorded $PM_{2.5}$ levels over 250µg/m^3 for less than 15min (N=13), a strong correlation was found between the UCB and SidePak devices in homes that were exposed to high levels of $PM_{2.5}$ ($R^2 = 0.95$), but not in homes where the levels of $PM_{2.5}$ were low where there was no correlation ($R^2 = 0.03$). Figure 3.4 A and B shows that at high levels there is a very good correlation but at levels less than 250µg/m^3 the correlation is worse.

Figure 3.4: UCB and Sidepak data side by side.

Data from house 43 (A) and house 7 (B)



Personal versus Static monitoring

17 individuals agreed to have personal monitors attached to their clothing. These data were collected on a separate day to the static monitoring in the home and was done in order to be able to approximate static monitoring with personal exposure levels. The average TWA for respirable dust gravimetric results was 2.46 greater than the average TWA for the same homes that had respirable particulate matter measured (n=6).

For the photometric device (SidePak) it was 1.2 times greater (n=3). For the UCB and the HOBO both personal average TWAs were less i.e. the levels measured using a static device were greater than when the devices were placed on a person. (HOBO was 1.26 times less (n=8); UCB was 1.87 times less (n=4)). The same pattern was seen with all 4 devices with regard to peak values and time above a 65 $\mu g/m^3$ or $250 \mu g/m^3$.

Transition Metal Content

20 filters (10 rural / 10 urban) were analysed for aluminium, arsenic, cobalt, chromium, copper, mercury, nickel, selenium and vanadium. Levels of arsenic, cobalt, mercury, nickel, selenium and vanadium collected on the glass fibre filters were <0.1µg (the limits of detection of the assay), aluminium, chromium and copper were detected in small quantities. The mean metal weight on the filter for all homes was Al = 13.40µg; Cr = 0.21µg; Cu = 0.48µg. The percentage mass of the filters were Al = 6.25; Cr = 0.037; Cu = 0.11. There was no significant difference in transition metal level content when compared by fuel type (wood and charcoal) in this study.

Endotoxin Content Analysis

Tables 3.5, 3.6 and 3.7 provide summary statistics of the measured total inhalable and respirable dust concentrations measured in the homes that also had endotoxin measured. The data are presented by primary fuel type of the home, and by measurement duration. The short duration samples (table 3.5) are an order of magnitude higher than the 24h samples (table 3.6) reflecting the much higher smoke concentrations during cooking events than at other times in the household. Arithmetic mean (AM) total inhalable dust concentrations in homes cooking with wood and maize were 4.73μg/m^3 and 5.11μg/m^3

Table 3.7 provides summary statistics of airborne endotoxin concentrations from samples collected using both respirable and total inhalable sampling heads and from both 24h and short-term samples. There is a clear gradient indicated here with charcoal producing the lowest household airborne endotoxin concentrations (AM: 33.6 EU/m3), with wood burning homes three times higher at 78.8 EU/m3. There are only two samples from Maize burning homes and as the range spans more than 100 fold no conclusions can be drawn.

Figures 3.5A and B are box plots of endotoxin concentrations per mass of particulate on the filter divided by fuel type and airborne endotoxin concentrations. They show an increase in endotoxin from charcoal, wood through to Maize. Some of the difference in airborne endotoxin concentrations between charcoal and wood burning may be due to the greater mass of particulate measured during the 4 short cooking period samples taken in wood burning homes. Correcting for this, figure 3.5B shows that the median endotoxin concentration per mass of particulate is broadly similar for wood and charcoal in Malawi.

Table 3.5: Summary statistics of PM concentrations (mg/m^3) by fuel type, sampling fraction and for short cooking interval samples.

Fuel	Size	N	Min	Max	AM	SD	Median
Wood	Resp	4	1.40	9.65	4.73	3.97	3.94
Maize	Resp	2	0.65	9.56	5.11	6.30	5.11

Table 3.6: Summary statistics of PM concentrations (mg/m^3) by fuel type and sampling fraction for 24h samples.

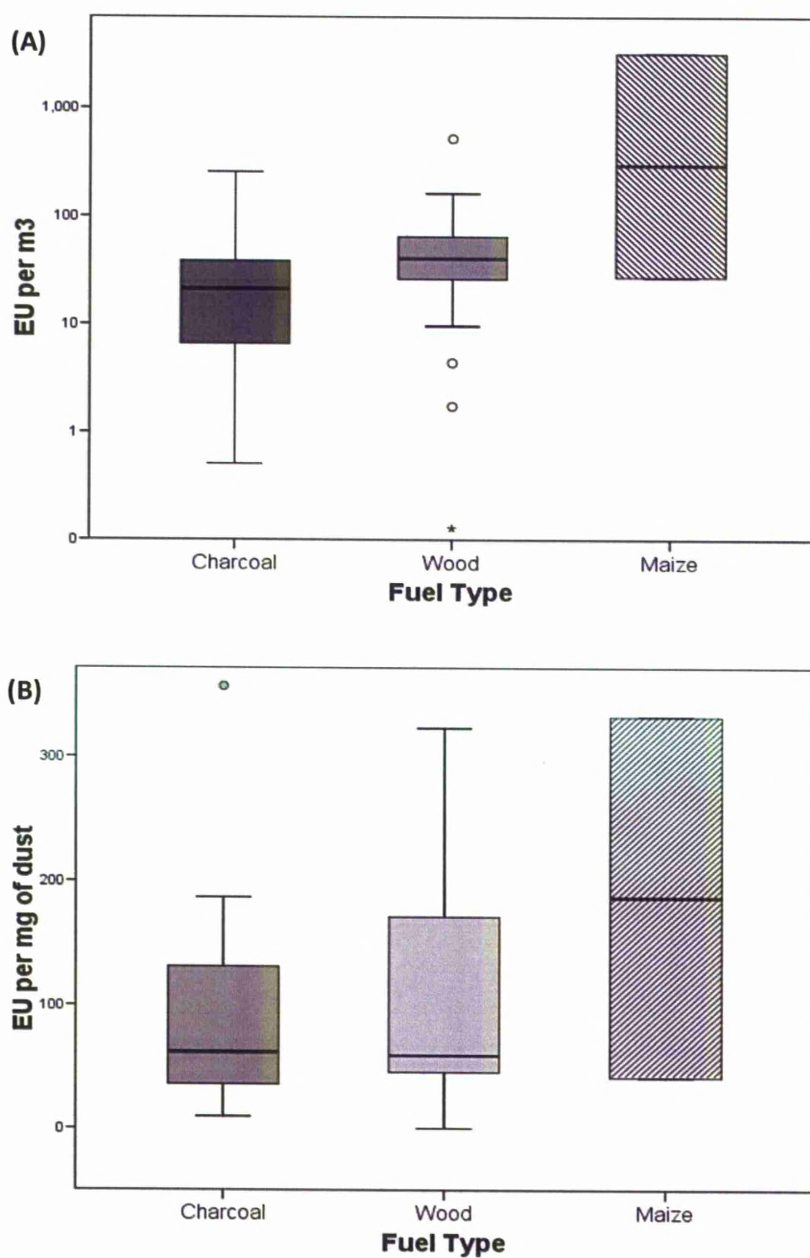
(TIP = Total inhalable particulates)

Fuel	Size	N	Min	Max	AM	SD	Median
Wood	TIP	4	0.43	0.81	0.65	0.18	0.68
Wood	Resp	9	0.03	0.70	0.32	0.25	0.24
Charcoal	TIP	2	0.20	0.32	0.26	0.09	0.26
Charcoal	Resp	17	0.04	0.72	0.25	0.17	0.23

Table 3.7: Summary statistics of Endotoxin concentrations (EU/m^3) by fuel type.

Fuel	N	Min	Max	AM	SD	Median
Charcoal	19	0.51	256	33.6	56.4	21.2
Wood	17	0.13	520	78.8	123	40.6
Maize	2	27	3170	1600	2220	1600

Figure 3.5: Box plot of airborne endotoxin concentrations (A) and of endotoxin per mass of particulate on the filter (B), subdivided by fuel type. *NB: the Y axis in A is a logarithmic scale and in B is a linear scale.*



3.5 Discussion and Conclusions

This is the first assessment of indoor air quality in Malawi. Concentrations of respirable dust in this sample were high and exceeded WHO recommended levels for indoor air of 25μg/m^3 in all homes sampled. In 80% of homes, the PM levels measured were four times greater than the WHO level for indoor air quality.

3.5.1 Air Pollution levels in Rural and Urban Areas

Particulate Matter

Respirable dust levels were similar in both rural and urban homes. This is different to results from other developing world countries where rural levels were significantly higher than those measured in urban households.[317] Total inhalable dust levels were higher in the rural environment. This may be explained by wood use, as implied by the multiple linear regression analysis, but also by the greater prevalence of domestic animals kept for food, or the rural environment being significantly dustier and homes being of more simple construction (figure 4.1 and 4.3).

Whilst $PM_{2.5}$ levels were not significantly different between the rural (wood burning) and urban (charcoal) homes in our study (Figure 3.2), data from Mozambique also suggest that homes that burn charcoal produce less PM pollution.[324] Data from neighbouring countries to Malawi are limited however similar results in single studies have been seen in Zimbabwe, Tanzania, Kenya and Mozambique. [318;324-326]

Carbon Monoxide levels

Homes in the urban environment experienced significantly higher levels of carbon monoxide (CO). This is almost certainly due to burning charcoal and perhaps due to urban homes being less well ventilated due to better construction compared to homes in the rural location. The CO

concentrations measured in this study were high and similar levels have been seen in other studies, where associations with health effects have been seen, although in these studies CO is potentially acting as a surrogate for other pollutants.[15;16;327] The median levels of CO recorded in this study is higher in the urban homes than homes using the traditional fire in the Guatemala RESPIRE study. [328]

In the UK there are up to 20 deaths each year from acute CO poisoning and although these statistics are not available for Malawi, the levels reported in this work suggest that acute CO poisoning is potentially an important cause of death in urban Blantyre, especially during the colder months when charcoal stoves are often used to heat rooms. [329]

Peak Levels

The peaks of PM exposure reached more than 30000μg/m^3 using the UCB-PM monitor and carbon monoxide levels were in excess of 300ppm. The environmental standard used to quantify air pollution is the time-weighted average (TWA). TWA has been used in this study in order to allow comparison with published data but it may underestimate the health impact of high peak levels to which individuals were exposed. The duration during which individuals are exposed to fine particulate matter, greater than 250μg/m^3, may be a better predictor of long-term lung damage. The length of time that indoor air concentrations of fine particulate matter exceeded the US Environmental Protection Agency ($PM_{2.5}$) 'hazardous' 24 hr mean level of 250μg/m^3 in rural homes was typically 1-2h per day. Whether duration above a certain threshold (e.g. 250μg/m^3) is more strongly associated with health effect than TWA levels needs to be tested by epidemiological work and in vitro toxicological studies.

This study has not addressed the subject of seasonal variation in exposure. There are three distinct seasons in Malawi (wet, dry and cold)

that alter cooking and heating practices; we sampled during the dry season. The questionnaire data showed that there is an increase in indoor cooking during the wet season and so there is likely to be a subsequent increase in exposure levels, which was not accounted for in this work.

3.5.2 Transition Metal and Endotoxin content of Smoke

Transition Metal Content

Transition metal content of biomass fuel smoke derived from charcoal and wood in this region of Malawi contains much lower levels of redox active fine particulates than levels reported in India and Mexico. The transition metal content of PM can cause inflammation and damage DNA, [330;331] and a biological hierarchy seems to exist in terms of tissue damage with low-valence transition metals being key to PM bioreactivity.[19] In India, where individual's burn dung cake, PM contains much higher levels of transition metals, including arsenic which has been shown to have a biological effect.[20;285] Levels reported from urban airborne PM in Mexico contained transition metal concentrations of 0.03ng/m^3 to 1000ng/m^3 and it is possible that the sensitivity of the assay used in this study requires larger quantities of PM to be collected and examined before definitive statements can be made about the constituents, and relative toxicology of biomass fuel smoke from Malawi.

Endotoxin Content

Endotoxin concentrations reported in this study are high and much higher than those found in a recent study measuring airborne endotoxin in 10 homes in northern California where mean concentrations were generally less than 1 EU/m^3. [21;332] They were also considerably higher than those measured from a large study of the homes of 332 children in Canada. [333] The mean airborne endotoxin concentration in the Canadian study was 0.49 EU/m^3, almost one hundred times less than the 24 hour

average levels measured in this study for charcoal burning. Health data from the Canadian study suggest that even at these low levels there is a statistically significant relationship between airborne endotoxin and respiratory illness in the first two years of life.

Data from this study in Malawi were compared to data from wood burning homes in Nepal and they were broadly similar.[21] In Nepal, however cow dung is burnt in many homes and the levels of endotoxin recorded are approximately five times greater (498 EU/m^3) suggesting clearly increased levels of endotoxin with movement down the energy ladder of unprocessed solid fuels. The only previous study of endotoxin concentrations in biomass burning homes carried out in Ladakh in India. [334] Comparison is difficult due to the small sample size of the Indian study but that study found average endotoxin levels in two homes of 24 and 190 EU/m^3 which are broadly in line with these data. The Indian homes were small portable tent-like structures with little in the way of ventilation or extraction of smoke generated from burning dung and crop residues.

These data from Malawi suggests much larger epidemiological studies are needed to examine the risks of living in biomass burning households and the effects of endotoxin exposure. Outside of the context of a randomised control trial, controlling for socio-economic confounders of both BMF smoke exposure and probable causes of high endotoxin levels is likely to be problematic. Interventions to tackle endotoxin exposure should also be evaluated in field studies.

3.5.3 Static versus Personal Monitoring

The data reported here were largely obtained using static monitors within participant's homes but 17 individuals allowed personal monitors to be used. Data from occupational settings comparing personal and static sampling indicate that personal sampling reveals higher exposure

concentrations than those estimated with static monitors as workers often interact closely with sources of pollution [335;336]. That was this study's findings; the gold standard measure of indoor air pollution (gravimetric sampling; using the apex pump and cyclone sampling head) was 2.46 times greater than static sampling in the same homes. However, the passive / diffusion devices (HOBO and UCB) actually had lower TWA values for the personal monitoring than for static. This may have occurred for several reasons; the exposures were measured on different days and therefore there may be significant between day variation, the diffusion and passive samplers may be affected by air movement when worn leading to falsely lower values and the HOBO and UCB were not designed to be worn on the person.

A wide variability was seen in terms of exposure results. This is likely to be due to the location of the air sampling device in relation to where cooking took place and it is probable that even in homes that had very low recorded levels the individual responsible for the cooking will have been exposed to very high peaks of exposure, that may not have been recorded due to the position of the air sampling device.

Women who are involved in cooking and fire-tending activities that involve close contact with the stove or fire therefore may have higher peak exposures than those reported in this chapter. This effect may be hard to detect in practice as when individuals wore monitors they were clearly identifiable in the community which in turn led to some criticism by neighbours and may have led them to modifying their usual cooking behaviours on the day of sampling.

The air sampling procedure itself could have lead to other changes in behaviour with both static and personal monitoring: There may have been more visitors during the sampling period to inspect the devices or enquire about the visit of strangers to the house, which in turn may have

lead to more cooking or alternatively cooking may have been done in a different location. It was said by one participant that she had cooked outdoors on the day of sampling as she did not want the equipment to be damaged. Also, as part of the 'community sensitisation' to the project, village meetings were held in order to explain why the work was being done and this itself may have lead to changes in behaviour as a result of increased education around the effects of biomass smoke on health.

3.5.4 Practical Issues of Air Sampling

No previous work has been done to measure air quality in Malawi and some methodological lessons were learned.

Air Sampling Devices

Location of the devices is key to recording an accurate exposure assessment, but it was difficult to get right. The cooking location often varied between meals and was dependent on the weather and time of day. Ideally exposure assessments should measure personal exposure rather than area or static household concentrations, but this is fraught with the problems outlined above, as well as the devices being relatively heavy and uncomfortable to wear.

Battery life of the SidePak meant that it was only possible to measure 15h and whilst in the urban environment we were able to return later in the day to change the batteries; this was not possible in the rural environment. The Apex pump only lasted for 22h which also limited the possible sampling time. If possible data should be gathered over extended time periods to take in to account the effects of day to day and seasonal variability and the probable disturbance to normal behaviour that occurs by a visit from a research team.

The ideal device would be one that is small enough to be able to be worn within the breathing zone, cheap and be able to measure and log real

time concentrations of biomass smoke while having low power requirements.

Security

Often several thousand pounds worth of equipment was left in participant's homes. In order to prevent both tampering and also theft the machines were placed in an adapted, locked cash box; this had the added advantage of reducing the noise level with the active samplers.

Training

Field workers and laboratory technicians were trained in how to load filter papers into the gravimetric devices, how to download the information to a computer and how to place the devices in participant's homes. The recording of information was critical to appropriate interpretation of results and this was often not given enough attention by fieldworkers. This was partly due to the fieldworker not being aware of how the information was used and more attempts to include all researchers in the interpretation of results would be good.

3.5.5 Health Effects

Evidence of varying degrees of certainty exists that biomass fuel smoke is associated with acute lower respiratory tract infections and low birth weight in children,[57;130] lung cancer,[80] COPD,[56] interstitial lung disease,[60] cardiovascular disease[52], cataracts in adults[86] and tuberculosis.[73] However, despite IAP from biomass fuel potentially affecting a larger number of individuals globally there are less data on biomass smoke specific exposures, both in terms of levels of exposure and health effects than on outdoor air pollution in industrialised countries.[337] Data from Kenya have explored the exposure-response relationship of biomass fuel and lower respiratory tract infections; [12] this has suggested that the benefits of reduced exposure to PM_{10} may be larger for average exposure less than about 1000-2000μg/m^3, but to date

there are no further data on this from Africa. Recent epidemiological data from outdoor air in the USA have shown that a decrease of $10\mu g/m^3$ in the concentration of fine particulate matter was associated with an estimated increase in mean (±SE) life expectancy of 0.61 ± 0.20 year. [29] The effect of air pollution indoors has been investigated more in the context of second hand tobacco smoke, nevertheless reductions in smoke levels have led to a reduction in both mortality and morbidity. [338;339]

If appropriate interventions were instituted in countries such as Malawi, with subsequent substantial reductions in exposures to PM and other constituents of biomass, the health benefits are likely to be large. Norman *et al* has estimated the burden of disease attributable to IAP from household use of solid fuel in South Africa (RSA). [340] They used published relative risks for ALRI in children under five, COPD and lung cancer, data from the RSA census to ascertain household fuel use and applied the WHO comparative risk assessment methodology in order to estimate the disease burden attributable to IAP. Norman *et al* corrected exposures for a ventilation factor, based on where cooking occurred in the home, the climate etc. It is likely that Malawi data would be corrected by a similar ventilation factor. It was estimated that only 20% of households were exposed to indoor smoke from biomass fuels as a result of this exposure approximately 2500 excess deaths occurred in RSA and loss of healthy life years were approximately 61000 disability adjusted life years (DALY). The population in RSA (49.2 million) is approximately 3.3 times greater than the population in Malawi (15.3 million) but in Malawi 95% of all citizens are exposed to biomass fuels and so the number of excess deaths per year is likely to be at least similar if not higher.

Four potential intervention categories have been identified that can reduce exposures and reduce the health impact: Cleaner burning fuels,

improved cook stoves, housing design and behavioural change. These data from Malawi indicate that intervention strategies that are appropriate for Malawi should be considered as a matter of urgency.

3.5.6 Conclusion

Levels of indoor air pollution in rural and urban Malawian homes were sufficiently high to be a cause for concern. These data will hopefully prove to be important as a baseline exposure assessment for Malawi and will inform health impact assessments. It also provides justification to try and reduce exposure levels and will help inform intervention studies aimed at reducing exposure levels in Malawi and other similar countries.

CHAPTER 4: SPIROMETRY IN RURAL AND URBAN POPULATIONS IN SOUTHERN MALAWI: A CROSS-SECTIONAL STUDY

4.1 Aim

The aims of this study were to:

I. Carry out a cross-sectional study of spirometry in two populations, one rural and one urban, in southern Malawi
II. Study whether spirometric values differ between individuals who a) burn different types of biomass fuel and b) are exposed to biomass fuel smoke for different amounts of time
III. Study the relationship between spirometric values and indoor air pollution data

The hypotheses were a) more than 95% of the population are exposed to BMF and b) individuals exposed to the most polluting types of biomass fuel and those with the highest levels of exposure will have the most impaired lung function.

4.2 Introduction

Indoor air pollution from biomass fuel is increasingly recognised as a major health concern in the developing world.[341] It ranks 10th amongst preventable risk factors contributing to the global burden of disease and is responsible for an estimated 36% of mortality from respiratory disease (mainly lower respiratory infections and chronic obstructive pulmonary disease (COPD)). [91;342]

The WHO Global Burden of Disease and Risk Factors project estimated COPD to be the tenth leading cause of disability-adjusted life years in all countries, regardless of their economic status. In developed countries COPD is predominantly associated with tobacco consumption however cigarette smoking rates remain low in developing countries compared to

Europe and America, especially amongst women in Southern Africa.[66;343] From meta-analysis data the prevalence of COPD in non-smoking women is estimated to be two to three times higher in rural areas where women are exposed to biomass smoke, compared with urban women who are exposed much less suggesting a causal link.[56] In countries of low and middle income it is estimated that 35% of people with COPD have developed the disorder after exposure to indoor smoke from biomass fuels. Exposure to biomass smoke may thus be the most important global risk factor for the development of COPD. [70;91;344]

Non-smoking, biomass exposed women are thought to develop COPD with clinical characteristics, quality of life and increased mortality similar in degree to that of tobacco smokers.[13;67] There have been few published spirometry data from Africa and none from Malawi. This study provides cross-sectional spirometry data from populations in Malawi for the first time and explores the relationships between these data and the type and extent of exposure to biomass fuel smoke.

4.3 Materials and Methods

4.3.1 Study Area

The study area is described in Chapter 2.1. Individuals from urban (Blantyre) and rural (Chikwawa) areas were sampled. Both study areas have a population of approximately 150,000 people (2008 census). Individuals from 6 villages in Chikwawa and from 15 townships in Blantyre participated in the project.

4.3.2 Study Design and Participants

This was a community-based cross-sectional observational study of adults aged 30 yrs and over. Household members and neighbours of individuals who took part in the air sampling study, described in Chapter 3.3 were invited to take part in this spirometry study. For practical and logistical reasons a convenience snowball sampling strategy was adopted in the two study settings of Blantyre and Chikwawa. Women were recruited preferentially as previous work has suggested that women are more exposed to biomass smoke and so more likely to develop the adverse health effects of IAP. [345]

4.3.3 Ethics

This is described in Chapter 2.3.

4.3.4 Questionnaire

Study participants completed a questionnaire which identified exposure to potential risk factors for COPD, including smoking, occupation and exposure to biomass fuels used in the home for cooking, heating and lighting, demographic data and an assessment of socioeconomic status. (See Appendix 5 for the Case Report Form). Chapter 2.6 describes how the socio-economic living status was calculated.Pack years of cigarette smoking were calculated; A pack year is the number of years of smoking multiplied by the average number of cigarettes smoked per day, divided

by 20 (the number of cigarettes in a standard packet). A validated questionnaire was used to obtain information about respiratory symptoms (cough, sputum, wheezing and shortness of breath) and previous respiratory diagnoses. [346]

4.3.5 Spirometry

Each participant's height and weight were measured in centimeters and kilograms respectively. Spirometry was performed, according to American Thoracic Society guidelines, using a Vitalograph® 2120 Spirometer handset and Spirotrac IV ® software (version 4.34).[311] Each manoeuvre was repeated at least three times in order to meet reproducibility criteria and the highest results from the best traces were reported.

Data from each patient were saved in the Spirotrac IV® database. The primary spirometry measurements used for analysis were the Forced Expiratory Volume in one second (FEV_1) and Forced Vital Capacity (FVC). Airway obstruction was diagnosed if FEV_1/FVC was less than 0.7.

There are no reference values available for Malawian adults and so spirometry results were compared to African-Americans (using the Knudson reference values on the Spirotrac IV Software ®) to give actual FEV_1/predicted FEV_1 ratios. [347]

Quality assurance

A panel of experts comprising two respiratory specialists and a respiratory physiologist reviewed all spirometry traces to confirm adherence to ATS/ERS acceptability criteria. All interviewers and spirometry operators were trained before the survey. Calibration of the flow-measuring device was done at least twice a day, using a 3 Litre calibration syringe.

4.3.6 Sample Size Calculation

The BOLD study reported a prevalence of COPD of 25% in Cape Town.[69] We estimated that 15% of adults in Malawi would have impaired lung function in a biomass exposed and 1% in a non-biomass exposed group. A sample size of 91 in each group would allow a significant difference to be detected with 90% power and 95% confidence between biomass exposed and non-exposed people. Previous studies using spirometry data indicate 10-15% of subjects produce poor quality expiratory maneuvers; therefore it was planned to collect data on at least 110 individuals per group. In the event very few individuals were not exposed to biomass fuel, and the groups were therefore compared according to the predominant fuels used, i.e., wood and charcoal.

4.3.7 Data Analysis

The clinical database (MS Excel, Microsoft Corp, USA) was analysed using SPSS 15 (Chicago, Illinois USA). Households were categorised according to the main fuel used for cooking: charcoal or wood. For categorical data the association with wood / charcoal use was assessed using Pearson $\chi 2$ tests and for continuous data Students unpaired t tests were used. Multiple responses to specific questions e.g. type of lighting material or cooking fuel were dichotomised (wood v charcoal, clean lighting v polluting lighting etc). Socioeconomic status was calculated from standard indices (type of water supply, type of roof material, type of window material, household density and specific possessions e.g. phone, bicycle, radio etc).

The primary outcome variable was FEV_1. In univariate analyses lung function data were analysed using Student t tests to compare means between two groups and linear regression to examine the relationship with continuous variables. Results from the univariate analysis that were potential confounders, and had a p value <0.1, together with other known

risk factors for impaired lung function (previous TB and smoking) were included in the multiple linear regression analysis.

Comparison was made between participants who had obstructed spirometry and non-obstructed spirometry, using the Mann-Whitney U test (for non-normally distributed data) to compare groups.

Correlation with Air Sampling Data

Additional exploratory analyses were performed. Data from individuals who performed spirometry and who had also taken part in the study described in chapter 3 and had exposure to biomass fuel smoke measured, were analysed by correlation and regression analysis (using Pearson's correlation coefficient and linear regression).

4.4 Results

4.4.1 Study Population (Table 4.1)

374 adults completed the questionnaire and performed spirometry. There were significant differences between the rural and urban participants in terms of type of cooking and lighting fuel, location of cooking, whether individuals heated their homes, home construction as well as other markers of socioeconomic status (Figures 4.1 and 4.2).

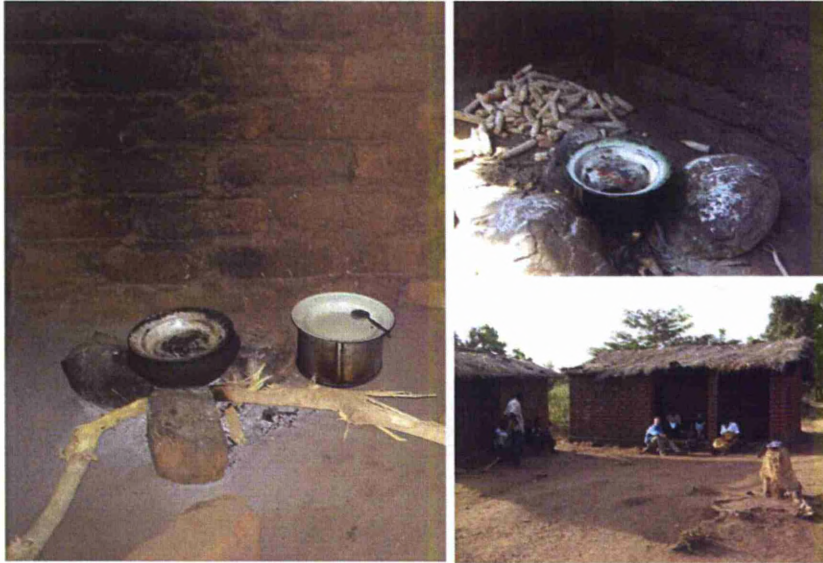
Study participants cooked for between 3 to 6 hours per day in a variety of indoor, veranda and outdoor locations, with different degrees of ventilation. Charcoal stoves were not vented and the wood fires were nearly all 3 stone fireplaces (Figure 4.1). Heating was reported at certain times of the year in the urban communities. Dung was very rarely used in this population but crop residues (in the form of maize husks) were used seasonally as both a starter and as a fuel. The type of wood varied but was all gathered locally.

There were 28 (7.5%) ex-smokers and 52 (13.9%) current smokers. The mean number of pack years was 5.89, (median 1.45 mode 0.15 (SD 13) – i.e. a very skewed distribution curve). The majority of current or ex-smokers had less than 1.5 pack years. Smoking was associated with male gender.

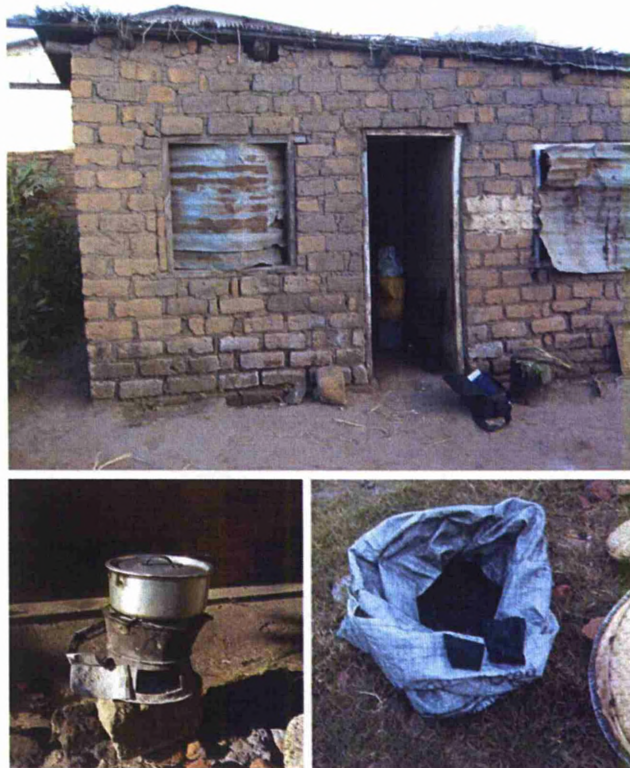
Table 4.1 summarizes the characteristics of the population by main cooking fuel type. There was a strong association with fuel type for home location, the type of lighting, location of cooking and the indices of socioeconomic status. 35% of urban homes and 18% of rural homes used 'low exposure' lighting i.e. electricity or paraffin lamps surrounded by glass. Strong associations were found between economic status, home location and type of cooking or lighting fuel used.

Figure 4.1: (A) Three stone wood burning fireplaces in rural Chikwawa. (B) Charcoal stove in urban Blantyre

(A)



(B)



The responses to the respiratory symptom questionnaire show that people who used wood self-reported more cough and respiratory tract infections than people who cooked with charcoal; they also were significantly older than the charcoal burning respondents (mean age 42.1yrs versus. 35.6yrs, $p<0.001$); they had a lower average body mass index (mean BMI: 21.4 versus. 22.6; $p<0.001$), but average height between the two groups was not significantly different. Individuals who burnt wood were also more likely to smoke tobacco (28.2% versus. 17.5%; $p=0.022$).

Table 4.1: Summary of demographic details of study population by type of cooking fuel. *374 adults took part in the study. There were clear differences in behaviours (e.g. cooking and lighting fuel) and socioeconomic indices (e.g. type of roof material or windows) between individuals who used different fuels. Numbers of respondents and percentages are shown except for continuous variables when the arithmetic mean and standard deviation are presented.*

Table 4.1: Summary of demographic details of study population by type of cooking fuel.

Demographic Details		Charcoal users N (%)	Wood users N (%)	P value
	Spirometry passed Quality Control (% of all people who performed spirometry)	141 (88)	156 (89.7)	NS
Home location	Rural	4 (2.8)	113 (72.4)	<0.001
	Urban	137 (97.2)	43 (27.6)	
	Female : Male	86 (61)	93 (60)	NS
	Age	35.6	42.1	<0.001
	Height	161.1	160.4	NS
	Body Mass Index	22.6	21.4	0.004
	Number of ex or current smokers	24 (17.5)	44 (28.2)	0.022
	Number of females who smoke	3 (3.5)	14 (15.1)	0.008
Main cooking location in the dry season	Inside	26 (18)	15 (10)	0.048
	Outside	114 (80)	141 (90)	
	Mix	1 (0.7)	0 (0)	
Main cooking location in the wet season	Inside	119 (84)	81 (52)	<0.001
	outside	22 (16)	75 (48)	
	Mix	0 (0)	0 (0)	
Main source of lighting	Simple paraffin lamp, candle, flaming torch	90 (63.9)	103 (67.3)	<0.001
	Hurricane lamp	15 (10.6)	31 (20.3)	
	Electricity	27 (19.1)	8 (5.2)	
	Mixture	9 (6.3)	11 (7.3)	
	No. of individuals who used fuel for heating	87 (61)	49 (31)	<0.001
	Rooms/Resident	0.88	0.86	NS
	Sum of assets	1.69	1.45	0.095
Type of window material	No windows	1 (0.7)	9 (6)	<0.001
	Space only	24 (17)	91 (58)	
	Glass	115 (82)	56 (36)	
	No. of Individuals that kept animals	34 (25)	108 (69)	<0.001
	No. of Individuals who slept in home with animals	26 (19)	77 (49)	<0.001
Roof Material	Grass	3 (2)	78 (50)	<0.001
	Corrugated material	138 (98)	78 (50)	

4.4.2 Spirometry (Tables 4.2, 4.3 and 4.4)

Acceptable traces were obtained from 330 (89%): 118 rural (72 female) and 213 urban (134 female) residents. FEV_1 and FVC were normally distributed. Results of the univariate and multivariate analysis are shown in Table 4.3. Lower spirometric values were associated with cooking with wood (mean FEV_1 2.43L versus. 2.78L; $p<0.001$; a 12.5% difference) as well as living in a rural location. Thirty three individuals were excluded from analysis because they used either exclusively electricity (n=7), wood and charcoal (n=21) or electricity and wood or charcoal (n=5). Very few individuals were not exposed to biomass fuel, and so groups were compared according to predominant fuels used. Therefore a comparison of wood and charcoal was made.

In the univariate analysis, smoking appeared to have a protective effect on lung function as did cooking indoors in the wet season. In the sample studied smokers had a higher mean FEV_1 but the majority of smokers were male which is not accounted for in the univariate analysis. However adjustment for confounding factors reversed the association, demonstrating that smoking still has a detrimental effect but that wood smoke and poverty are probably more important as a cause of impaired lung function than smoking in this population. The type of lighting used and heating the home were not significantly associated with lung function impairment.

Table 4.2: Summary of Respiratory Questionnaire and Spirometry results *(divided by fuel type). Participants who used charcoal had a higher FEV_1 and less cough compared to the participants who used primarily wood*

		Charcoal users N (%)	Wood users N (%)	P value
Responses to respiratory symptom questionnaire	Cough	4 (3)	25 (16)	<0.001
	Phlegm	9 (6)	14 (9)	NS
	Breathlessness	15 (11)	21 (13)	NS
	Asthma	10 (7)	12 (8)	NS
	Respiratory infection	13 (9)	23 (15)	0.145
	Previous TB	13 (9)	6 (4)	0.061
Number of individuals with airway obstruction (FEV_1/FVC <70%)		20 (14.2)	25 (16.0)	NS
Number of individuals with evidence of restriction (FEV_1/FVC > 0.70 and an FVC or FEV1 < 80% predicted)		2 (1.4%)	6 (3.8)	NS
Spirometry values (SD)	FEV_1 (ml)	2780 (680)	2430 (670)	<0.001
	Percent of predicted FEV1	106%	99%	0.008
	FVC (ml)	3490 (870)	3190 (830)	0.002
	Percentage predicted FVC	111%	109%	0.119
	FEV_1/FVC ratio	79.80 (7.48)	76.54 (9.11)	0.001

In the multivariate analysis (Table 4.3) FEV_1 was significantly associated with age, height, gender and previous TB, despite this not being a significant variable in the univariate analysis. Non-significant associations were seen with cooking with wood and lower socioeconomic status. When stepwise linear regression was used cooking with wood (p=0.03), economic status (p=0.037) and previous TB (0.019) were each selected as significantly predictive of FEV_1. This analysis is presented in Appendix 6 page 281 – 284.

45 (13.6%) of all participants had COPD (defined by an FEV_1/FVC <70%); 23 were women and a total of 23 of these COPD subjects were lifelong non-smokers. The overall frequency of 'restriction' in this sample was 2.7%. Using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification, 14 (31%) of these individuals are GOLD stage 2 or above. [348] Using the respiratory questionnaire we have shown that a further 44 individuals who have no evidence of airway obstruction but on the basis of symptoms, are potentially at risk (previously referred to as GOLD Stage 0 in 2001). Table 4.4 summarizes the characteristics of the patients with airway obstruction.

Table 4.3: Summary statistics of univariate and multivariate associations with FEV_1. *A list of categorical variables was tested for association with differences in FEV_1. The results of the univariate analysis are shown in column 3. These results were included in a multivariate model (Column 5) and non-contributory factors were excluded. NB: For Social Living Index (SLI) the figures represent the mean difference between each of the 3 groups (Low Medium and High) i.e. Medium SLI had a mean FEV_1 200ml greater than the Low SLI group and 200ml lower than the High SLI group. Therefore Low SLI had a mean FEV_1 400ml lower than High SLI*

Variable		Mean FEV_1 in (ml)	Unadjusted mean difference in FEV_1 (ml) (95% CI)	P value	Adjusted mean difference in FEV_1 (ml) (95% CI)	P value
Age (per year)		-	-20 (-10, -30)	<0.001	-10 (-20,-10)	<0.001
Gender	*Female* *Male*	2340 3020	690 (560, 820)	<0.001	340 (191, 500)	<0.001
Height (per cm increase)		-	50 (40, 60)	<0.001	40 (30, 50)	<0.001
Smoking (tobacco)status	*Never smoked* *Current or Ex smoker*	2540 2780	230 (50, 410)	0.013	-120 (-270, 30)	0.109
Cooking material	*Charcoal* *Wood*	2780 2430	-340 (-490, -190)	<0.001	-120 (-290, 50)	0.174
Home location	*Rural* *Urban*	2420 2680	260 (100, 410)	0.001	0.00 (-180, 180)	0.988
Heat home with fuel indoors	*No* *Yes*	2560 2620	60 (-210, 90)	0.42	-	-
Type of lighting	*Electric or hurricane lamp* *Smokey light source*	2580 2600	20 (-140, 180)	0.84	-	-
Cooking location in wet season	*Outside* *Inside*	2460 2640	180 (20, 340)	0.027	-60 (-190, 80)	0.422
Sleeping in the same room as animals	*No* *Yes*	2700 2350	-350 (-510, -200)	<0.001	-80 (-210, 5)	0.237
Economic status	*Low* *Medium* *High*	2370 2500 2730	200 (105, 295)	<0.001	80 (-10, 170)	0.093
"Previous TB"	*No* *Yes*	2600 2520	-80 (-230, 39)	0.626	-290 (-530, -5)	0.016

Table 4.4: Summary of the characteristics of individuals who had obstructive lung function. *Figures in brackets are percentages unless otherwise stated. For continuous data mean values are presented.*

Characteristics		Charcoal users (%)	Wood users (%)
	No. obstructed (% of all individuals)	20 (14)	25 (16)
	Female	10 (50)	13 (52)
	Age (SD)	42 (11)	48 (14)
	Smoker	7 (35)	15 (60)
Home location	*Rural*	20 (14)	25 (16)
	Urban	10 (50)	13 (52)
Economic status	*Low*	1 (5)	14 (56)
	Medium	7 (35)	7 (28)
	High	12 (60)	4 (16)
	"Previous TB"	3 (15)	2 (8)
Spirometry results (SD)			
	FEV_1 (ml)	2550 (530)	2190 (600)
	FVC (ml)	3850 (900)	3500 (840)
	FEV1/FVC	0.65 (0.6)	0.62 (0.8)
GOLD Stage	1	17 (12)	14 (9.0)
	2	3 (2)	10 (6.4)
	>3	0 (0)	1 (0.6)

4.4.3 Correlation with Air Sampling Data

In a sub-group of individuals who performed adequate spirometry air sampling was carried out in their homes: 51 had a measure of their carbon monoxide exposure (using a HOBO); 36 individuals had gravimetric respirable PM exposure results; 24 individuals had SidePak and 34 individuals had UCB photometric assessments of respirable PM.

No statistically significant correlation was seen between any of the exposure measures that are listed in Table 3.3 and FEV_1. Figure 4.2 is one illustrative scatter plot of these data exploration.

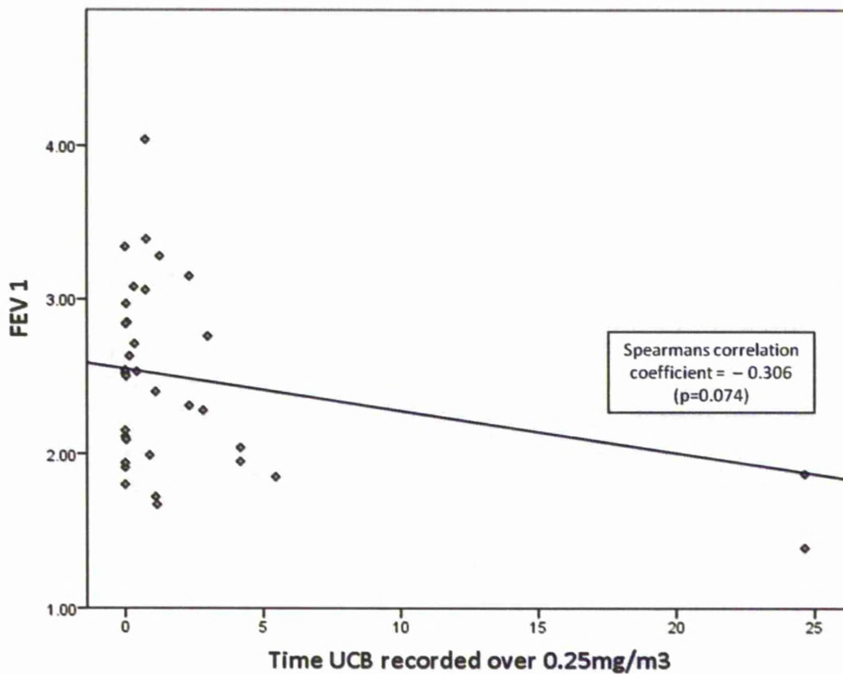


Figure 4.2: Scatterplot of Time UCB monitor recored > 0.25mg/m^3 against FEV$_1$. *This is an illustrative plot of all the exploratory analyses that took place. No correlation was observed between FEV$_1$ and any air pollution indice.*

Table 4.5 shows the mean value (and standard deviation) for each air sampling variable compared by whether the lung function was obstructed or not. Obstructed lung function was not associated with any of the measures of air pollution described in chapter 3.

Table 4.5: Mean values of 24 hour air sampling parameters according to presence of airway obstruction.

Figures in brackets are standard deviations. Mann-Whitney U test was used for for all variables.

	Spirometry	N	Mean (SD)	p value
Respirable Gravimetric PM TWA (mg/m^3)	Normal	18	0.24 (228)	0.822
	Obstructed	5	0.30 (233)	
SidePak >TWA (hours)	Normal	17	0.16 (0.24)	0.972
	Obstructed	6	0.23 (0.35)	
SidePak mean time >0.25mg/m^3 (hours)	Normal	17	1.31 (1.80)	0.718
	Obstructed	6	1.13 (1.71)	
UCB TWA (mg/m^3)	Normal	27	0.25 (0.48)	0.680
	Obstructed	8	0.26 (0.25)	
UCB mean time >0.25mg/m^3 (hours)	Normal	27	1.83 (4.41)	0.230
	Obstructed	8	7.00 (10.97)	
CO TWA (ppm)	Normal	37	3.69 (4.76)	0.316
	Obstructed	10	1.24 (0.90)	
CO mean time > 5ppm (hours)	Normal	37	2.84 (3.39)	0.123
	Obstructed	10	0.67 (1.06)	

4.5 Discussion and Conclusions

This is the first assessment of spirometry in Malawi. These data demonstrate that wood smoke and lower socioeconomic status were independently associatated with impaired lung function. The data also show COPD to be common, affecting 13.6% of the study population. Smith et al suggested over 25 years ago that respiratory health was likely to be affected by biomass fuel smoke and more recent data have shown an association of wood smoke with impaired lung function.[59;64;349-352] Orozco-Levi et al have reported that wood or charcoal alone independently increase the risk of COPD with odds ratios of 1.8 and 1.5 respectively.[63] In this study a 12.5% difference in FEV_1 between individuals who cook with wood and charcoal was found.

The severity of COPD found in this study was predominantly mild disease (GOLD stage I and II). The BOLD study in Cape Town reported a prevalence of COPD of 16.7% in women and 22.2% in men (GOLD Stage II).[69] Nevertheless, given the constraints of the sampling strategy used in our study, the prevalence of COPD of 13.6% found warrants further investigation.

4.5.1 Indoor Air Pollution and Poverty

The interacting effects of poverty, malnutrition and IAP make the association between different cooking fuels used and impaired lung function hard to dissect, as reliance on wood for domestic energy is itself a marker of poverty. Wood smoke however is associated with impaired pulmonary function and compared to charcoal it is known that wood smoke contains multiple toxic compounds and individuals exposed to wood smoke develop COPD with the same clinical, radiological and functional characteristics as tobacco smokers and have a similar prognosis.[9;67;353] Aside from the particulate matter, toxic inorganic and organic compound content there is also a high concentration of airborne

endotoxin in biomass smoke, with wood smoke containing more endotoxin than charcoal.[21] The levels of endotoxin reported from homes in Malawi are between 100 and 1000 times higher than homes in North America and in many Malawian households the levels exceed the European Union health-based guidance limit value for the workplace. [333]

The association of good lung function with cooking indoors was reversed after adjustment for confounding factors as it was also associated with charcoal use and affluence. Blantyre is also located at a higher altitude and has cooler average temperatures, resulting in more indoor cooking.

The rural population in this area of Malawi is significantly poorer than the urban population and it is likely that this influences lung function. The rural population were of a similar height but had a significantly lower body mass index indicating that nutrition is not as good in the rural population. Maleta et al have reported that 70% of Malawian children are stunted,[354] and rates of lower respiratory tract infections are higher in a malnourished population, which in turn is a risk factor for impaired lung function. [355]

Early life exposures to IAP are likely to contribute to lung development, potentially both *in utero* as well as during growth and development in children. In particular low birth weight, strongly associated with poverty, leads to impaired lung development and increased rates of airway obstruction. Biomass smoke itself is also associated with low birth weight.[57;108;356;357] Poverty is increasingly recognized as a risk factor for impaired lung function and our study adds weight to this conclusion. [358-360]

4.5.2 Influence of Tuberculosis and HIV

Reported previous treatment for tuberculosis was predictive of impaired lung function in the multivariate analysis; this is consistent with studies

elsewhere looking at the effect of TB on lung function. [70;361-363] Recent epidemiological studies in India and Nepal link biomass smoke exposure to an increased risk of TB, and suggest that kerosene used in simple lamps is an independent risk factor.[74;75;364] In Malawi TB rates are approximately 400 per 100000 of population, largely due to HIV (prevalence rate 15%).[365;366] It is known that HIV itself is a risk factor for the development of COPD,[367] therefore the association with TB in this study may reflect both of these risks. A potent mix of disease processes and exposures is likely to be contributing to an as-yet un-described burden of poor respiratory health in 700 million biomass-exposed individuals in Africa.

4.5.3 Study Limitations

This study had a number of limitations: Although a random sampling strategy would have been ideal this was not possible because denominator data could not be obtained. There were major practical challenges and cultural sensitivities to how a study like this could select participants. The most acceptable and appropriate strategy possible was therefore adopted and this proved highly successful in recruiting participants with refusal to participate being very unusual. Thus although selection bias with the symptomatic or worried well being more likely to participate, the high recruitment rates will have helped to minimize this.

Different multivariate analyses produced discrepant results; when all univariate factors were included in a simple model wood smoke and low socioeconomic status were not significant in the final model (Table 4.3). When a stepwise approach was taken however, wood smoke and poverty were significant with other variables being rejected. This is consistent with other studies suggesting that fuel type and economic status are important variables in determining FEV_1. Given the limitations of the sampling strategy, the data have been analysed using a multiple

regression analysis in order to produce a list of important variables to be considered in planning further randomly sampled studies to assess the main risk factors for impaired lung function.

TB was not a microbiological or a radiological confirmed diagnosis in this study but the national programme is well coordinated and centrally regulated using the WHO DOTS strategy. Lastly it was not possible to measure 'smoke exposure' in all the participant's homes. However it is demonstrated in Chapter 3 that respirable dust concentrations of IAP in this region of Malawi are high. The concentrations of respirable dust in all of the homes sampled exceeded WHO recommended levels.[368] Averaged over 24 hours the concentrations of respirable dust in all of the homes sampled exceeded the WHO recommended level for outdoor fine particulate matter ($PM_{2.5}$) air quality ($25\mu g/m^3$). In 80% of homes the mean particulate matter levels measured were four times greater than this recommendation. There were also significant differences in the levels of respirable dust and carbon monoxide in homes that burn wood and those that burn charcoal. These measured levels suggest that there is high biomass smoke exposure occurring in the same population that performed spirometry in this study and suggests that the observed differences seen in lung function may be related to the differences that were measured in charcoal and wood burning households.

4.5.4 Measures of Exposure to Indoor Air Pollution

There currently is not a household air pollution exposure equivalent of pack years. As presented in Chapter 3, the peak exposure levels in this population are high.[368] An appropriate exposure measure may be the duration that individuals are exposed to $PM_{2.5}$ above a certain threshold e.g. $> 250\ \mu g/m^3$, rather than time weighted average (TWA) levels, as this may be a better long-term predictor of lung damage. This hypothesis

needs to be tested both epidemiologically and in laboratory based *in vitro* toxicological studies.

This study has begun to explore the relationship between lung function and biomass fuel exposure. Chapter 3 assessed levels of exposure in urban and rural Malawi whilst this chapter has assessed lung function in the same population. In order to attempt to define the exposure-response functions of biomass smoke and lung function a future study will have to have a much larger sample size and air sampling measures will have to be carried out in many more people. As in this study, health and socioeconomic factors will also have to be included and it is likely that despite greater numbers being studied the relationship will be difficult to fully describe, given the limitations of 24h or 48h air sampling to describe a lifetime of exposures and insults to the lung, that are represented by measuring FEV_1.

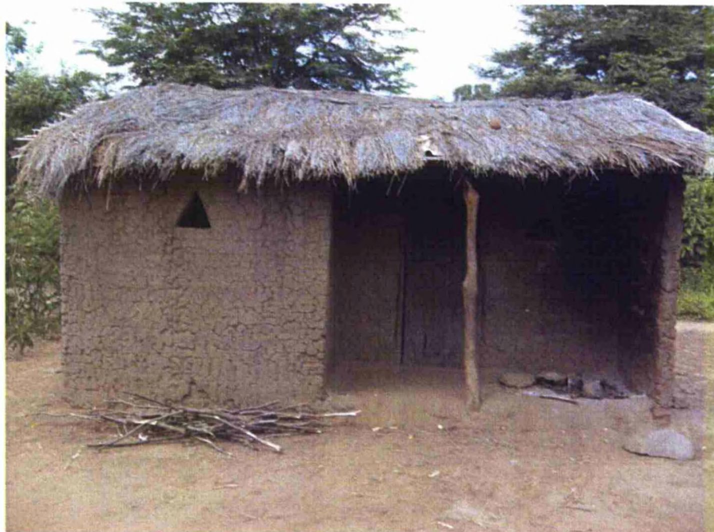


Figure 4.3: Rural home in Chikwawa, southern Malawi

4.5.5 Conclusion

This study has shown that an important burden of impaired lung function exists in both rural and urban Malawian adults. Wood fuel burning may be an important reversible cause of the burden of lung disease, either by a change of fuel or by improved stove technology. Our analysis has emphasized however, that the causes of impaired lung function in poor communities are complex and air quality is only one of several important contributing risk factors.

CHAPTER 5: RELATIONSHIP BETWEEN REPORTED SMOKE EXPOSURES AND PARTICULATE MATTER IN ALVEOLAR MACROPHAGES.

5.1 Aim

The aim of this study was to test the hypothesis that there is an association between reported smoke exposure and the amount of particulate material (PM) observed in alveolar macrophages (AM).

5.2 Introduction

Over ninety-five percent of Malawian adults are exposed to BMF smoke in their homes; [368] cigarette smoking is uncommon. The burning of BMF generates large quantities of particulate matter, with the peak indoor concentration of PM_{10} (particulate matter <10 μm in aerodynamic diameter) often exceeding 2000 $\mu m/m^3$.[13] The health effects associated with this exposure are outlined in section 1.2. The mechanisms underlying these associations are not completely understood but are explored in Chapter 6 and 7. [369]

Alveolar macrophages (AM) are the pre-dominant resident pulmonary phagocyte and are critical regulators of the pulmonary immune milieu. Particulate loading of lung macrophages provides a direct measure of inhaled pollutants derived from biomass smoke. There are no published data on the relationship between reported environmental source of exposure and the particulates in AM obtained from lung samples.

5.3 Materials and Methods

5.3.1 Subject Recruitment, Consent and Questionnaire

Volunteers were recruited to a pneumonia study involving bronchoscopy and a questionnaire assessment of smoke exposure.[251] Participants attending a Queen Elizabeth Central Hospital (QECH) were asked about their fuel use for cooking, heating and lighting (See Appendix 5). All subjects gave informed written consent for their personal information to be recorded, to HIV testing and to undergo a bronchoscopy.

Consenting adults completed a questionnaire. This ascertained type of energy used at home, cigarette smoking and domestic and occupational exposure to smoke.

HIV Testing and Management

HIV testing was performed using two rapid tests (Serocard and HIV-Gold, Trinity Biotech, Bray, Ireland) and CD4 cell counts (Facscount; Becton Dickinson, Johannesburg, South Africa, RSA). Patients who tested positive for HIV were referred to the anti-retroviral clinic at QECH.

5.3.2 Bronchoscopy and BAL

Bronchoscopy and lavage (BAL) was performed according to standard methods described in Chapter 2.3. At the time of bronchoscopy, all individuals were asymptomatic and had a normal full clinical examination. They also had a normal chest X ray. BAL cytospin preparations were stained with Giemsa and viewed with transmitted illumination, described in Chapter 2.10.2.

5.3.3 Particulate Content in Alveolar Macrophages

The methods involved in cytospin preparation, image acquisition and analysis are explained in Chapter 2.10 and 2.11 (Method One).

After assessing the variability of the means in five different individuals using 25, 50, 75 or 100 cells it was found that there was no significant difference in the calculated mean PM load when either 50, 75 or 100 cells were compared. Therefore in this study, images of 50 consecutive macrophages in diagonally distributed high power fields were recorded from each subject. The geometric mean of PM load within the cytoplasm of 50 cells was used in the analysis. Digital colour images of 50 AM per subject with an intact cell wall were obtained using a Leica DMRB microscope at 1000 x magnification under oil immersion (Figure 2.7). 50 cells produced a reliable estimate of the median surface area of PM.

The method used for calculation of PM within each cell is described in detail in Chapter 2.10: Method One. Images of slides were analysed as described by Kulkarni et al. (2005). Briefly, each AM image was processed using Paint Shop Pro software (Paint Shop Pro 8; Jasc Software, Eden Prairie, MN, USA). For the analysis of PM within the cytoplasm the Scion image grabber and software (Scion Image, Frederick, MD, USA) was used (see Figure 2.8).[313] The percentage area of the cytoplasm occupied by PM in 50 cover-slip adherent AM was calculated. The geometric mean of these 50 values, i.e. the PM load for each subject, was used in analysis. The geometric mean was used in the analysis because the distribution of PM loading of alveolar macrophages was not normally distributed.

5.3.4 Data Analysis

The clinical database (MS Access, Microsoft Corp, Redmond, WA, USA) was verified and analysed using Stata version 8.2 (Stata Corp, College Station, TX, USA). A logarithmic transformation was performed for mean percentage of cytoplasmic area occupied by particulate load in ANOVA. In logistic regression analysis, PM load was examined as a

binary variable by dividing the group into high and low particulate groups about the median value for particulate load.

5.4 Results

5.4.1 Subject Recruitment and Questionnaire

Forty men and seventeen women were recruited to the bronchoscopy study (Table 5.1). Men and women did not differ in the prevalence of AIDS, median CD4 count or seroprevalence of HIV; all participants reported using biomass fuel for cooking and heating at home. Women used more charcoal in cooking and fuel for heating, whereas men used simple tin lamps significantly more often.

Despite being asymptomatic at the time of bronchoscopy participants may have still had an AIDS defining illness prior to their bronchoscopy (Severe presumed bacterial infections i.e. pneumonia is WHO Clinical Stage 3).

5.4.2 Bronchoscopy and Particulate Content in Alveolar Macrophages

All 57 BAL samples yielded adequate specimens for examination. There were no complications associated with bronchoscopy. The log transformed particulate load data from all 57 subjects are shown in Figure 5.1, presented according to the lighting and cooking exposures of the subjects from whom the macrophages were obtained. There was a significant difference between the PM load in groups divided according to predominant lighting form used at home (Comparison of the means; ANOVA $p=0.0009$) and type of cooking fuel ($p=0.0078$). Male subjects had higher PM load (non-paired t-test on log transformed particulate score; $p=0.0001$), used more smoky tin lamps and candles ($\chi 2$ test; $p<0.0001$) and used more wood for cooking ($p=0.004$) than females as shown in Table 5.1.

	Male	**Female**	**P value**
Number	40	17	
Age (SD)	34 (10.6)	29 (8.7)	NS
HIV Seropositive	18 (45%)	9 (53%)	NS
CD4 median cells/µl (range)	218 (7-796)	290 (0-997)	NS
AIDS	8 (20%)	3 (18%)	NS
Previous lung disease or TB treatment (%)	13 (33%)	6 (36%)	NS
Cooking	N (%)	N (%)	($\chi 2$) P = 0.03
Wood & Charcoal	25 (63)	5 (30)	
Charcoal	14 (35)	7 (41)	
Other	1 (2)	5 (29)	
Lighting			($\chi 2$) P = 0.02
Paraffin tin lamp	26 (65)	3 (18)	
Hurricane lamp	6 (15)	0 (0)	
Candle	4 (10)	3 (18)	
Electric	4 (10)	10 (59)	
Heating with biomass fuel	21 (55)	6 (36)	NS
Tobacco ever smoked	8 (20)	2 (12)	NS
Symptoms	*N (%)*	*N (%)*	
Cough	4 (10)	2 (12)	NS
Dyspnoea	2 (5)	3 (18)	NS
Wheeze	1 (2.5)	0 (0)	NS
Sputum production	0 (0)	0 (0)	NS
Mean percentage cytoplasm filled by particulate (sd)	8.38 (8.8)	2.97 (2.72)	P <0.01

Table 5.1: Details of 57 subjects attending for bronchoscopy by gender

Forward stepwise logistic regression (inclusion at $P < 0.2$) was used to determine variables significantly associated with high particulate load defined as being higher than the group median value (4.5% cytoplasmic area). The variables significantly associated with high PM load in alveolar macrophages were use of tin lamps (corrected OR = 6.5; P =0.013) and age (corrected OR = 1.1 per year; P = 0.03). Several of the reported exposures are co-variant, as might be expected in this small study. In particular, there was a significant association between cooking with wood and use of tin lamps in poor households (of the 29 subjects reporting use of tin lamps, 24 also cooked with wood; regression R^2 = 0.36). Removal of cigarette smokers from the analysis prior to multivariate analysis showed that cooking with wood was the most significant predictive factor (corrected OR = 5.4; p = 0.025) of PM carbon load.

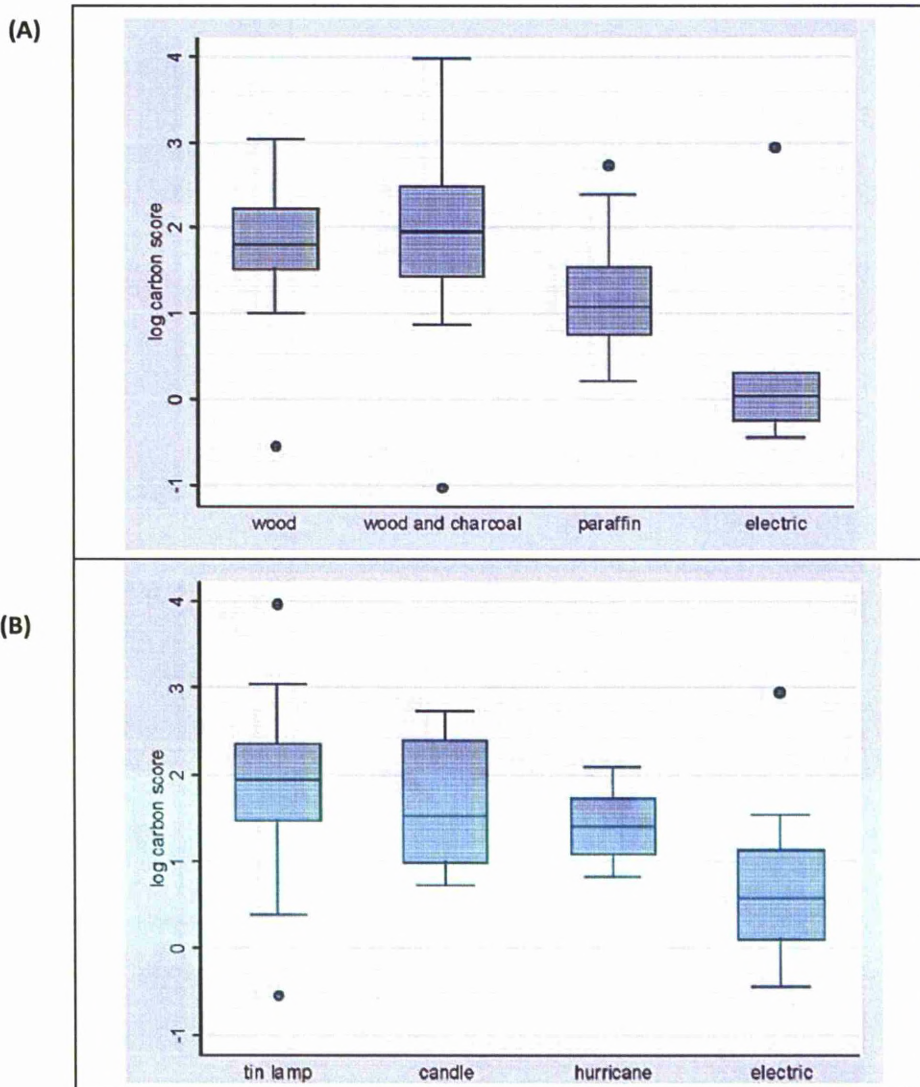


Figure 5.1: PM load associated with use of cooking methods (A) and common lighting methods (B) in Malawi. *Log transformed PM data are plotted for each common form of lighting and cooking. Particulate load is defined as the geometric mean of the percentage area of the cytoplasm occupied by particulate, in 50 cover-slip adherent alveolar macrophages. There was a significant difference in the AM particulate load seen in subjects reporting use of different types of lighting (Anova P < 0.001) and cooking (Anova P = 0.008). Median is represented by bar, box represents the 25th and 75th percentile, whisker represents the lower and upper adjacent values and dots represent outliers.*

5.5 Discussion

There is the first description of PM load from bronchoalveolar lavage derived alveolar macrophages in African adults in the literature. These data demonstrate that PM load is significantly associated with different types of exposure. It also shows that Malawian men, from urban Blantyre, have significantly higher levels of PM in alveolar macrophages compared to women. PM load was most strongly associated with the use of primitive paraffin lamps and is consistent with observations of Malawian domestic life: men being more likely to sit inside with a lamp at night while women cook outside.

These data add to work done in British children. Kulkarni et al demonstrated that PM in alveolar macrophages from induced sputum was inversely related, in a dose-dependent manner, with lung function; an increase of $1.0\mu m^2$ of PM content in AM was associated with a reduction of 17 percent in the percentage of the predicted FEV_1.[100] Kulkarni et al also demonstrated that for every of $1.0\mu g/m^3$ increase in PM_{10} there was an associated $0.10\mu m^2$ increase in the carbon content of airway macrophages.

The mechanism by which PM in alveolar macrophages affects function is varied but phagocytosis, oxidative burst and receptor regulation are all affected. This has been discussed in Chapter 1.6, 1.7 and Chapters 6 and 7.

5.5.1 Strengths and Weaknesses of study

First, a significantly greater number of men compared to women enrolled for bronchoscopy: Some of the reasons for this may include employment differences or greater male autonomy with regard to decision making and therefore increased freedom to volunteer.

Second, participants did not have a direct measure household PM_{10} in their homes, using air sampling equipment. A questionnaire was used as an assessment of their exposure instead, although this is a valid indirect measure of exposure that has been used elsewhere, it is a relatively rough assessment of exposure.[64;370] Domestic air sampling is also problematic as discussed in Chapter 3; it is unlikely that a single 24h measurement in a home will provide a good indication of the lifetime exposure to PM and that more complex exposure reconstruction methods coupled to lifetime cooking, occupational and smoking histories would be required to produce estimate lifetime exposures more accurately.

Thirdly, the nature of the particulate material found in the macrophages has not been described however previous work has shown that material from alveolar macrophages is morphologically identical to aggregates from smoke emissions to which the subjects were exposed.[371] Finally, these images are cross-sections i.e. two dimensions only of a 3D structure. However the distribution of PM within a macrophage, as shown by confocal microscopy, does not imply that this estimate of PM load is going to be significantly altered. [372]

5.5.2 Lifespan of Alveolar Macrophages and Effect of PM on function

The length of time that PM is retained within alveolar macrophages is unknown. In studies on fire-fighters after the World Trade Centre collapse, PM remained in alveolar macrophages for many months after exposure; black material was present in airway macrophages up to 10 months after exposure. In experiments on healthy volunteers insoluble particles were present in airways up to 3 months after instillation.[373;374] In animal models, the amount of particulate-pigmented material in lung macrophages has been shown to reflect the inhaled dose, as well as having a significant effect on macrophage function.[43;76;279;375] However,

Kulkarni *et al* demonstrated that in children who had asthma the amount of PM present was much lower than age and sex matched controls.[100] This suggests that in disease states the macrophage may have a significantly shorter lifespan. This is particularly relevant in individuals with HIV.

5.5.3 Conclusion

The work presented in this chapter associates PM load in alveolar macrophages with reported exposures and is an important finding because it enables AM function to be assessed in the context of exposure dose *in vivo*. This is explored further in Chapter 6. The data presented in this chapter are also important because BMF smoke exposure has been associated with a significant burden of human disease and the mechanisms behind this association are not known.

Published literature has focused on the effects of tobacco smoke and vehicle emissions, but further studies are needed to define the extent and nature of biomass fuel use, the effect of cooking and lighting choices on indoor air pollution, the mechanism by which BMF smoke affect human AM function and the health benefits to be obtained in Africa by effective interventions resulting in clean indoor air.

CHAPTER 6: AN *IN VITRO* MODEL OF PARTICULATE MATTER EXPOSURE IN THE ALVEOLUS

6.1 Study Aims

The aims of this study were to:

I. Reproduce the *in vivo* alveolar macrophage appearance in the laboratory by challenging macrophages with particulate matter (PM) *in vitro*.
   a. To explore a dose-dependent response in the phagocytosis of PM by Human Alveolar Macrophages (HAM) and Monocyte Derived Macrophages (MDM) *in vitro*.
   b. To explore a time-dependent response in the phagocytosis of PM by HAM and MDM *in vitro*.

II. To measure the effect of HAM and MDM exposure to PM *in vitro* on inflammatory cytokine (IL-6 and IL8) production.

6.2 Introduction

Exposure to biomass fuel (BMF) smoke has both acute as well as chronic health effects. The strongest epidemiological evidence of an association between BMF smoke and disease is the development of acute lower respiratory tract infections in children and chronic obstructive pulmonary disease in women. [55;56;342]

The pulmonary effects are likely to be explained by inhalation of smoke into the lung. *In vivo*, human studies have demonstrated that acute exposure to high levels of smoke from a forest fire elicits a pulmonary inflammatory response sufficient to raise circulating IL-8 levels and consequently to alter bone marrow production of neutrophils, raising the peripheral neutrophil count.[280;291;376;377] Chronic exposure to air pollution has been shown to lead to the development of atherosclerotic disease,

with acute peaks of outdoor air pollution associated with out of hospital cardiac deaths. [378-380]

In Chapters 3 and 4 it has been shown that the levels of biomass smoke in Malawi are high and that lung function is influenced by several different factors including the type of fuel that is used in the domestic environment. In Chapter 5 it was demonstrated that deposition of PM in alveolar macrophages (AM) is associated with the type of biomass fuel exposure, it is likely therefore that AM are associated with the pathogenesis of the diseases that are associated with BMF exposure.

Pulmonary defence mechanisms against infection, the effect of pulmonary inflammation, the role that alveolar macrophages, IL-6 and IL-8 play and the response that AM have to PM are discussed in Chapter 1.6 and 1.7.

6.2.1 PM Load in Alveolar Macrophages

Particulate loading of lung macrophages provides a direct measure of inhaled pollutants derived from BMF smoke.[100] Chapter 5 demonstrates that PM load in alveolar macrophages (AM) can be utilised as a biomarker of PM exposure. [381]

In order to improve understanding of the pathogenesis of disease laboratory models are needed. This chapter develops a model of the macrophages after exposure to PM and assesses the cytokine (IL-6 and IL-8) response after such an exposure. We also compare monocyte derived macrophages (MDM) and human alveolar macrophages (HAM) in their response to PM.

6.3 Materials and Methods

6.3.1 Cell Culture of Monocyte Derived Macrophages and Human Alveolar Macrophages

Buffy coat was obtained from the UK National Blood Transfusion Service and Monocyte Derived Macrophages (MDM) were extracted from whole blood using the methods described in Chapter 2.7.1. MDM were cultured in 24 well plates and cells that were 12 days old (+/- 2 days) were used in the assays.

Human Alveolar Macrophages (HAM) were obtained from healthy volunteers who underwent bronchoscopy and bronchoalveolar lavage (BAL) at the Royal Liverpool University Hospital. Bronchoscopy and the isolation of HAM and their culture are outlined in Chapter 2.6 and 2.7.2. Cells were used between day 3 and day 5 post BAL.

6.3.2 Particulate Matter Challenge of Cells in vitro

MDM and HAM were treated with PM at specific doses. Fine Carbon Black (FCB) was used in time and concentration experiments. It was kindly donated by Professor Donaldson's laboratory (University of Edinburgh); it is a well-described material, which can be easily sterilised. The method for addition of PM to MDM in culture is explained in Chapter 2.9.

Fine Carbon Black Dose

Indoor air pollution in Malawi and elsewhere typically occurs in a series of short exposures (approximately 5 hours per day, in two or three cooking periods) over many days; for this reason we compared 5-hour exposures to fine carbon black. Escalating doses of FCB were added to cells in a 24 well plate in duplicate. They were then incubated for 5 hours.

Dose-Dependent Response

A 2000μg/ml FCB solution was sonicated for 30 minutes. Using this stock solution, concentrations of 2.5μg/ml, 10μg/ml and 20μg/ml of FCB were produced by dilution with complete media. The media was aspirated and discarded from all the wells on the plate and 500μl of each dose of FCB were pipetted into duplicate wells. 500μl of complete media was added to duplicate control wells. The plate was incubated for 5 hours at 37°C. The cells were then fixed and stained using the Fields B stain as outlined in Chapter 2.10. Images from each well were acquired and analysed using Image SXM (Chapter 2.11). Data were presented as the mean PM load for duplicate wells for each dose.

After 5 hours, culture medium from FCB exposed cells was collected, spun at 13000 rpm for 8 minutes at 4°C (in order to remove excess PM) and the supernatant collected (frozen at -20°C) for cytokine analysis by ELISA. Cells were washed 2x 1ml RPMI 1640 and then fixed ready for microscopic examination.

Time-Dependent Response

20μg/ml FCB was added to all treatment wells and 500μl of complete media into the control wells. The plates were incubated at 37°C. At each time point (0, 0.5, 1, 2, 4, 6, 12 and 24 hours) the treatment was aspirated from duplicate wells and the wells were washed three times with PBS. The duplicate wells were then fixed with paraformaldehyde (400μl) for 6 minutes. The paraformaldehyde was aspirated and the wells washed twice with PBS. 1ml PBS was added into the duplicate wells and the plate returned to the incubator until the next time point. Once all the time points had been met on the plate and the control wells fixed with paraformaldehyde, the PBS was aspirated and the plates left to dry at room temperature for 15 minutes. 400μl of methanol was added to each well and the plates incubated for 10 minutes at 37°C on an orbital shaker.

The cells were stained using Fields B stain (Chapter 2.10). Images were acquired and analysed using Image SXM (Chapter 2.11). Data were presented as the mean PM load for duplicate wells for each time point.

6.3.3 Cell Staining techniques of MDM and HAM

This method is described in Chapter 2.10.2. Briefly, after the culture medium had been removed MDM and HAM were fixed with paraformaldehyde and air dried for at least 10 minutes. Methanol was added for a further 10 minutes and the cells were stained pink / red colour with Fields B (red) stain. The well was rinsed three times with tap water (3 x 1ml) and then air-dried.

6.3.4 Image Acquisition, Analysis and Calculation of PM load in MDM and HAM

The development of this method is described in detail in Chapter 2.11. Cells were examined using a Leica DMRB microscope and images were acquired with a DFC300 camera and Leica Firecam image acquisition software.

Images were stored and subsequently examined for their PM load using Image SXM software (described in Chapter 2.10 and Appendix 3).

6.3.5 ELISA Cytokine analysis

ELISA methodology is outlined in Chapter 2.12 and Figure 2.13.

Standards and samples were prepared in duplicate. Values were calculated using a standard curve. An adequate standard curve was defined as one that had an $R^2 > 0.95$. Duplicate samples had to have a coefficient of variation of less than 15%.

6.3.6 Analysis Methods

PM loading of macrophages is not normally distributed. Log of PM load is normally distributed therefore the geometric mean was used in this analysis. Each individual's cells were cultured in different wells of the

culture plate, therefore giving several results for each participant, therefore the mean value of the PM loads was used in the analysis and the standard deviation calculated from these values.

For IL-6 and IL-8 results the mean values from 2 wells for each dose was calculated and was used in the analysis. The student t-test was used to compare MDM and HAM cytokine values

6.4 Results

6.4.1 Dose-dependent response

Five different samples of MDM and HAM were used. From each sample cell culture wells were used to obtain a PM load for each individual.

Figure 6.2A shows a dose-dependent response was observed. MDM and HAM became more saturated with FCB particles with increasing dose (Figure 6.2B). An increase in dose of FCB caused an increase in ingestion by MDM and HAM i.e. an increase from 2.5μg/ml to 20μg/ml caused an increase in mean PM load, 12.2 % to 33.3 % in MDM, 7.3% to 38.8% in HAM (Figure 6.2). There was no statistically significant different phagocytosis of PM by MDM or HAM.

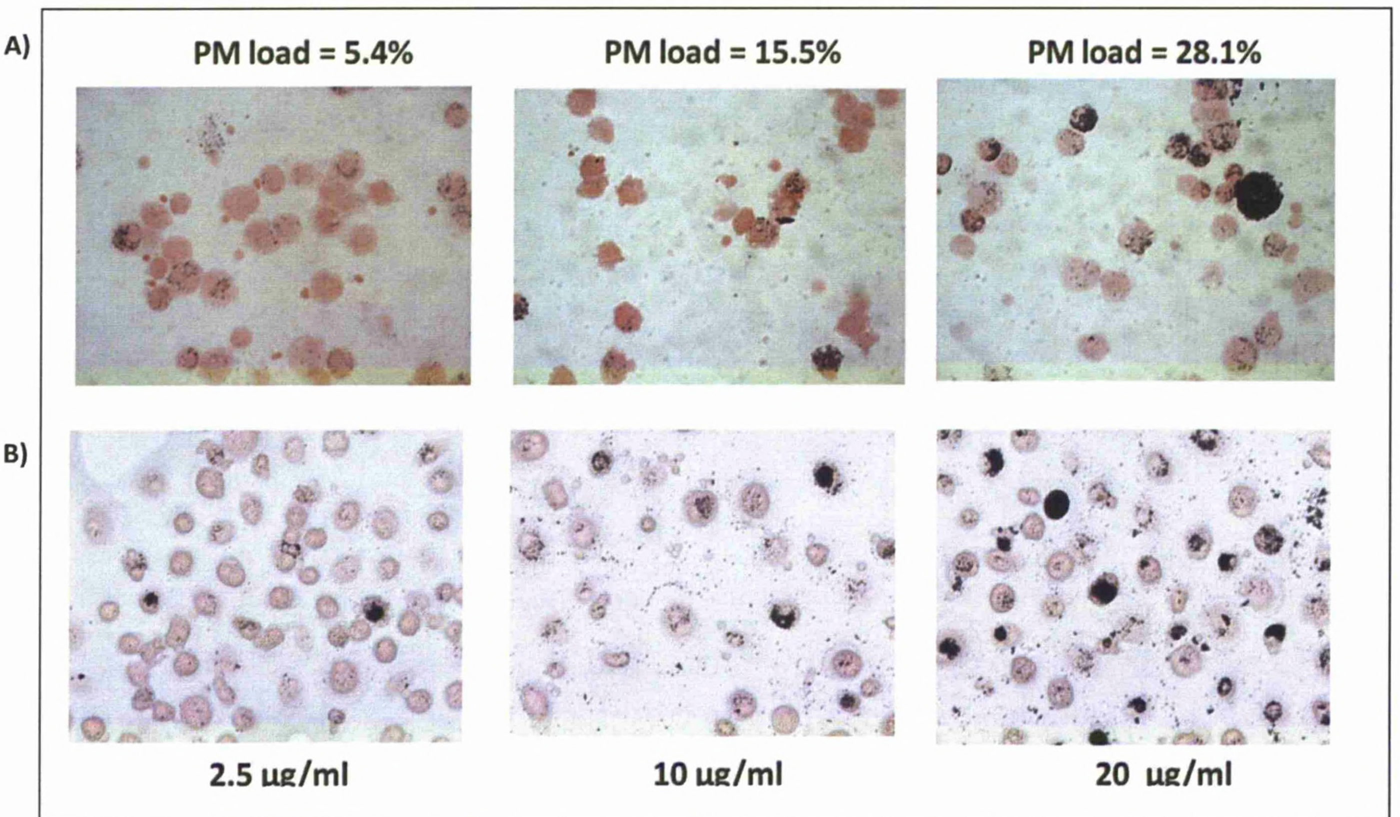


Figure 6.1: Human Alveolar Macrophages from Liverpool and Blantyre.

***A)** Shows sample images taken from the BAL cytospins (stained with Hemacolor) of 3 individuals who had been exposed to BMF smoke **in vivo.** The PM load, calculated by Image SXM software, is indicated above (as a percentage of cytoplasmic area) **B)** Shows HAM from 3 individuals from Liverpool who were given 3 different doses of FCB **in vitro** in order to recreate a similar appearance as the HAM that had been exposed to PM (from a BMF source) in vivo.*

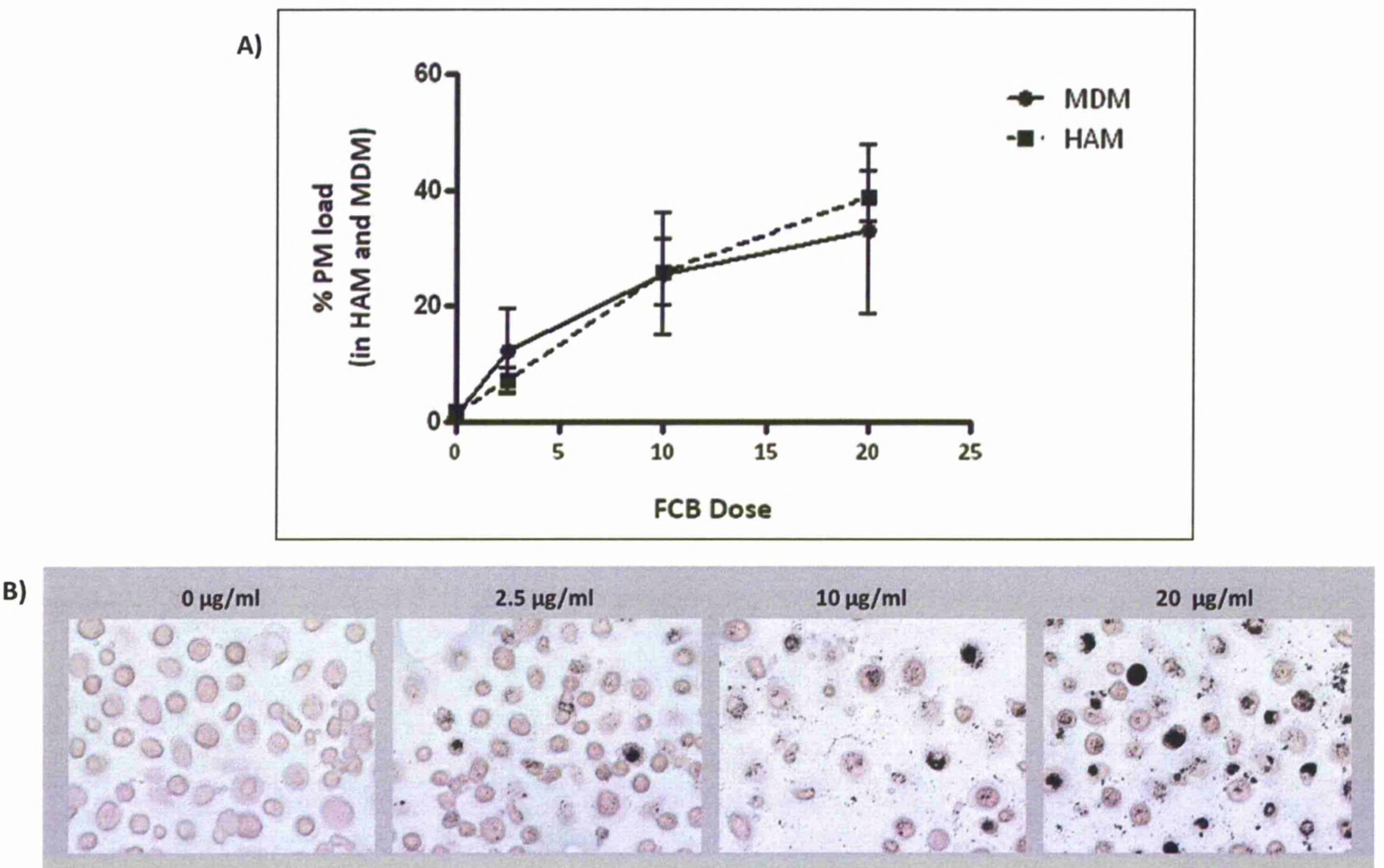


Figure 6.2: Dose-dependent response to the exposure of MDM and HAM to FCB.

A) *Data are means of the PM load for duplicate wells for each dose of FCB; 0.0µg/ml, 2.5µg/ml, 10.0µg/ml and 20.0µg/ml.* ***B)*** *Representative images of HAM exposed to varying doses of FCB. 0.0µg/ml, 2.5µg/ml, 10.0µg/ml and 20.0µg/ml.*

6.4.2 Time-Dependent Response

The time dependency of FCB uptake was studied using MDM samples derived from two individual donors.

A time-dependent response was observed, as shown in figure 6.3. The carbon scores obtained using Image SXM showed an increase from 1.95 % at 0.5 hours to 71.8% at 24hours.

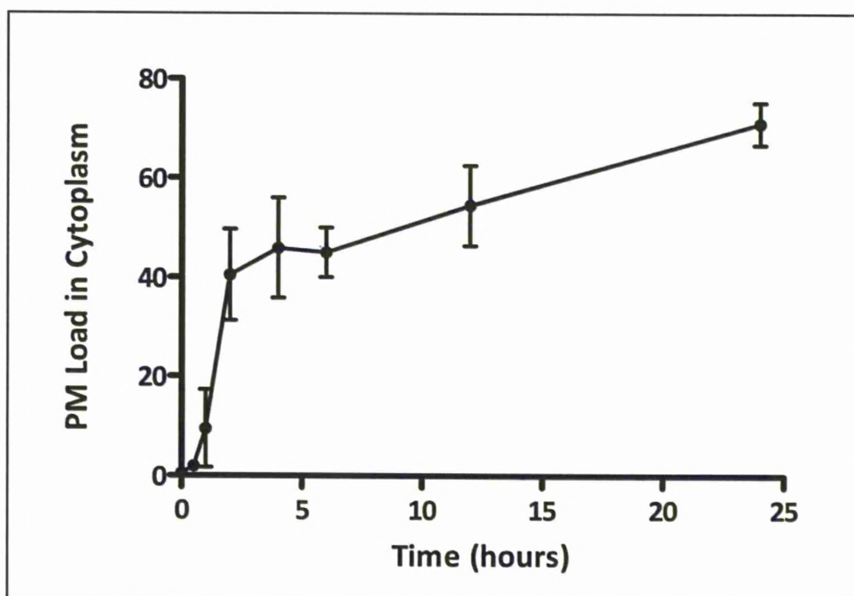


Figure 6.3: Time-dependent responses to the exposure of MDM to 20µg/ml FCB. *Data are means of PM load for duplicate wells for each incubation time; 0.0, 0.5, 1.0, 2.0, 4.0, 6.0, 12, 24.0 hours. Standard deviation bars are shown.*

6.4.3 Inflammatory Cytokine Response

MDM and HAM samples used in the analysis were obtained from 5 different individuals and experiments were done in duplicate. The mean and standard deviation of the mean values for each participant are shown in Figure 6.4.

A dose dependent increase in both IL-6 and IL-8 is seen. The difference between MDM and HAM responses is statistically significant for all doses of FCB ($p<0.05$). HAM had a higher baseline production of cytokinesand a greater response in pg/ml, although a similar fold increase was observed.

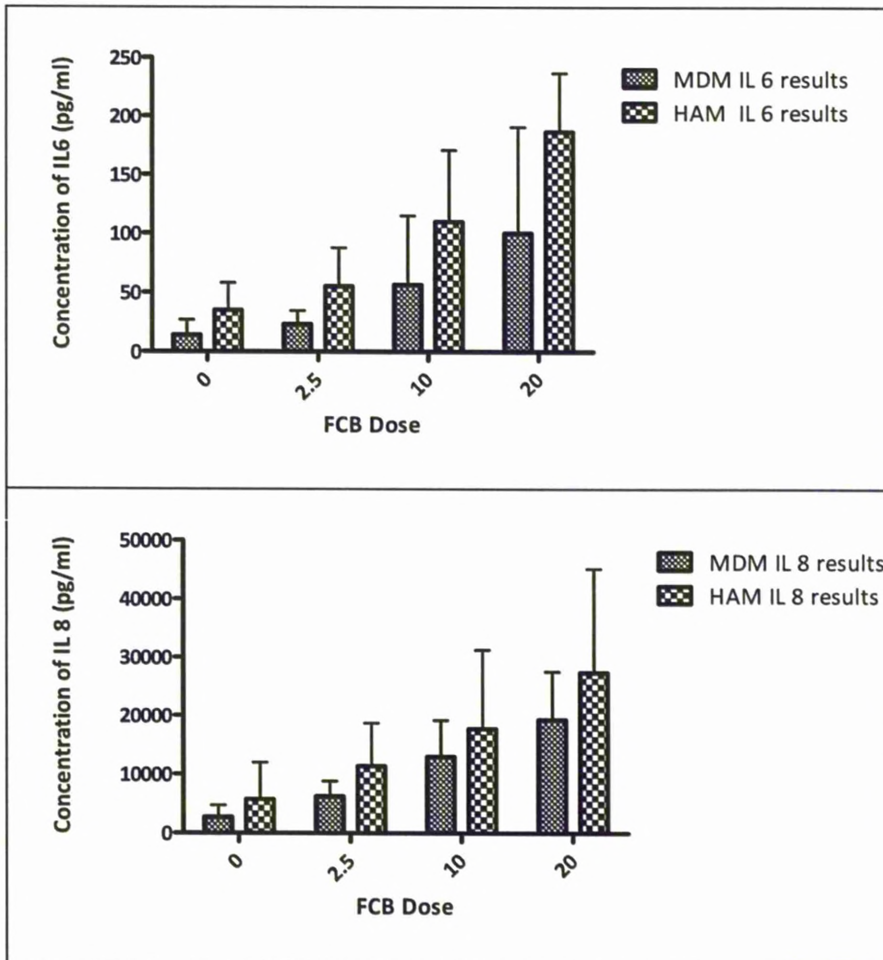


Figure 6.4: IL-6 and IL-8 response to increasing doses of FCB

6.5 Discussion

The key finding of the work performed in this chapter are:

i. *In vitro* MDM and HAM ingest FCB in a dose-dependent manner.
ii. *In vitro* MDM ingest FCB in a time dependent manner.
iii. *In vitro* MDM and HAM both show a dose-dependent IL-6 and IL-8 in relation to exposure to FCB with the highest levels of secretion of both cytokines seen in HAM compared to MDM.

The cytokine response of MDM and HAM and the differences between the two cell types demonstrated by this study are novel findings.

The strengths of this work are that MDM had a similar morphological appearance to HAM and their uptake of PM was demonstrated to be almost identical. Therefore, in many *in vitro* experiments MDM can be used, without the need for an invasive bronchoalveolar lavage that has to be carried out to obtain human alveolar macrophages. However, the cytokine response differences observed between HAM and MDM make MDM a less suitable model in order to try and clarify the mechanisms of lung damage and increased susceptibility to infection that is observed in individuals who are exposed to BMF smoke.

This study also demonstrates that Image SXM software can accurately quantify the ingestion of FCB ingestion by MDM and HAM and shows that it is an important tool in order for begin to describe how pulmonary defence mechanisms in a population at risk of HIV related pneumonia are affected.

The model developed in this chapter is an *in vitro* model of an *in vivo* situation and therefore has a number of limitations. Firstly, with regard to the cells used, the maturity of the MDM was an estimate and could only be determined by the morphology of the cells. Although the MDM

were morphologically and behaviourally representational of HAM they are unlikely to have been expressing the same surface receptors. Secondly, the method of exposure i.e. PM *in vivo* is delivered to the alveolar macrophage by inhalation and there are a number of cells involved in processing antigen in the alveolus; FCB used in this study was delivered to the macrophages in solution. However, inhaled PM will be end up in solution in alveolar lining fluid. Finally, the data presented in this chapter is derived from cross-sectional images i.e. two dimensions of a three dimensional cellular structure. Nevertheless, the distribution of PM within a macrophage, as shown by confocal microscopy, does not imply that the calculated PM load is going to be significantly altered by using two dimensional as opposed to three-dimensional images. [372]

There is an extensive literature on the mechanisms by which PM affects health. This work has been carried out in either MDM, cell lines (e.g. U937) or animals models. [228] Work done in the 1980s on mice demonstrated that an increased number of particles rather than mass of particles lead to a greater production of macrophages and that these macrophages are derived from the bone marrow.[382;383] More recently it has been demonstrated that the size of particle that is inhaled produces a different effect in terms of lung damage and inflammatory response and that inhalation of ultrafine carbon particles triggers a biphasic pro-inflammatory process in the lung.[228;384] The data presented in this chapter show that there are differences between the MDM and HAM model of exposure and that using HAM as opposed to MDM or animal models has advantages, as well as disadvantages.

The experiments in this chapter used fine carbon black. Several studies have demonstrated that the carbonaceous core of PM is not as pro-inflammatory as other components of combustion-derived particles, demonstrated using components of diesel particulates that have been

extracted by methanol.[385] This effect has been observed in the mechanisms by which alveolar macrophages protect the lung from infection. Rats exposed to diesel exhaust particles by intratracheal instillation decreased LPS-induced TNF-α and IL-1 production from alveolar macrophages. In contrast, carbon black did not exhibit this inhibitory effect. This result supports the hypothesis that exposure to diesel exhaust particles increases the susceptibility of the lung to infection by depressing the antimicrobial potential of alveolar macrophages. This inhibitory effect may be due to adsorbed organic chemicals rather than the carbonaceous core of the diesel particles.[386] It has also recently been demonstrated *in vivo* in mice that acute high level loading of AM with ultrafine carbon black PM does not increase the susceptibility of mice to pneumococcal infection.[44] Therefore future studies of susceptibility to infection should focus on unprocessed biomass PM.

Conclusions

This study demonstrates a valid model for exploring the effect of biomass derived PM on macrophage function and that PM loading of macrophages exhibits a dose and time dependency. This work also has shown that HAM and MDM secrete IL-6 and IL-8 in a dose dependent manner, with HAM secreting a greater concentration than MDM. This finding is important because these two cytokines are important in orchestrating human lung defence in response to both infectious disease as well as to PM.

Further work needs to be carried out using alveolar macrophages and field derived PM (from wood, charcoal, dung and crop residues) in order to continue to describe the complexity of pulmonary defence mechanisms involved in the ingestion of environmental particles and the association with respiratory infection.

In Malawi, shown in Chapter 3, women are exposed to high levels of indoor air pollution from BMF smoke, for approximately 5 hours per day and the findings of dose and time related uptake of PM and related cytokine secretion provide evidence of potential mechanisms of the observed adverse effects, triggered by downstream cytokine effects. This study provides further evidence that high smoke levels are having potentially adverse health effects.

CHAPTER 7: THE IMPACT OF PARTICULATE MATTER BURDEN ON HUMAN AIRWAY DERIVED MACROPHAGE PHAGOCYTE FUNCTION

7.1 Study Aims

The aim of this work was to:

Explore the association between particulate matter loading in human alveolar macrophages (HAM) and three different HAM functions:

i. Phagocytosis
ii. Oxidative burst
iii. Proteolytic function

7.2 Introduction

Cross-sectional and case-control studies have shown an association between BMF smoke exposure and a number of adverse health outcomes including lower respiratory tract infections (LRTI) in children and COPD in women. [341;387] These epidemiological studies alone do not establish causal link. Therefore studies of biological mechanisms are needed to compliment other sources of data on the health effects of BMF exposure.

Longitudinal studies can help establish causality but for outcomes like LRTI and COPD particularly, these require long periods of follow up, large numbers of participants and a significant associated expense. Another approach is to understand the mechanisms by which BMF could cause disease thus providing support for the biological plausibility of a causal relationship

An example of this type of health effect is the association of tuberculosis (TB) incidence with BMF exposure. There is biological plausibility of an association as the alveolar macrophage (AM) has a key role in both the processing of PM as well as in the pathogenesis of TB and there is also

evidence from other smoke exposures (i.e. tobacco smoke) of an association between PM exposure and TB. However, epidemiological studies have many potential confounders e.g. poverty or overcrowding, that can be difficult to adjust for and consequently the case that PM exposure from BMF leads to a greater incidence of TB has not yet been fully made.[73-75;388] Laboratory based studies can assist in answering this type of question by demonstrating a clear biological effect.

This chapter focuses on such mechanistic evaluation. Specifically, the effects of biomass fuel derived particulate matter load on three aspects of alveolar macrophage function (phagocytosis, oxidative bust, and proteolysis) are explored.

7.3 Methods

Recruitment, ethics, bronchoscopy and BAL have all been described previously in Chapter 2.

HAM were grown in 6 well plates at a concentration of 3 x 10^6 cells per well. Culture media (10% FCS RPMI plus 2mM L-glutamine and Penicillin-Streptomycin-Neomycin Solution) was changed after 4 hours and the cells were left overnight before performing the assay.

7.3.1 Reporter Bead Assay

We used a modification of a macrophage function assay, recently described Professor David Russell (Cornell University).[197] Chapter 2.13 and Figure 2.15 describe the background to this assay.

The assay assesses the proteolytic and oxidative burst function of alveolar macrophages as well as the ability to phagocytose beads, by assessing the number of AM associated with beads using flow cytometry (demonstrated in Figure 7.2).

Flow Cytometry Analysis strategy

Figure 7.1, 7.2 and 2.15 shows the rightward movement of reporter fluor intensity indicating the degree of proteolysis (or intraphagosomal oxidation depending on the beads used) in those with 'calibration high' signal. Figure 7.1 shows a histogram overlay showing cells with no exposure to beads (blue) and those with proteolysis beads (red).

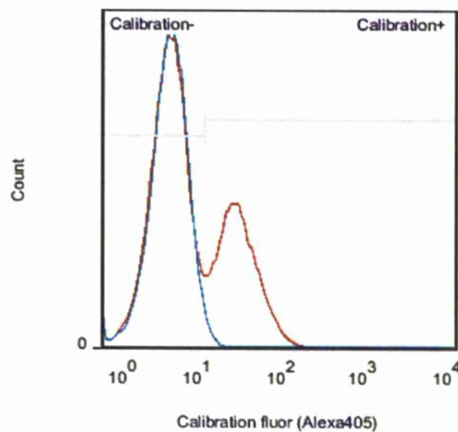


Figure 7.1: Flow Cytometry Analysis Strategy. *Histogram overlay showing cells with no exposure to beads (blue) and those with proteolysis beads (red). The same method was used for oxyburst beads.*

Results were expressed as a ratio of substrate fluorescence to calibration fluorescence demonstrated in Figures 2.16 and 7.2, (otherwise it would simply be a measure of the number of beads ingested).

Oxidative bust activity index was calculated by:

$$\frac{\text{Mean Fluorescence Intensity (MFI) of substrate DCDHF from cells ingesting beads (FL1)}}{\text{MFI of calibrator beads (Alexa 633) from cells ingesting beads (FL4)}}$$

Proteolysis activity index was calculated by:

$$\frac{\text{(MFI) of substrate DQ Bodipy combined with BSA from cells ingesting beads (FL1)}}{\text{MFI of calibrator beads (Alexa 594) from cells ingesting beads (FL4)}}$$

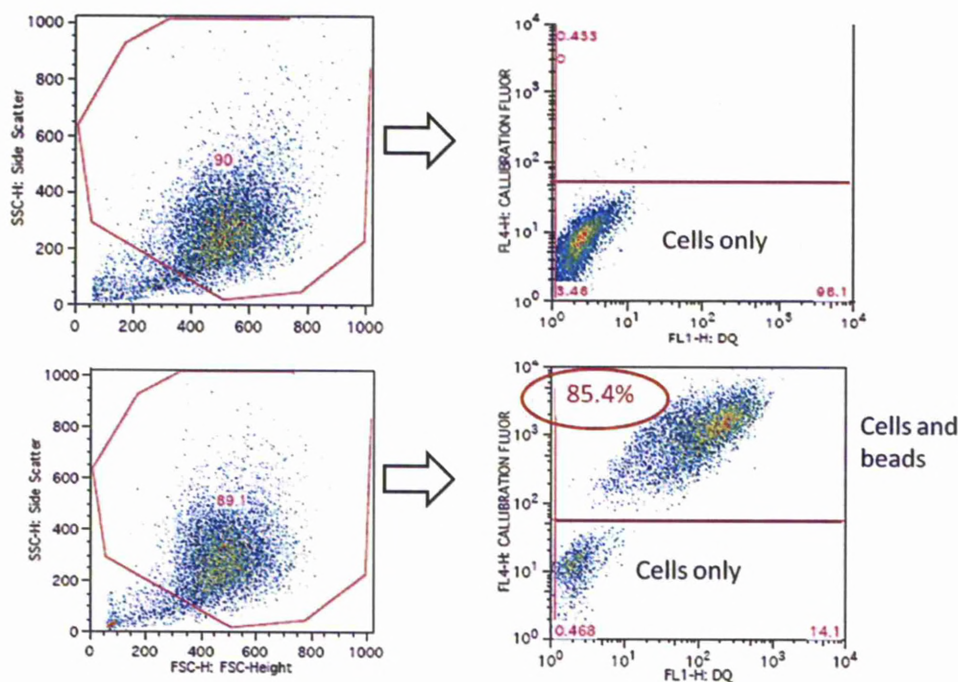


Figure 7.2: Alveolar macrophage gating strategy and Phagocytic Index. *Each dot represents an event (i.e. one cell). The rightward shift of reporter fluor intensity (FL1) indicates the degree of intraphagosomal proteolysis. 85.4% is the percentage of cells associated with beads and was used as a measure of phagocytosis.*

7.3.2 Exploratory Analyses of the Relationship of PM load in HAM with HIV status, Lung Function and Air Quality.

PM load was calculated for all patients, in our group, who had a bronchoscopy during the study.[279] HIV testing was performed as outlined in Chapter 5.3.1.

During the bronchoscopy study described in this Chapter, the air quality in the homes and lung function (spirometry) was measured in a convenience sample of the participants who underwent a bronchoscopy and BAL.

Air sampling was performed within 6 weeks of the bronchsocopy date. It was performed using the UCB photometric device and the HOBO® Carbon Monoxide monitor, as detailed in Chapter 3.3.4.

Spirometry traces were performed and examined as outlined in Chapter 2.6 and Chapter 4.3.5. The traces were reviewed by a 4 experts (SJ, PA, SBG and DGF). FEV_1 was examined by PM load in HAM.

7.3.3 Analysis

The distribution of PM load in HAM in the population sampled was non-Gaussian (Figure 7.3) therefore it was converted to a logarithmic scale. The log PM load was divided into 3 groups (low, medium and upper tercile PM load) according to the 33rd and 66th percentiles of all the individuals who had a PM load calculated, using Image SXM software.

Proteolysis, oxidative burst and phagocytosis were all examined by PM load in HAM cytoplasm. ANOVA was used to assess if there was a difference between terciles of PM load.

7.4 Results

7.4.1 Study Population

75 participants had a bronchoscopy and BAL performed. The characteristics of the study population and whether the flow cytometry assay was performed are summarised in Table 7.1, divided according to level of PM loading of HAM.

Flow Cytometry assays were not performed in all participants because either cell counts from BAL were too low or contamination of cell culture occurred or the BAL was performed before the reporter bead assays protocol was fully developed.

Characteristic of BAL Participants		**PM loading of HAM (by Tercile)**		
		Low (%)	***Medium (%)***	***Upper (%)***
	Female)	12 (46)	11 (44)	9 (38)
	Age (SD)	31 (7)	36 (10)	39 (11)
Smoking Status	Non-Smoker	17 (68)	18 (72)	20 (83)
	Ex-Smoker	8 (32)	6 (24)	3 (13)
	Current Smoker	0	1 (4)	1 (4)
Fuel Used for cooking	Charcoal	17 (68)	16 (64)	13 (54)
	Wood	6 (24)	8 (32)	10 (41)
	Electricity	2 (8)	1 (4)	1 (4)
	HIV positive	10 (42)	9 (37)	4 (17)
Flow cytometry assay performed?	Proteolysis	20 (77)	17 (68)	13 (54)
	Oxidative burst	19 (73)	20 (80)	16 (67)

Table 7.1: Characteristics of All Participants who took part in Bronchoscopy study (by PM load in HAM).

7.4.2 PM loading of HAM

The PM load in HAM (on cytospin slides) ranged from 0.1% to 28.1%. The mean PM load was 3.21% (SD 4.77) and the median value was 1.30.

The 33rd and 66th percentile were 0.4 and 2.6 % PM load in HAM respectively.

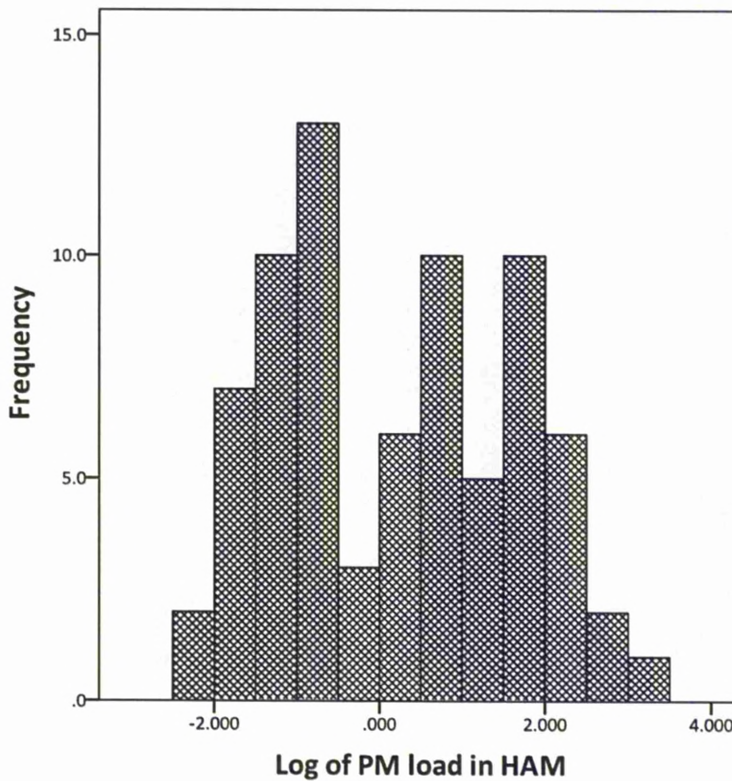


Figure 7.3: Log of PM load in HAM. *PM load was not normally distributed therefore the log transformed values (shown in this figure) were used in the analysis.*

7.4.3 Reporter Bead Results

Phagocytosis

There was no significant difference in the phagocytosis of either DQ beads (after 180 minutes) or Oxyburst beads after 60 minutes in HIV negative participants (shown in Figure 7.4). For DQ beads n=30 and for Oxyburst beads n=35.

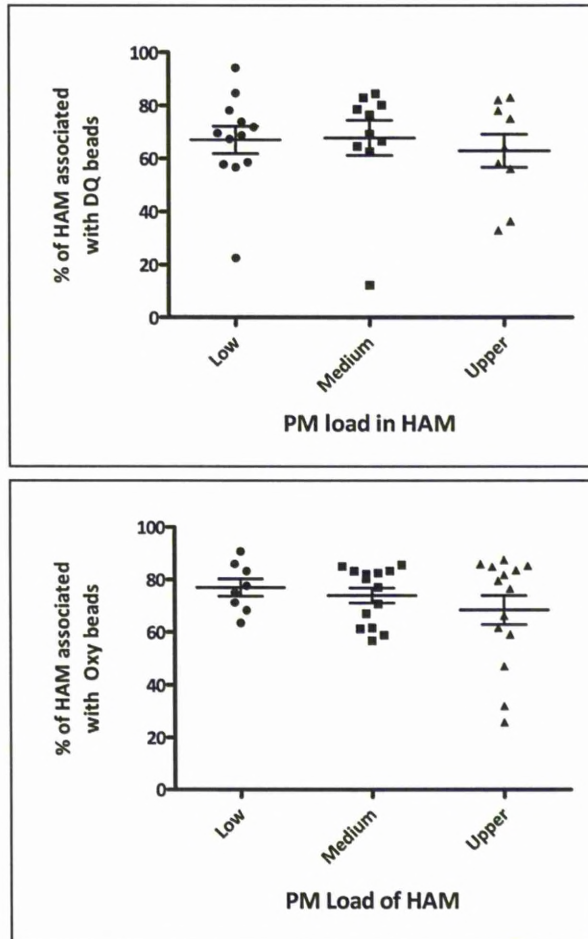


Figure 7.4: Phagocytosis by PM load in HAM. *ANOVA p=0.84 for DQ beads and p=0.48 for Oxyburst beads.*

Oxidative Burst

38 BAL samples from HIV negative participants had the oxidative burst assay performed. There was a significant difference in the oxidative burst activity between the low and upper tercile of PM loading in HAM. For each tercile the number of participants was 11, 14 and 13 for low, medium and upper tercile respectively.

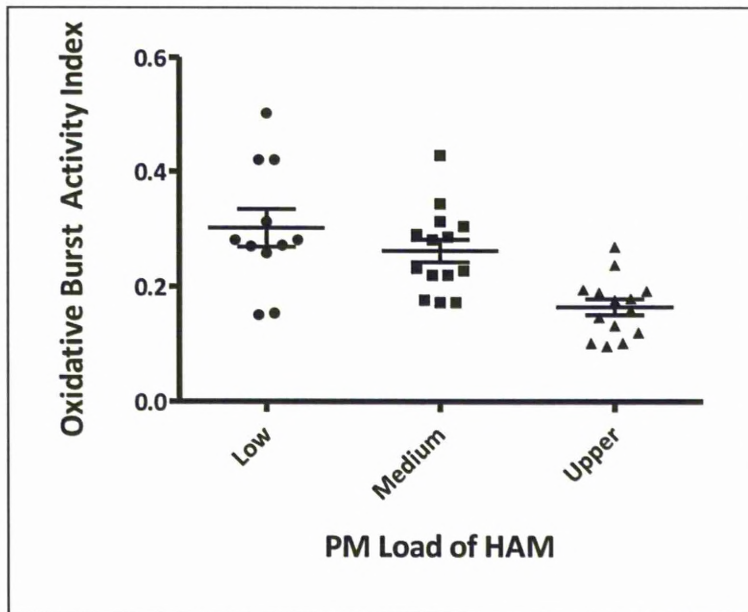


Figure 7.5: Oxidative burst by PM load in HAM. *ANOVA p=0.003*

Proteolysis

31 BAL samples from HIV negative participants had the proteolysis assay performed. There was no significant difference in the proteolysis activity between the low and upper tercile of PM loading in HAM. For each tercile the number of participants was 13, 10 and 9 for low, medium and upper tercile respectively.

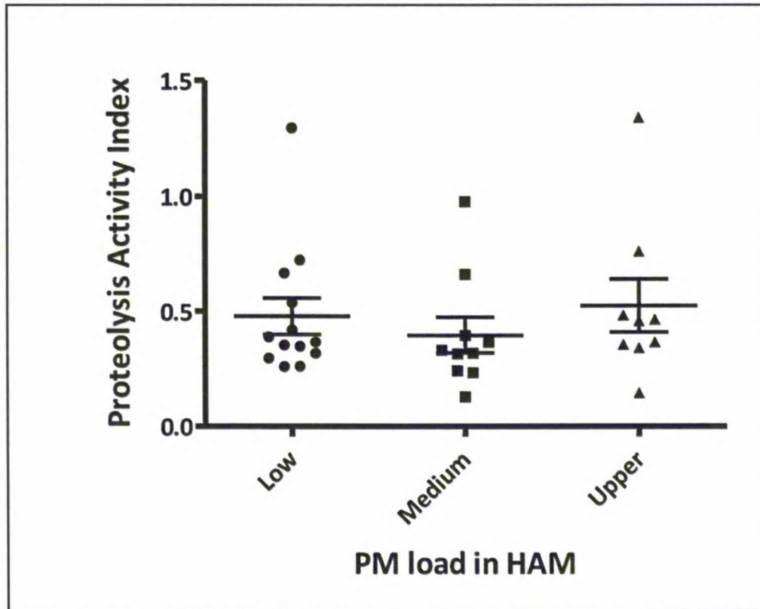


Figure 7.6: Proteolysis by PM load in HAM. *ANOVA p = 0.62*

7.4.4 Exploratory Analyses of the Relationship of PM load in HAM with HIV status, Lung Function and Air Quality.

HIV status

The mean PM load in HIV negative participants was 4.08% (SD=5.5) and in HIV positive participants was 1.74%. (SD=2.32). There was a significant difference between the log transformed PM load in HIV negative individuals compared to HIV positive participants; p=0.031 (students t test).

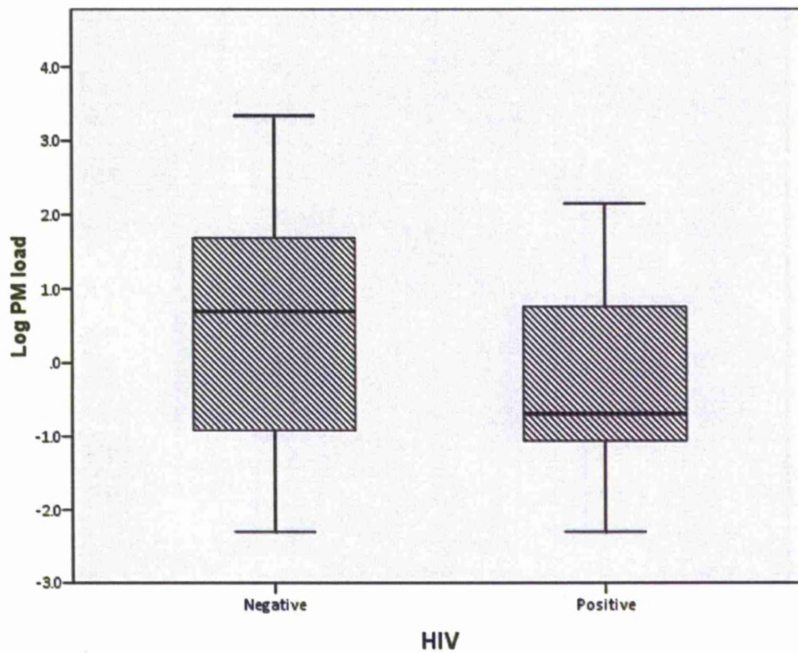


Figure 7.7: Box plot of HIV disease and PM load. *There was a significantly different PM load in HIV positive and HIV negative individuals (p = 0.031)*

Air sampling

32 individuals who had a PM load in HAM calculated also had air sampling performed. There was not a significant correlation between any of the outcome variables listed in Table 3.3 (HOBO time > 5ppm, HOBO peak value, HOBO Time weighted average (TWA), UCB peak value or UCB TWA). The time (in minutes) that the UCB device recorded PM levels greater than 250μg/m^3 is demonstrated in figure 7.8; there was no significant correlation with this outcome variable either.

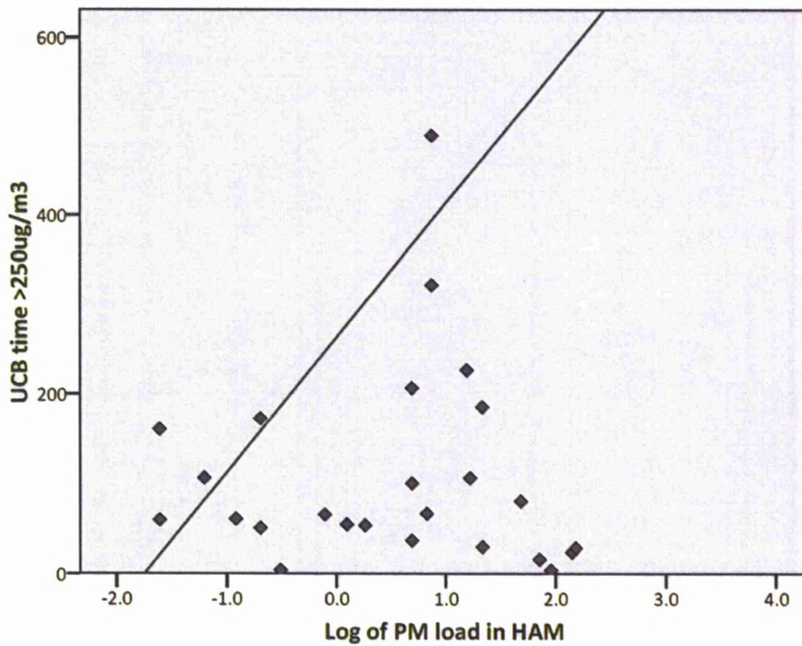


Figure 7.8: Scatter Plot of UCB measured Respirable dust above 250μg/m^3 against the log of PM load within HAM. *R^2 = 0.112. There was not a statistically significant correlation (Pearson) between the two variables. In 5 homes the UCB malfunctioned and so these points are not shown. Time in minutes is on the y axis.*

Spirometry

30 individuals who had a BAL performed also had spirometry data. Three of the spirometry traces were rejected after review because they did not reach ATS/ERS standard – the traces were not of high enough standard for interpretation.

No correlation was seen between PM load in HAM and FEV_1.

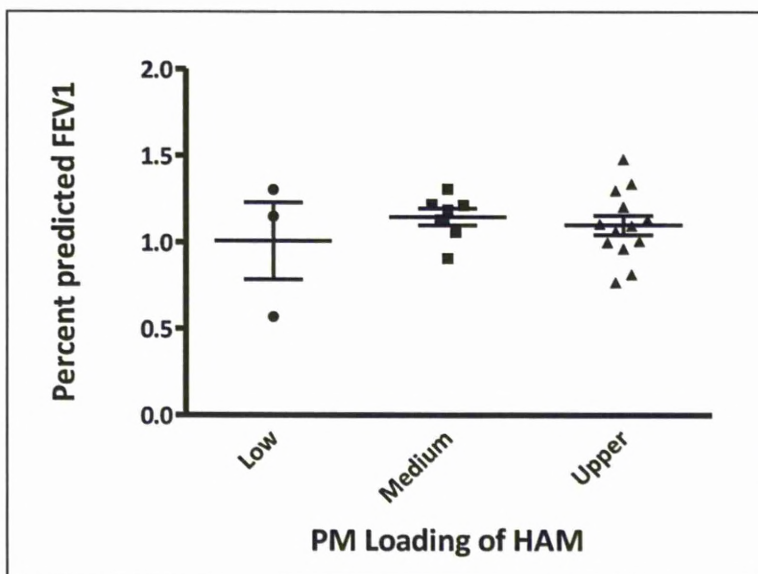


Figure 7.9: Box Plot of percentage predicted FEV1 against the Log of PM load within HAM. *R^2 = 0.0001. There was not a statistically significant correlation (Pearson) between the two variables*

7.5 Discussion and Conclusion

7.5.1 Alveolar Macrophage Function and the Relationship to PM load

This chapter describes the first study of human alveolar macrophage function in individuals who are routinely exposed to household air pollution from biomass fuel.

The main finding is that although proteolytic function and phagocytosis of beads does not appear to be affected by PM load at the levels seen in this study, oxidative burst activity is reduced in HAM that have a higher PM load. This is an important finding that provides insight into the mechanisms by which BMF smoke exposure causes disease.

Phagocytosis

Phagocytic activity was studied to test the hypothesis that the relationship between biomass smoke exposure and lower respiratory tract infections is due to diminished phagocytic ability in biomass particulate loaded macrophages. The data presented in this chapter provide no support for this hypothesis. As such they are consistent with the findings presented in chapter 6 that *in vivo* macrophages continue to be capable of phagocytosis at high levels of PM exposure.

The effect that PM has on phagocytosis is not entirely clear. Zhou *et al* demonstrated that concentrated ambient particles caused increased binding of *S. pneumoniae*, and reduced killing as a result of decreased internalization of bound bacteria.[43] However with tobacco smoke the effect on mouse macrophages from BAL was a reduced complement-mediated phagocytosis of *S. pneumoniae*, although the ingestion of unopsonized bacteria or IgG-coated microspheres, similar to the ones used in this chapter, was not affected by tobacco smoke.[300] Lundborg et al have shown that ultrafine carbon particles do seem to impair phagocytic capacity of silica particles by macrophages when they are

added to cells *in vitro* and although the same group have demonstrated that diesel particulates impair the phagocytosis of different organisms. [389;390] The result reported in this chapter are similar to that observed for IgG-coated beads by Phipps *et al.*[300] These different studies, using PM derived from different sources, on different models have yet to clarify the effect that BMF has on the phagocytosis of organisms and more work is required in order to clarify the defect.

Oxidative Burst

A reduction in the ability of HAM to mount an oxidative burst response at higher levels of PM exposure is an important finding. Wood smoke has been shown to increase intracellular reactive oxygen species suggesting that this is a potential mechanism by which chronic lung damage from BMF smoke exposure occurs.[391] Also, other non-BMF derived PM has been shown to cause substantial intracellular oxidant stress within AM, which may contribute to subsequent cell activation and production of proinflammatory mediators that are observed after PM exposure; [392] for example it has been demonstrated previously that PM derived from diesel particulates effects respiratory burst in AM and the work shown in this chapter supports this finding. [393]

Macrophage elimination mechanisms for many organisms (including *M. tuberculosis*) include the generation of reactive oxygen intermediates (ROIs).[394] The data presented here help to support biological plausibility hypotheses and a greater understanding of cellular mechanisms can provide insights into new prevention approaches and potential treatment strategies. The relevance of reduced oxidative burst activity needs further exploration but this is the first study performed in humans that has assessed AM function in the context of BMF smoke exposure and a functional deficit has been demonstrated.

Proteolysis

Proteolytic function is more difficult to interpret. Proteolytic activity was studied to test the hypothesis that the relationship between biomass smoke exposure and COPD is due to increased proteolysis in biomass particulate loaded macrophages. Proteolysis might have been expected to be increased in cells that have a higher PM load, as this would provide a potential mechanism for the development of COPD in individuals who are routinely exposed to PM from household air pollution; no such increase was observed in this study. However, activated macrophages are proteolytically less active than resting macrophages, in order to potentially maximize the antigen sampling and presenting capacity of activated macrophages, therefore what was observed may reflect a balance between these two responses and actually represent impaired function. [395]

Strengths and Limitations

This study is weakened by relatively low number of participants in the study; 38 participants took part in the oxidative burst assay. Also, the spread of PM in HAM is not normally distributed i.e. it is skewed to the low PM load in HAM range. When the data were analysed as a continuous variable (using the log transformed values for oxidative burst activity index) there was not a significant (negative) correlation ($p=0.051$, using Pearson's correlation coefficient). However this work was repeated at MLW in early 2010 (Dr Jamie Rylance correspondence) and the findings for all assays are the same as reported in this chapter.

The reporter bead assay is a new way of assessing AM function and there is little published material that has used it. Further data are required from controlled models (either *in vitro* or in animals) using the reporter bead assays in order to be able to interpret the results with more clarity. Nevertheless, it is a robust assay that enables phagolysosome function to

be examined in the context of other clinical variables.

7.5.2 Relationship of PM load in HAM with HIV status, Lung Function and Air Quality

HIV

These data show that HIV negative individuals have a higher PM load in AM compared to HIV positive individuals, this has also been observed in previous pilot studies.[202] It is likely that there are several variables that are contributing to this observed result. The two most significant risk factors for pneumonia in this population are HIV and exposure to biomass fuel smoke (although there are few data supporting this from African adults) and this difference in PM loading implies that these two risk factors potentially interact.

There are several potential explanations of this observed difference in PM loading: It maybe that macrophage lifespan is shorter in HIV positive individuals resulting in less time for PM to accumulate. HIV positive individuals may be exposed to lower levels of air pollution, although this was not a sick population (CD4 cell count was always greater than 200) and they were not obviously cooking or lighting their homes differently by questionnaire, so this explanation seems unlikely. Phagocytic activity for PM between HIV negative and positive individuals may be different leading to a reduced uptake of PM by HIV positive macrophages. However this finding has not seen in other studies looking at phagocytosis of *S. pneumoniae* or indeed the phagocytic data from this chapter, where there was no difference observed between HIV positive and negative individuals in their ability to phagocytose beads.[272] However, it is likely that in the context of cigarette smoking in HIV infection, phagocytosis is affected. [396] Whether this is the same in individuals exposed to BMF smoke remains to be seen.

When the same analysis was carried out on the population studied in Chapter 5 the result was that there was not a significant difference between log PM load in HAM HIV positive and negative participants (HIV positive: 1.34 (SD 0.96) v HIV negative: 1.57 (SD 1.01); p=0.38). The population sample in Chapter 5 had a higher proportion of men who took part in the bronchoscopy study and men had a higher average PM load, perhaps as a result of kerosene lamp exposure. Therefore it is possible that the lack of a significant difference in the data set examined in Chapter 5 reflects the effect other potential confounders.

The finding demonstrated in Figure 7.7 certainly warrants further investigation of the relationship between HIV and BMF smoke exposure.

Air sampling

This small data set does not show a significant correlation between measured air pollution and PM load in HAM. It potentially highlights the importance of personal air sampling as opposed to air sampling that is only performed in the cooking area.

Air sampling data collected in this way is a one off measure and a 24-hour snap shot of exposure therefore is not fully representative of total exposure. Chapter 5 demonstrated that reported smoke exposure was associated with PM load in macrophages and PM loading in macrophages has been demonstrated to have an association with exposure, therefore it is also possible that the sample size examined in this exploratory analysis is simply too small to pick up a signal. [100;381]

Different BMF use may well confound results as the appearances of wood exposed macrophages as opposed to charcoal or kerosene-exposed macrophages are likely to have different appearances. It is unlikely to be possible to study a population exposed solely to one type of pollutant and so future studies would need to try and take into account multiple exposures and include thorough exposure histories from participants,

especially in the 30 days leading up to alveolar macrophage samples being obtained. In order to do this PM load in induced sputum AM would enable a greater number of participants to take part, especially in the predominantly wood exposed rural population, rather than using PM load in BAL derived AM, that are more difficult and costly to obtain.

It is possible that what this data actually demonstrates is that sampling the air is actually a lot poorer for measuring total exposures than using PM load in AM, and that PM load is actually the only 'true measure' of exposure over a time period. This is because PM load represents the accumulation of exposures, from a variety of sources and that HOBO or UCB simply measure a snapshot and this is may not be informative enough of total exposure. It is of interest to note in the data that there was one individual who had their exposures measured and PM load measured twice, 8 months apart. The air sampling results were significantly different but the PM load in this individual was almost exactly the same.

Spirometry

Valid spirometry was only performed in 27 individuals and so in this sample it was not possible to detect a significant correlation.

Using spirometry as a outcome measure is a good way of assessing a health effect because the test is reproducible, sensitive enough to pick up a biologically significant difference between groups and is relatively easy and cheap to perform This exploratory examination however was not able to demonstrate an association between lung function and PM load in HAM.

A study appropriately designed to assess spirometry and PM load would potentially be a good way to assess the dose-response curve to BMF smoke. However in order to do this clarification of the relationship

between exposure levels and PM load would also need to be examined further.

7.5.3 Conclusions

This chapter demonstrates a deficit in HAM oxidative burst function in association with PM load and this is likely to in part explain some of the increase in susceptibility to infection observed in BMF exposed individuals. More work is required to be able to elucidate these mechanisms further.

This chapter has demonstrated that the application of both Image SXM methodology for calculating the PM load in HAM and the reporter bead assay for assessing HAM function are valid tools to explore these mechanisms further.

The effect that BMF has on HIV infected individuals AM function requires more investigation. This chapter has demonstrated a difference in PM loading in HIV positive and negative individuals. This is helpful in generating a number of potential hypotheses that require further examination.

The relationship of PM load in HAM with both measured exposures and spirometry requires new studies in order to clarify the importance of PM load as a biomarker of exposure and the influence that it has on the development of impaired lung function in biomass smoke exposed populations.

CHAPTER 8: FINAL DISCUSSION

8.1 Introduction

The use of biomass fuel is a major cause of global mortality and morbidity. There is great demand for data on the health effects of household air pollution and the work carried out during this thesis has both contributed to this literature.

This thesis describes five related studies that were designed in order to describe the levels of pollution, to examine the health effects of BMF exposure on respiratory health in Malawi and to explore the potential mechanisms involved in susceptibility to pneumonia.

Three hypotheses were tested in these studies. They were that:

1. Indoor air pollution levels in Malawian homes are high.
2. BMF smoke exposure is associated with impaired lung function.
3. Particulate matter impairs the function of alveolar macrophages.

In the preceding six chapters data have been presented and discussed addressing each of these three hypotheses. In this final discussion the major findings are summarised, the implications of this work for potential intervention trials, for future air sampling work and for epidemiological work addressing the burden and causes of impaired lung function are discussed, the limitations of the studies are outlined, suggestions are made for further work that needs to be done and finally, concluding remarks are made.

8.2 Major findings

The studies carried out during this thesis has resulted in three conclusions that support each of the general the hypotheses:

Firstly, in Chapter 3, it has been shown that the levels of IAP associated with biomass fuel burning in Malawi are high; 80% of homes sampled exceed the WHO level for outdoor air quality for $PM_{2.5}$ levels by at least four times and levels of endotoxin that individuals are exposed to far outweigh what is seen in homes in industrialised countries.

Secondly, in Chapter 4, data have been presented that suggests that wood smoke (more than charcoal) together with lower socioeconomic status is associated with impaired lung function.

Thirdly in Chapter 7, PM load in human alveolar macrophages (HAMs), obtained from individuals who are exposed to household air pollution from BMF on a daily basis, has been shown to be associated with a deficit in HAM oxidative burst function.

In addition to these findings data have been presented in Chapter 5 demonstrating that PM load in HAMs is associated with reported exposures and in Chapter 6 a valid model for exploring the effect of biomass fuel derived PM on macrophage function has been described. Also in Chapter 6, the PM load of alveolar macrophages has been shown to exhibit a dose and time dependency and data presented on cytokine secretion shows that HAM and MDM secrete IL-6 and IL-8 in a dose dependent manner, with HAM secreting a greater concentration than MDM.

8.3 Implications

This thesis has potentially important implications on several areas of research in Malawi and in the wider region of Southern Africa. It is also likely that some of the findings presented here are relevant to other areas of the world where burning biomass fuel for household energy is also a major risk factor for impaired health.

There is currently a significant and an appropriate interest in establishing intervention studies based around alternative cook stoves; the Global Alliance for Clean Cookstoves (GACC) was launched in September 2010 with the aims of saving lives, improving livelihoods and combating climate change through the development of a thriving global market for clean and efficient cookstoves (http://cleancookstoves.org/).

8.3.1 Implications for Intervention Trials

This thesis has implications for an intervention trial should one be based in this region of Africa.

Many of the questions around health effects of household burning of BMF would be best answered by intervention trials. It can be argued that no further health effect data are required in order to justify action to reduce pollution levels that are found in homes in Malawi and so any further work should only be done, if possible, in the context of an intervention trial.

As outlined in Chapter 1.2.2 only two cook stove intervention trials have reported any data and although there is an intervention trial being carried out in West Africa (Ghana) this had not started recruitment at the time of writing (June 2011) and will not report for another five years. Ghana is likely to be significantly different from Malawi and other parts in the region of Southern Africa in terms of food and cooking practices, fuels used as well as local customs. The intervention being trialled in Ghana may also not be applicable in terms of acceptance, cost, building

materials etc and its widespread use outside of Ghana and West Africa may not be practical.

This thesis will be of potential benefit to an intervention trial either based in Malawi or in one of its neighbouring countries in several ways. By demonstrating in Chapter 3, that BMF smoke exposure levels are high in southern Malawi this thesis has firstly demonstrated that the problem is highly relevant and likely to be detrimental to health, adding more weight to the need to perform a trial aimed at reducing exposures in order to improve health.

Data presented in Chapters 3 and 4 regarding cooking location make the possibility of an education campaign about smoke exposure a realistic alternative or at the very least an important add-on to any stove intervention trial. Many households, especially in the rural environment could potentially move almost exclusively to outdoor cooking for the majority of the year and simple strategies to improve ventilation could have a dramatic effect on exposure levels. A public health education program around the effects of polluted air could be instituted at relatively low cost, especially in comparison to an intervention trial and this kind of program would essentially support social marketing programs aimed at encouraging alternative stoves to be adopted. Data from this thesis can be used to advocate these kinds of health improvement programs.

The potential detrimental effect that wood compared to charcoal smoke exposure has on lung function is discussed in Chapter 4. The higher $PM_{2.5}$ exposure levels observed with wood and the health consequences associated with this level of exposure, should contribute to the discussion over which type of interventions should be trialled. This should also help to inform a public debate around the merits of one fuel type over another, as well as the type of stoves that should be supported.

The use of charcoal is controversial in Malawi and the impact on both the local and global environment that occurs as a result of charcoal use can be significant but the data presented here and elsewhere could significantly inform the debate over the potential advantages and disadvantages that cooking with charcoal has.

The data presented in Chapter 5 demonstrate that PM load in alveolar macrophages is potentially an important biomarker of BMF smoke exposure. Monitoring exposures and correlating this to a health effect can be both difficult and expensive. The use of PM load in alveolar macrophages from induced sputum is a realistic biomarker of success of an intervention and the image analysis methodology described in Chapter 2.11 and carried out in Chapter 7 show that this can potentially be done very quickly and cheaply and could be an important aspect of an intervention trial.

8.3.2 Implications for future air sampling work

Because the exposure levels demonstrated in Chapter 3 were high, future air sampling work should be focussed around interventions to reduce exposures.

Chapter 3 describes the smoke exposures of a sample of southern Malawian homes from rural and urban locations. This was the first time such work has been reported in Malawi and significant capacity was established in terms of personnel and expertise as a result of doing the work at the MLW. In Chapter 3.5.4 an attempt has been made to give practical advice on air sampling. It is hoped that this work may help other researchers planning on carrying out similar work in the future.

The toxic components of BMF fuel smoke have not yet been fully described. Much of this work could be done by analysis of different fuels burnt in a controlled environment in a laboratory (i.e. in a burn chamber) in order to obtain samples for analysis for a variety of different

compounds. The samples collected could then also be used in order to carry out *in vitro* analysis on the toxicity of fuels. The development of this kind of biobank of smoke material could then be used in the mechanism studies described in Chapter 6 as well as outlined in Chapter 8.5.3.

The benefits of this research into the toxicity of BMF smoke are that it could potentially lead to results that inform an intervention. For example if it is found that different types of wood are more toxic than others or that poorly manufactured charcoal is more toxic than well manufactured charcoal etc it could lead to appropriate changes in fuel regulation at governmental level. i.e. Addressing both stoves as well as fuel are important – as recently stated by GACC.

8.3.3 Implications for future studies on the causes of impaired lung function

Chapter 4 suggests that the prevalence of airway obstruction in Malawi may be high and a key implication of this work is to highlight this fact to health care providers as well as to study the burden of disease in more detail.

In order to be able put preventative measures in place for COPD, given its irreversible nature it is important not only to ascertain the prevalence of disease, but also to try and find the most relevant causes of COPD. The study in Chapter 4 suggests two factors (indoor air pollution and poverty) that require further exploration by a well conducted burden of disease study.

8.3.4 Implications for future studies on the mechanism of lung damage from biomass fuel smoke

In Chapters 2 and 6 a model for examining the effects of BMF on alveolar macrophage function has been described. This model will enable basic research questions of macrophage function to be examined in the context of smoke exposure at doses that are similar to those experienced by individuals *in vivo*. Samples of BMF smoke from many global locations can be compared for their toxicity using this model, as suggested in 8.3.2.

The development of Image SXM (with Dr Stephen Barrett) provides a freely available tool that can potentially be used to process large samples of, for example, induced sputum for PM load in order to monitor exposure levels in the context of a cookstove intervention. Or alternatively, in mechanism studies BAL derived HAM PM load can be associated with different macrophage function assays.

These are important implication of this thesis as it may improve our ability to answer questions of both biological mechanism as well as the success of practical interventions to reduce exposure.

8.4 Limitations of Thesis

There are a number of limitations to this thesis, most of which have been outlined in each separate chapter.

In Chapter 3, different numbers, of different types of air sampling devices lead to not all homes being sampled in the same way. Whilst the author does not believe that this lead to bias in the results it is clearly sub-optimal. Ideally the same devices should have been used in all homes sampled.

As mentioned in Chapter 4, a random sampling strategy would have been the ideal. The sampling methodology used in order to describe the burden of COPD accurately is extremely important; the BOLD study makes this clear and the data in Chapter 4 is weakened by the methodology used.[69;397] However, it was not possible to carry out such robust epidemiological sampling in the context of this study and the main strength of this work is in highlighting which potential variables should be looked at further.

The key limitation of Chapter 6 was the particulate used in the *in vitro* work i.e. fine carbon black (FCB) and the assumptions made about visual appearance and dose. However, FCB was used in order to establish the model and more work is on going to explore the differences observed with other BMF smoke derived PM. In terms of dose used and the appearances at microscopy there are arguments for and against and as stated in Chapter 6.5 any *in vitro* model of an *in vivo* situation has a number of limitations and the results need to be interpreted in this light.

The weakness in Chapter 7 is primarily the distribution of PM load in HAM as it is significantly skewed to low PM loads. As explained in Chapter 7.5.1 the study was also weakened by low number of participants in the study. These experiments need to be repeated in order to ascertain whether the results obtained are real.

8.5 Future work

During the field and laboratory work of thesis the author has been involved in trying to establish a network of researchers in order to develop plans for an intervention trial based in Malawi. The BREATHE-Africa consortium was formed to bring together individuals, groups and organisations from across the world together with a common interest in delivering health benefits to people in Africa through improved energy provision.

Several grant applications have already been submitted in order to be able to advance areas of work in this field and future work has been divided into four key themes:

1. Exposure and biomarkers
2. Health effects
3. Mechanisms
4. Interventions

8.5.1 Exposures and Biomarkers

Work carried out in all of the studies of this thesis highlights that more work is required in order to define exposure in the most relevant manner (TWA or peak exposure or duration or position monitoring relative to smoke source etc). Grigg et al have demonstrated, using induced sputum samples, that PM load in HAM is associated with impaired lung function in UK children and in Chapter 5 PM load in HAM is associated with smoke exposure in adults.[100;381] Improvement of the PM load method (from Chapter 2) has made it quicker and more objective. Therefore, expanded implementation to compare sputum and bronchoalveolar lavage, to associate this marker with air quality measurements and physiological parameters (including HIV load) and to utilise the PM load as a measure of exposure in other experiments are necessary.

8.5.2 Health effects

Experiments are needed in order to evaluate the effects on family health (including birth outcome, pneumonia, lung function measurements in adults and children, and tuberculosis) of IAP from BMF smoke. Cross sectional studies have confirmed an association of ALRI incidence with IAP but the longitudinal studies required to determine the strength of association between IAP and low birth weight, perinatal mortality and tuberculosis have not been carried out in Africa.

8.5.3 Mechanisms

Dose-Response Curve

A recent conference organised by the US National Institutes of Health held in Washington, DC, in May 2011 outlined the importance of establishing valid dose-response curves to exposure.[398] It was highlighted that lessons should be learnt from tobacco smoke exposure in terms of dose response. Pope et al (2009) have demonstrated that even at relatively low levels of fine particulate exposure from either air pollution or second-hand cigarette smoke is sufficient to induce an adverse biological response.[399] The exposure-response relationship between cardiovascular disease mortality and fine particulate matter is relatively steep at low levels of exposure and flattens out at higher exposures. It is important therefore to identify similarities and differences between tobacco toxins and the various components of indoor air pollution.

Biological Plausibility

Another priority for research is to strengthen the basis of biological plausibility of interactions of indoor air pollution with cancers and infectious diseases, particularly diseases affecting the lung. As stated by Sumi Mehta in Washington in May 2011, even a small increase in susceptibility to or the severity of tuberculosis would have a great effect

in terms of public health because of the huge burden of that disease, especially in the context of HIV related TB in Africa. [398]

HIV Disease

In Africa where HIV infection is common, BMF is the main household energy source. The most important effect of HIV infection in Africa is to cause increased bacterial infections, pneumonia and TB. HIV infection is associated with mild airway obstruction and loss of gas transfer, with severe impairment occurring in the presence of *Pneumocystis jiroveci* infection.[400] HIV is also associated with the accelerated development of COPD and it is likely, although not proven, that HIV infection is a significant contributor to airway disease in much of the adult population of Africa.[367;401] Both BMF use and HIV are associated with an increase in the incidence of pneumonia [55;116] and both particulate matter exposure and HIV result in increased pulmonary inflammation.[290;402;403] It is possible therefore that by causing pulmonary inflammation the two major risk factors for pneumonia in African adults (HIV and BMF smoke) may actually demonstrate previously unrecognised synergy. However, the influence of BMF smoke on HIV-infected individuals has not yet been clarified.

An important area of future work therefore is to explore whether BMF use has an adverse effect on pulmonary defence in HIV-infected adults by causing altered macrophage function, increased lung inflammation and increased HIV replication in alveolar macrophages. Some potential methods to be able to answer this question and others have been described in this thesis.

8.5.4 Interventions

There are now several non-governmental organizations introducing improved cookstoves in Africa, India and Guatemala. However, very little of this work is being done as part of a randomized control trial. The

Partnership for Clean Indoor Air (PCIA) has documented both success and failure in community uptake of various different interventions offered. Therefore planning for interventions must involve communication of proven, long-term multiple parameter gains for the community before changes in cooking behaviour will occur. This could include innovative side-benefits e.g. mobile phone charging on cook stoves using thermoelectric devices.

Potential interventions in Malawi need evaluation using measures of community uptake and acceptance as well as validated biomarkers. Interventions need to be chosen that have a high likelihood of both health and environmental impact as well as community uptake and they then need to be implemented in long-term longitudinal studies.

8.6 Concluding remarks

Collectively, the data from this thesis suggest that exposure to biomass fuel represents a significant burden to respiratory health in Malawi. Levels of smoke exposure are high; it is highly likely that this level of exposure detrimentally affects lung function as well as making individuals more at risk of developing pneumonia. The findings presented in this thesis may have important implications for future intervention trials and developing a greater understanding of the mechanism by which biomass fuel exposure increases susceptibility to pneumonia.

The paucity of intervention trials on such a significant global risk factor for poor health is shocking. The formation of the GACC and the health working group established within this organisation will hopefully lead to a change in the attitude of the global health community to one where clean cookstoves are seen as a worldwide health priority and are vigorously supported, with research being actively funded. Overall the current situation is increasingly encouraging.

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Appendix 1: Ethics letters (COMREC)

UNIVERSITY OF MALAŴI

Principal

Prof. R.L. Broadhead, MBBS, FRCP, FRCPCH, DCH

Our Ref.: MC/COMREC/16

College of Medicine
Private Bag 360
Chichiri
Blantyre 3
Malawi
Telephone: 01 671 911/01 674 377
Fax: 01 674 700/01 674 740
Telex: 43744

11th September, 2006

Dr Steve Gordon
Wellcome Trust Research Laboratories
P O Box 30096
Blantyre 3

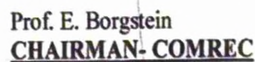
Dear Dr Gordon,

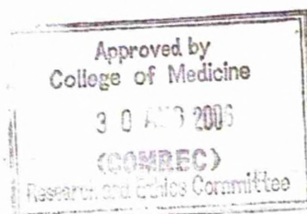
P.05/06/460 – The effect of biomass fuel smoke on pulmonary defence in a population at risk from HIV-related pneumonia

I write to inform you that COMREC reviewed and approved your proposal which you submitted at its meeting of 30th August, 2006.

As you proceed with the implementation of your study I would like you to take note that all requirements by the college are followed as indicated on the attached page.

Sincerely,





Prof. E. Borgstein
CHAIRMAN- COMREC

EB/tck

Appendix 1: Ethics letters (LSTM)

LIVERPOOL SCHOOL OF TROPICAL MEDICINE (Affiliated to the University of Liverpool)

Pembroke Place
Liverpool L3 5QA
Telephone: 0151·705 3100
Fax: 0151·705 3370
http://www.liv.ac.uk/lstm

4 October 2006

Dr Stephen Gordon
LSTM

Dear Dr Gordon

Re: Research protocol The effect of biomass fuel smoke on pulmonary defence in a population at risk from HIV-related pneumonia (06.45)

Thank you for your letter of 2nd October 2006 responding to the points raised by the Research Ethics Committee. The protocol now has formal ethical approval from the Chair of LSTM Research Ethics Committee.

The approval is for a fixed period of three years or for the duration of the grant, renewable annually thereafter. The committee may suspend or withdraw ethical approval at any time if appropriate.

Approval is conditional upon:

- Submission of ethical approval from other ethics committees
- Notification of all amendments to the protocols for approval before implementation.
- Notification when the project actually starts
- Provision of an annual update to the committee. Failure to do so could result in suspension of the study without further notice.
- Reporting of all severe unexpected adverse events to the Committee
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements will result in withdrawal of approval. The Committee would also like to receive copies of the final report once the study is completed.

Yours sincerely

Dr D Lalloo
Chair, Research Ethics Committee

Liverpool School of Tropical Medicine
An international centre of excellence in the field of tropical medicine and tropical health systems
A Company Limited by Guarantee
Registered Number 83405, England and Wales

Appendix 1: Ethics letters (Sefton LREC)

Sefton Local Research Ethics Committee
1 Arthouse Square
61 - 69 Seel Street
Liverpool
L1 4AZ

Telephone: 0151 296 7539
Facsimile: 0151 296 7536

12 February 2007

Dr Stephen B Gordon
Clinical Senior Lecturer in Tropical Respiratory Medicine
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Dear Dr Gordon

Full title of study: **The effect of biomass fuel smoke on pulmonary defence in a population at risk from HIV-related pneumonia**
REC reference number: **06/Q1501/192**

The Research Ethics Committee reviewed the above application at the meeting held on 31 January 2007.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, **with the proviso that the title on the consent form be changed to be the same as on the patient information sheet**.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application	5.2	13 December 2007
Investigator CV		22 November 2007
Protocol		22 November 2007
Letter from Sponsor		18 December 2007

Appendix 1: Ethics letters (Sefton LREC)

Advertisement		22 November 2007
Participant Information Sheet	2	22 November 2007
Participant Consent Form	2	22 November 2007
C.V. for Supervisor		13 December 2007
Letter From Funder		
Statement of Indemnity Arrangments		18 December 2007

Research governance approval

You should arrange for the R&D Department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q1501/192	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Peter Owen

Dr Peter Owen
Chair

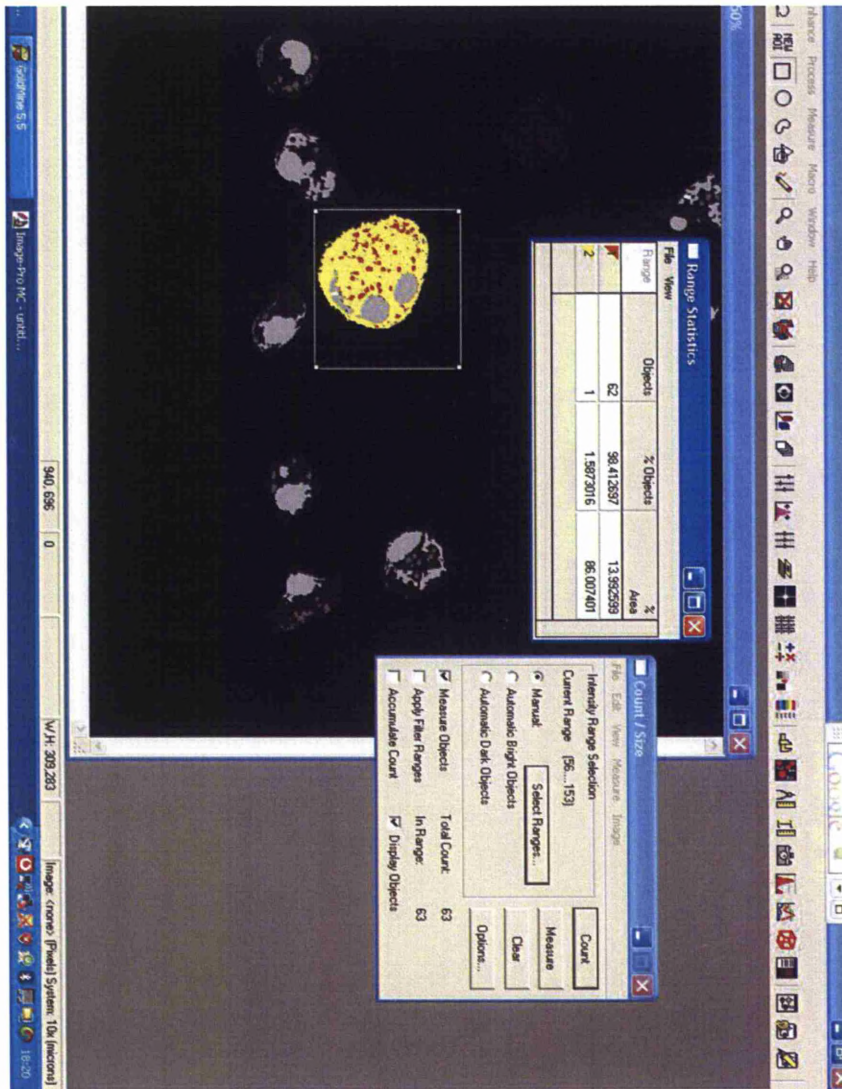
Email: sue.culshaw@liverpoolpct.nhs.uk

Enclosures: *Standard approval conditions*

Copy to: *Ms Sian Roberts*
Liverpool School of Tropical Medicine
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA
R&D Department for NHS care organisation at lead site

Appendix 2: Image Pro Plus analysis software.

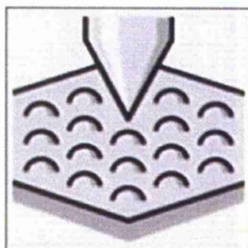
PM was correctly identified by the program however the steps to achieve this were not a significant advantage over the methods outlined by Kulkarni et al. Also the program was not able to batch process cells from one individual reducing the power of the assay. Other programs (Image J, Digital Pixel Imaging and Andor IQ) were assessed with limited success.



Appendix 3: Image SXM Software.

Particulate Matter Analysis using Image SXM

An outline of the analysis routines and algorithms



Introduction

Image SXM is a version of the public domain image analysis software NIH Image that has been extended to handle the loading, display and analysis of scanning microscope images (hence the acronym SXM). In addition, it contains routines that have been written specifically to process and analyse images of cells taken from light microscopes and to quantify the amount of particulate matter in macrophage cells.

It is assumed that each image comprises three objects of interest (cytoplasm, nuclei and particulate matter) and background. For each object, an algorithm is required that produces an image in which the object stands out from the others. Ideally, the algorithm must be able to detect all objects of the same type, but discriminate against any object of a different type, despite variations in image brightness, contrast or colour saturation, and variations in the colour of objects from one image to another. The algorithms that achieve this are described in the appendix.

In the following pages the process by which the particulate matter is quantified is outlined and the effects of user input are explained. These notes are not intended to be comprehensive documentation, but should be enough to give the user an idea of how the processing is carried out and allow the user to use reasonable judgement in selecting input options.

To find more information about Image SXM, see the Image SXM home page:

http://www.ImageSXM.org.uk

If you have any problems using Image SXM, please let me know:

S.D.Barrett@liv.ac.uk

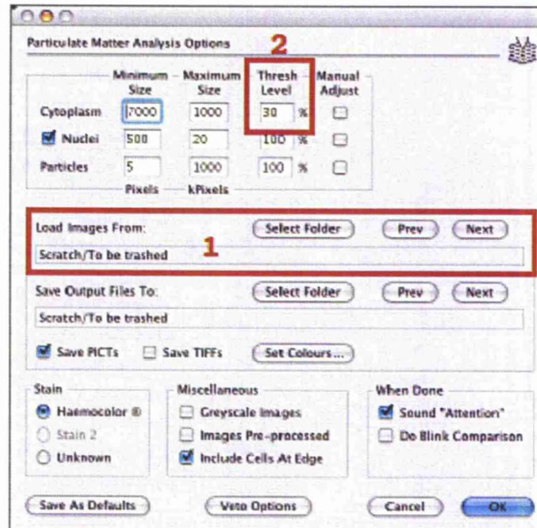
- 1 -

Appendix 3: Image SXM Software:

The Analysis Process

The raw (TIFF) images are loaded from a folder selected by the user [1], or a set of folders within the folder selected by the user. The images are processed folder by folder. Firstly, images within the selected folder (the top folder) are processed. Secondly, images within any sub-folders are processed.

A maximum of 256 images in each of 32 folders can be processed at a time.



For each image, the following process is carried out...

Cells
- create an image that makes cells look dark (*cf* background)
- check histogram of pixel values and note lowest and highest values
- threshold at value specified in dialog box [2]
  - [threshold at low values (~ 10%) to include most of cytoplasm]
  - [threshold at mid values (~ 50%) to include only denser region]
- optionally allow user to adjust threshold manually

Nuclei
- create an image that makes nuclei look dark (*cf* particulate matter)
- threshold at fixed level (adjusted by user-specified value in dialog box)
- optionally allow user to adjust threshold manually

Particles
- create an image that makes particulate matter look dark (*cf* nuclei)
- threshold at fixed level (adjusted by user-specified value in dialog box)
- optionally allow user to adjust threshold manually
- invert the image and threshold again to find interior 'holes' in pm

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Appendix 3: Image SXM Software:

Calculations

- identify pixels inside cell outline = C
- identify pixels inside nuclei outline = N
- identify pixels inside particle outline = oP
- identify pixels inside pm interior holes = iP

- P = oP − iP (subtract interior holes from pm)

- reject pixels inside N but outside C
- reject pixels inside P but outside C

- N = N + iP (count interior holes as nuclei)
- Cy = C − N (cytoplasm = non-nuclear cell)

- count pixels in P
- count pixels in N
- count pixels in Cy

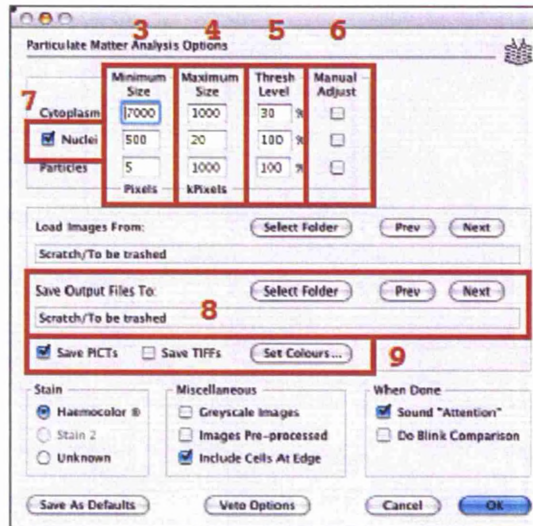
- particulate matter = P/Cy

Appendix 3: Image SXM Software:

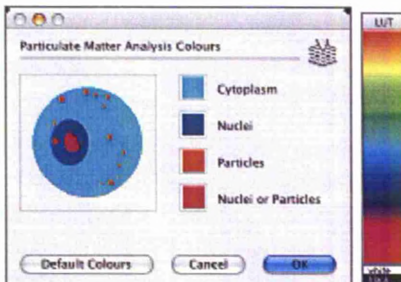
Selecting Options

Lower [3] and upper [4] limits to the sizes of objects that are included in the analysis can be set. The sizes are specified in pixels for the minimum size and thousands of pixels for the maximum size.

Thresholds for each object are set here [5]. The value for cytoplasm affects the measured areas of cells that have spread out (see p2). The values for nuclei and particles are percentages of the values calculated by the program, and should only be changed from 100% if the values calculated by the program are consistently wrong.



Thresholds calculated by the program can be manually adjusted by the user on an image-by-image basis [6]. The threshold level is displayed in the Info window and the values before and after user adjustment are recorded in the log file. If the user is unhappy with an image even after making these manual adjustments, press the 'V' key to veto that image from the calculation of the mean value of the relative area of particulate matter. Nuclei can be ignored completely in the analysis by unchecking this item [7].



Output files (see p6) are saved to a folder selected by the user [8]. Optionally, colour 'maps' showing which pixels were identified as cytoplasm, nuclei or particulate matter can be saved as PICT or TIFF images [9]. The colours used can be specified in a separate dialog box [9] (left).

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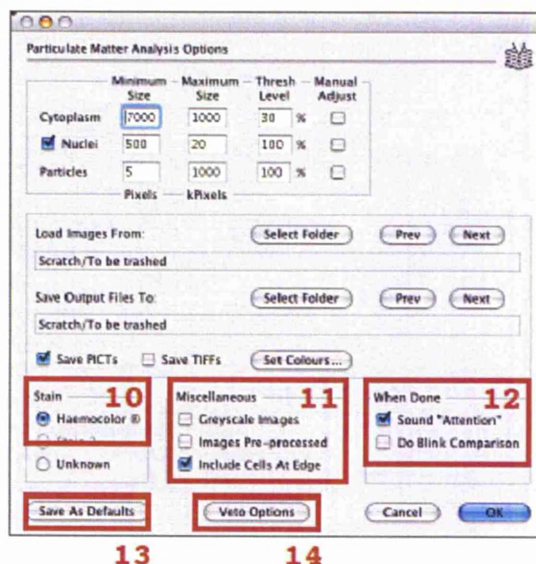
Appendix 3: Image SXM Software:

If the cells have been stained using Haemocolor® then select this item [10]. These analysis routines may not work with any other stain unless the code is modified — email Steve Barrett <S.D.Barrett@liv.ac.uk> if you use a different stain.



Select the appropriate items [11] if the images are greyscale (rather than 24-bit or 36-bit colour TIFFs) or have been pre-processed to remove the background and cell nuclei. Cells touching the edge of an image can be excluded from the analysis if you think that this will effect the calculation of the relative area of particulate matter.

The blink comparator [12] can be selected to launch when the analysis is complete so that you can carry out a visual check of the results.

The Save As Defaults button [13] saves the Preferences file with the current settings of the dialog box so that these will be the default values next time Image SXM is launched.

Some images may be vetoed from the calculation of the average value of P/Cy [14]. See p6 for details.

Appendix 3: Image SXM Software:

Output files

Three text files are generated for each analysis run

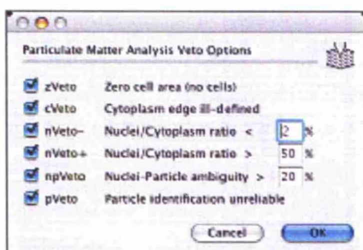
Log-yymmddhhmm.txt
PM-A-yymmddhhmm.txt
PM-G-yymmddhhmm.txt

where yymmdd are the date and hhmm the time at which the analysis ended. The log file contains full information of the analysis parameters and the areas (in kPixels) of cytoplasm, nuclei and particulate matter in each image. The PM-A and PM-G files contains just the (arithmetic and geometric) mean P/Cy values and their standard deviations for each folder of images analysed. If the analysis fails to complete due to an error, the file 'Log-Crash.txt' records the results for all folders analysed up to the time at which the error occured.

Some images may be vetoed from the calculation of the average value of P/Cy. These are indicated in the log file with the following flags:

Flag	Reason for veto
zVeto	zero cytoplasm area (no cells)
cVeto	cytoplasm edge ill-defined
nVeto-	nuclei too small as fraction of cytoplasm
nVeto+	nuclei too large as fraction of cytoplasm
npVeto	discrimination between nuclei and PM ambiguous
pVeto	identification of particulate matter ambiguous
uVeto	veto by user when manually inspecting images

Each veto can be enabled/disabled individually and the criteria fine-tuned in the Veto Options dialog box.



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Appendix 3: Image SXM Software:

Using the Analysis Routines

I would recommend that you start by analysing just a few typical images to establish whether or not the default settings are appropriate. Either copy a few images into a test folder, or analyse one image at a time by pressing the shift key before selecting the Particulate Matter Analysis item from the Cells menu (the menu item changes to 'PM Analysis for Single Image'). You can then analyse the rest of your images in one of two ways:

i) Run the analysis on one or more folders containing your images with all the settings at the default values. On automatic, the analysis will take a few seconds per image. The time remaining to complete the analysis is indicated in the Info window in the bottom left corner of the screen. Go and have a cup of tea.

When the analysis is complete, use the Blink Comparator to check each image with the output 'map' showing where pixels were identified as cytoplasm, nuclei and particulate matter. The Blink Comparator toggles between the original image and its corresponding map so that you can judge whether or not the identification was satisfactory.

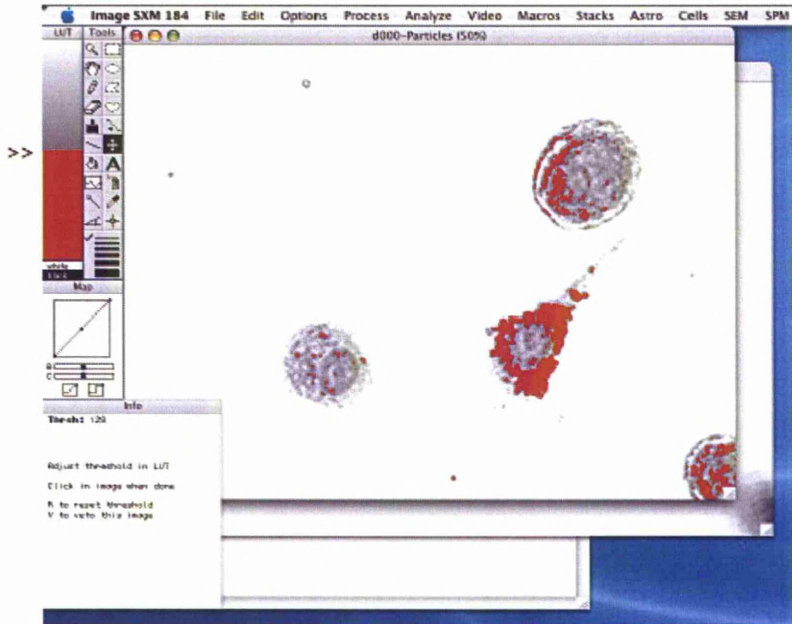
Note whether or not there is any consistent under/overestimate of the areas of any of the objects. If there is, adjust the threshold levels (see p4) and run again. Lowering (raising) a threshold level will count more (less) pixels and hence increase (decrease) the measured area of an object.

If there is no consistent under/overestimate of the areas but some objects in some images have been identified incorrectly, either:
(a) remove the files from the folder and re-run the analysis, or
(b) re-run the analysis with the Manual Adjust option selected and either adjust the threshold until you are happy with the area measured or veto the image.

ii) Run the analysis with the Manual Adjust options selected. For each image, the analysis will pause and allow you to adjust the thresholds that determine the areas of cytoplasm, nuclei and particulate matter. Adjust the threshold until you are happy with the area measured or veto the image. Both the automatic and the manually adjusted threshold values are recorded in the log file.

Appendix 3: Image SXM Software:

Manual adjustment of a threshold is carried out by dragging the red/grey boundary line the the look-up table (LUT) window whilst looking at the effect this has on the pixels in the image.



When you are happy with the threshold, click in the image. An image can be vetoed by pressing 'V' at any time during manual threshold adjustment.

Dr Steve Barrett
S.D.Barrett@liv.ac.uk **v2 July 2007**

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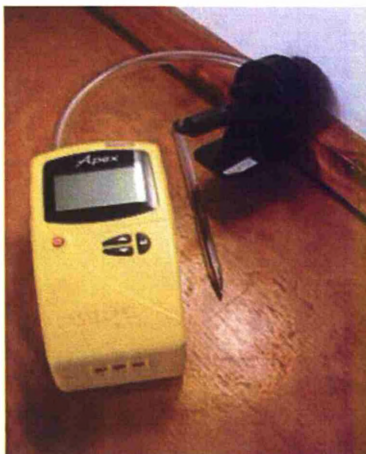
Appendix 4: Air Sampling Equipment

Carbon Monoxide monitor – A HOBO



Gravimetric analysis – An Apex pump and a cyclone sampling head for respirable dust

Photometric analysis – A University of California, Berkeley sampler and a TSI Sidepak.

Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study	**Bronchoscopy, Spirometry**
Individ No. BM _ _ _ / _	**& Air sampling CRF**

STUDY DETAILS

Participant Details:

Detach this form and do not enter details to database

1	Participant Name		
2.	Contact details (e.g. Telephone number)		
3.	Directions to Property – brief outline (Draw map below if necessary)		

Page 1 of 12

Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study	**Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __	**& Air sampling CRF**

STUDY DETAILS

INTERVIEW

4.	Interview Date	dd____/mmm____/yyyy____	
5.	Interviewer Name		
6.	Consent Obtained?	☐ Yes ☐ No	
7.	Consent obtained by:	☐ Marie K ☐ Duncan F ☐ Rose M ☐ Francis K ☐ Other ________	
8.	What investigations have been done? *Tick and date each one has been done*	HIV test ☐ ________ CXR ☐ ________ Bronchoscopy ☐ ________ Spirometry ☐ ________ Air sampling ☐ ________	

HOUSEHOLD / PARTICIPANT DETAILS

9.	Individual Number	BM __ __ __	
10.	Home Location		
11.	Home Description	☐ Rural ☐ Urban	
12.	Gender	☐ Male ☐ Female	
13.	Village		
14.	Year of Birth	yyyy____	

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __ **& Air sampling CRF**

Type of Biomass Fuel Use and Smoking

15.	What is the main type of cooking fuel	1.☐ Wood open fire 2.☐ mbaula (charcoal stove) 3.☐ Electricity 4.☐ Other ______	
16.	Where do you mostly cook?	1.☐ Inside 2.☐ Outside 3.☐ Other ______	
17.	Is there a difference in where you cook depending on the season etc?	☐ Yes ☐ No	
18.	Do you heat your house (separate to when you are cooking)?	☐ Yes ☐ No (go to Q 20)	
19.	If yes, how do you heat it?	1.☐ Wood open fire 2.☐ mbaula (charcoal stove) 3.☐ Electricity 4.☐ Other ______	
20.	Do you light your house?	☐ Yes ☐ No (go to 22)	
21.	If yes; what is the main way you light it?	1.☐ simple paraffin/kerosene lamp, candle or flaming torch 2.☐paraffin lamp with glass (hurricane lamp) 3.☐ Electricity 4.☐ Other ______	
22.	Are there other sources of smoke in your home?	☐ Yes ☐ No	
23.	If yes; What other sources of smoke are there? (Specify)		

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __ **& Air sampling CRF**

Type of Biomass Fuel Use and Smoking (continued)

24.	Is the smoke in your home less or more or about the same as when you were growing up?	☐ less ☐ more ☐same	
25.	Do you think that changes in smoke levels affects your health?	☐ Yes ☐ No – Go to **27**	
26.	If yes – could you tell me in what ways?		
27.	What is your job title? And what is the main thing you do or make at work?		
28.	Have you ever smoked?	☐ Yes, current smoker – Go to **29 & 30** ☐ Yes but stopped – Go to **31** ☐ No – Go to **32**	
29.	If yes, when did you start - how many **years** ago?	________ (now go to 30)	
30.	How many do you smoke a day?	________ (now go to 32)	
31.	If stopped, how long ago (years) did you stop smoking?	________ (now go to 32)	
32.	Does anyone smoke whilst in your house?	☐ Yes ☐ No	

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study	**Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __	**& Air sampling CRF**

	DETAILS OF HOME		
33.	Number of rooms in house (not necessarily partitioned by a door)	______	
34.	Residents in Household	______	
35.	How many adults sleep in the property?	______	
36.	How many children (<16 yrs old) sleep in the property?	______	
37.	Type of Roof Material	☐ Grass ☐ Corrugated material ☐ Tiles	
38.	Type of Windows	☐ No windows ☐ Space Only ☐ Glass	
39.	Does the household own animal for food?	☐ Yes ☐ No	
40.	If yes; what type(s) of animals?	☐ Chickens ☐ Cows ☐ Goats ☐ Other ______	
41.	Do animals sleep in the same room as people?	☐ Yes ☐ No	
42.	Does anyone in the house hold own any of the following?	☐ Car ☐ Motorbike ☐ Bicycle ☐ Radio ☐ Fridge ☐ Television ☐ Phone ☐ Computer	
43.	Type of Water supply	☐ piped to property ☐ piped to communal area ☐ well, bore hole or drawn from river etc	

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study	**Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __	**& Air sampling CRF**

	Respiratory Symptoms Questionnaire		
44.	Do you cough several times most days?	☐ Yes ☐ No	
45.	Do you bring up mucus and phlegm most days?	☐ Yes ☐ No	
46.	Do you get out of breath more easily than others your age?	☐ Yes ☐ No	
47.	Have you ever had asthma?	☐ Yes ☐ No	
48.	Have you had a respiratory infection in the last 12 months?	☐ Yes ☐ No	
49.	If yes, how many times?	________	
50.	Have you seen a doctor because of a chest problem in the last 12 months?	☐ Yes ☐ No	
51.	If yes, how many times?	________	
52.	Have you had to be admitted to hospital due to chest illness?	☐ Yes ☐ No	
53.	If yes, how many times?	________	
54.	Have you ever been treated for TB?	☐ Yes ☐ No	

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __ **& Air sampling CRF**

NB: Keep a print out of spirometry results in participants file

Spirometry checklist:

SVC :	**Maximal inspiration**	**Y**	**N**
	Good start	**Y**	**N**
	3 attempt	**Y**	**N**
	≤ 0.15 L difference between 2 highest	**Y**	**N**
FVC :	**Volume time:**		
	Good start (E V< 5% FVC/0.15 L)	**Y**	**N**
	Smooth line curve	**Y**	**N**
	≥6 sec/plateau ≥1sec/cannot/shouldn't blow	**Y**	**N**
	<6 sec but FEV1 >80% predicted	**Y**	**N**
	2 out of 3 highest differ ≤ 0.150 L	**Y**	**N**
	Trace lines are the same pattern	**Y**	**N**
	Flow Volume:		
	Sharp increase	**Y**	**N**
	Peak	**Y**	**N**
	Steady decrease to reach base	**Y**	**N**
	3 the same pattern	**Y**	**N**

SPIRONETRY RESULTS:
Pre bronchodilator

60	**Date spirometry performed**		
61	Height (in Centimeters)	________	
62	Weight (in Kilograms)	________	
63	**Predicted Values:**	FVC/FEV1/FEV6 ___/___/___	

64	SVC predicted / SVC test	/	
65	1st Attempt	FVC/FEV1/FEV6 ___/___/___	
66	2nd Attempt	FVC/FEV1/FEV6 ___/___/___	
67	3rd Attempt	FVC/FEV1/FEV6 ___/___/___	
68	FEV1/FVC ratio		
69	FEV1/FEV6 ratio		

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM __ __ __ / __ **& Air sampling CRF**

SPIROMETRY RESULTS:
Post Salbutomol

70	SVC		
71	1st Attempt	FVC/FEV1/FEV6 ___ /___/____	
72	2nd Attempt	FVC/FEV1/FEV6 ___ /___/____	
73	3rd Attempt	FVC/FEV1/FEV6 ___ /___/____	
74	FEV1/FVC ratio		
75	FEV1/FEV6 ratio		

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM _ _ _ / _ **& Air sampling CRF**

AIR SAMPLING DATA

HOME I.D = ______

76.	**1st visit date and start time**		
77.	**Home location**		
78.	**Fuel(s) used**		
79.	**Location of cooking:**	1.☐ Inside main living area ___ 2.☐ Elsewhere in main house ___ 3.☐ Inside separate building ___ 4.☐ Outside ___	
80.	**Location of samplers**		

Home I.D:

Sampling data

Test	Apex	SP	UCB	HOBO
Carried out				
Instrument #				
Sampling head				
Filter number				
'Zero time'				
Start time				
Flow rate (start)				
Run time				
Flow rate (end)				
End time				

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Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Return visit date and time	
Cooking and fuel use details over sampling period	
Notes	

Appendix 5: Case Report Form used in Chapters 3, 4 and 7

Biomass Study	**Bronchoscopy, Spirometry**
Individ No. BM _ _ _ / _	**& Air sampling CRF**

History and Physical Examination findings

90.	Where did the hear about bronchoscopy Have they had one before?		
91.	Respiratory Symptoms?		
92.	PMHx		
93.	Social and family history		
94.	Drug History		
95.	Review of Systems		
96.	BP		
97.	Resp Rate / Heart Rate / Temp		
98.	Any features of: *Clubbing, Jaundice, Anaemia,* *Oedema* *KS lesions* *Other*		
99.	Respiratory Examination		

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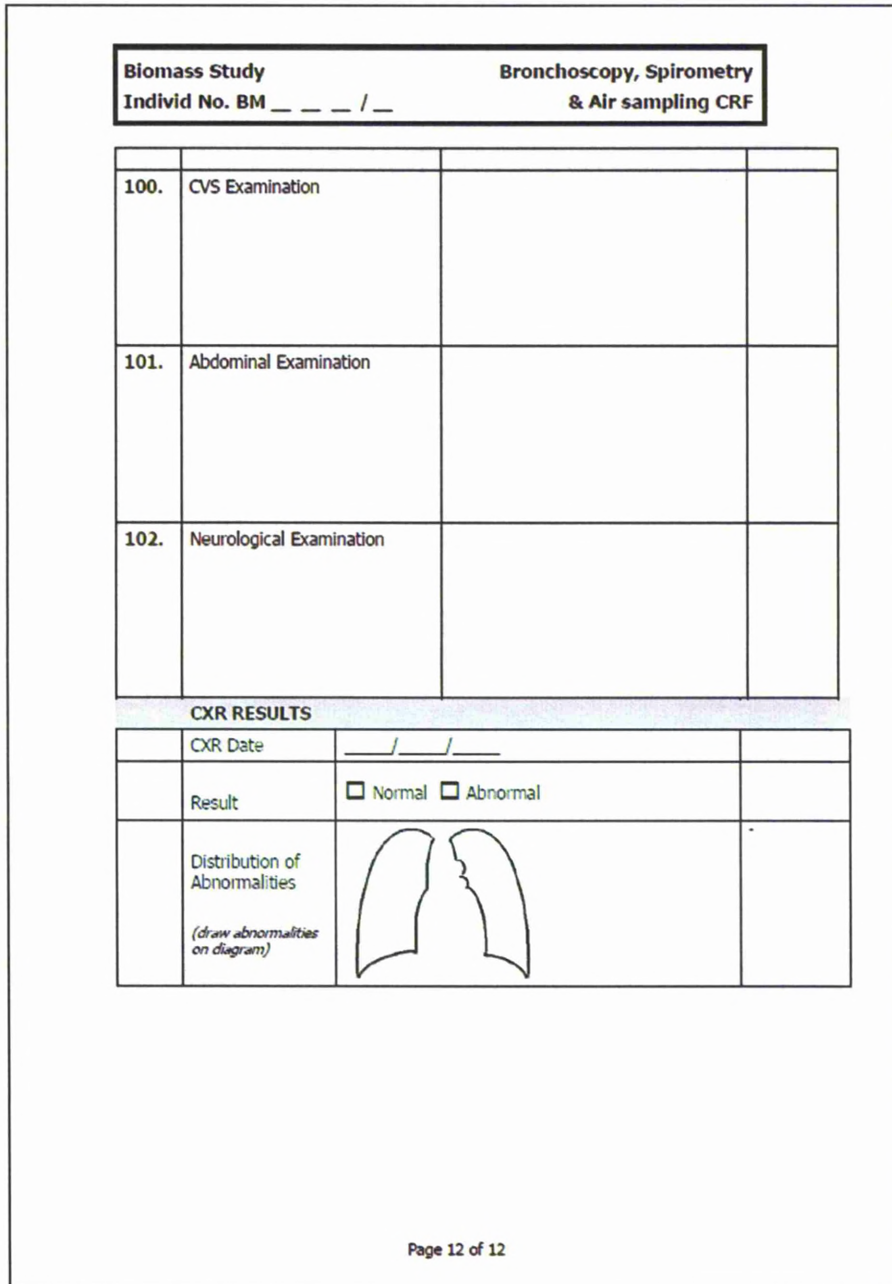
Appendix 5: Case Report Form used in Chapters 3, 4 and 7



Biomass Study **Bronchoscopy, Spirometry**
Individ No. BM _ _ _ / _ **& Air sampling CRF**

100.	CVS Examination		
101.	Abdominal Examination		
102.	Neurological Examination		

CXR RESULTS

	CXR Date	____/____/____	
	Result	☐ Normal ☐ Abnormal	
	Distribution of Abnormalities *(draw abnormalities on diagram)*		

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Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Total Inhalable Dust

Variables Entered/Removed

Model	Variables Entered	Variables Removed	Method
1	wood/char		Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).

a Dependent Variable: GMTWA

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.635(a)	.403	.337	406.529

a Predictors: (Constant), wood/char

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1005412.803	1	1005412.803	6.084	0.036(a)
	Residual	1487394.833	9	165266.093		
	Total	2492807.636	10			

a Predictors: (Constant), wood/char
b Dependent Variable: GMTWA

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta	B	Std. Error
1	(Constant)	204.000	181.805		1.122	.291
	wood/char	607.167	246.166	.635	2.466	.036

a Dependent Variable: GMTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Total Inhalable Dust (continued)

Excluded Variables(b)

Model		Beta In	t	Sig.	Partial Correlation	Collinearity Statistics
		Tolerance	Tolerance	Tolerance	Tolerance	Tolerance
1	mainlite	-.380(a)	-1.406	.197	-.445	.817
	heathome	.062(a)	.227	.826	.080	.995
	smoked	-.016(a)	-.056	.956	-.020	.917
	smokegrp	-.016(a)	-.056	.956	-.020	.917
	windows	-.300(a)	-.622	.551	-.215	.306

a Predictors in the Model: (Constant), wood/char

b Dependent Variable: GMTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Respirable dust

Variables Entered/Removed(a)

Model	Variables Entered	Variables Removed	Method
1	lighting		Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).

a Dependent Variable: GMTWA

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.403(a)	0.162	0.126	165.623

a Predictors: (Constant), lighting

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	122191.359	1	122191.359	4.455	0.046(a)
	Residual	630911.237	23	27430.923		
	Total	753102.596	24			

a Predictors: (Constant), lighting

b Dependent Variable: GMTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Respirable dust (continued)

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval for B	
		B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
1	(Constant)	118.276	55.208		2.142	.043	4.070	232.481
	lighting	145.650	69.010	.403	2.111	.046	2.892	288.407

a Dependent Variable: GMTWA

Excluded Variables(b)

Model		Beta In	t	Sig.	Partial Correlation	Collinearity Statistics
		Tolerance	Tolerance	Tolerance	Tolerance	Tolerance
1	wood/char	.208(a)	1.039	.310	.216	.907
	heathome	-.019(a)	-.095	.925	-.020	.997
	smoked	-.037(a)	-.175	.863	-.037	.848
	smokegrp	-.108(a)	-.521	.607	-.110	.871
	windows	-.210(a)	-1.019	.319	-.212	.857

a Predictors in the Model: (Constant), lighting

b Dependent Variable: GMTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Carbon Monoxide

Variables Entered/Removed(a)

Model	Variables Entered	Variables Removed	Method
1	wood/char		Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).

a Dependent Variable: COTWA

Model Summary

Mode l	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.539(a)	0.290	0.276	4.72370

a Predictors: (Constant), wood/char

ANOVA(b)

Mode l		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	465.365	1	465.365	20.856	.000(a)
	Residual	1137.978	51	22.313		
	Total	1603.344	52			

a Predictors: (Constant), wood/char
b Dependent Variable: COTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 3 –Mutivariate Analysis of air sampling data: Carbon Monoxide (continued)

Coefficients(a)

Model	Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
1 (Constant)	7.971	1.056		7.547	.000	5.850	10.092
wood/char	-6.113	1.339	-.539	-4.567	.000	-8.800	-3.426

a Dependent Variable: COTWA

Excluded Variables(b)

Model		Beta In	t	Sig.	Partial Correlation	Collinearity Statistics
		Tolerance	Tolerance	Tolerance	Tolerance	Tolerance
1	heathome	.052(a)	.434	.666	.061	.999
	smoked	-.162(a)	-1.367	.178	-.190	.974
	smokegrp	-.170(a)	-1.422	.161	-.197	.957
	windows	-.106(a)	-.734	.466	-.103	.669
	lighting	-.039(a)	-.309	.759	-.044	.892

a Predictors in the Model: (Constant), wood/char
b Dependent Variable: COTWA

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 4 – Enter Mutivariate Analysis (presented in Table 4.3) of Spirometry data (FEV_1)

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	0.716(a)	0.512	.495	.49142

a Predictors: (Constant), smokegrp, tb, placewt, age, aslproom, height, SLI, gender, wood/char, homeloc

ANOVA(b)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	71.755	10	7.175	29.713	.000(a)
	Residual	68.342	283	.241		
	Total	140.097	293			

a Predictors: (Constant), smokegrp, tb, placewt, age, aslproom, height, SLI, gender, wood/char, homeloc
b Dependent Variable: preFEV1

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta	B	Std. Error
1	(Constant)	-3.183	.760		-4.186	.000
	age	-.013	.003	-.205	-4.556	.000
	gender	.340	.076	.241	4.481	.000
	height	.038	.005	.454	8.394	.000
	homeloc	.001	.094	.001	.015	.988
	wood/char	-.119	.087	-.086	-1.364	.174
	placewt	-.055	.069	-.038	-.804	.422
	SLI	.079	.047	.090	1.686	.093
	aslproom	-.079	.066	-.054	-1.186	.237
	tb	-.291	.120	-.104	-2.422	.016
	smokegrp	-.123	.076	-.075	-1.610	.109

a Dependent Variable: preFEV1

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 4 – Stepwise Mutivariate Analysis:

Variables Entered/Removed(a)

Model	Variables Entered	Variables Removed	Method
1	height	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
2	age	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
3	gender	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
4	SLI	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
5	tb	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).
6	wood/char	.	Stepwise (Criteria: Probability-of-F-to-enter <= .050, Probability-of-F-to-remove >= .100).

a Dependent Variable: preFEV1

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.598(a)	.358	.356	.55497
2	.659(b)	.435	.431	.52178
3	.682(c)	.465	.459	.50862
4	.698(d)	.488	.481	.49825
5	.704(e)	.496	.487	.49521
6	.710(f)	.504	.494	.49203

a Predictors: (Constant), height
b Predictors: (Constant), height, age
c Predictors: (Constant), height, age, gender
d Predictors: (Constant), height, age, gender, SLI
e Predictors: (Constant), height, age, gender, SLI, tb
f Predictors: (Constant), height, age, gender, SLI, tb, wood/char

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 4 – Stepwise Mutivariate Analysis (Continued):

ANOVA(g)

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	50.163	1	50.163	162.871	.000(a)
	Residual	89.934	292	.308		
	Total	140.097	293			
2	Regression	60.872	2	30.436	111.795	.000(b)
	Residual	79.225	291	.272		
	Total	140.097	293			
3	Regression	65.076	3	21.692	83.853	.000(c)
	Residual	75.020	290	.259		
	Total	140.097	293			
4	Regression	68.351	4	17.088	68.832	.000(d)
	Residual	71.746	289	.248		
	Total	140.097	293			
5	Regression	69.469	5	13.894	56.655	.000(e)
	Residual	70.628	288	.245		
	Total	140.097	293			
6	Regression	70.617	6	11.769	48.616	.000(f)
	Residual	69.480	287	.242		
	Total	140.097	293			

a Predictors: (Constant), height
b Predictors: (Constant), height, age
c Predictors: (Constant), height, age, gender
d Predictors: (Constant), height, age, gender, SLI
e Predictors: (Constant), height, age, gender, SLI, tb
f Predictors: (Constant), height, age, gender, SLI, tb, wood/char
g Dependent Variable: preFEV1

Appendix 6: Mulivariate Logistic Regression Analysis (Outputs from SPSS)

Chapter 4 – Stepwise Mutivariate Analysis (Continued):

Coefficients(a)

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta	B	Std. Error
1	(Constant)	-5.534	.638		-8.675	.000
	height	.051	.004	.598	12.762	.000
2	(Constant)	-4.593	.618		-7.429	.000
	height	.049	.004	.578	13.085	.000
	age	-.017	.003	-.277	-6.272	.000
3	(Constant)	-2.935	.730		-4.023	.000
	height	.038	.005	.448	8.324	.000
	age	-.017	.003	-.282	-6.544	.000
	gender	.305	.076	.217	4.031	.000
4	(Constant)	-3.113	.716		-4.345	.000
	height	.037	.004	.433	8.180	.000
	age	-.016	.003	-.252	-5.854	.000
	gender	.319	.074	.227	4.299	.000
	SLI	.138	.038	.156	3.632	.000
5	(Constant)	-3.349	.721		-4.648	.000
	height	.038	.004	.448	8.443	.000
	age	-.015	.003	-.240	-5.564	.000
	gender	.311	.074	.221	4.218	.000
	SLI	.146	.038	.167	3.866	.000
	tb	-.256	.120	-.091	-2.135	.034
6	(Constant)	-3.223	.718		-4.487	.000
	height	.038	.004	.450	8.530	.000
	age	-.014	.003	-.219	-4.970	.000
	gender	.313	.073	.222	4.264	.000
	SLI	.094	.045	.107	2.097	.037
	tb	-.282	.120	-.101	-2.354	.019
	wood/char	-.157	.072	-.113	-2.177	.030

a Dependent Variable: preFEV1

Appendix 7: Questions asked for preliminary survey into biomass fuel use (Chapter 5).

Question	Responses	
How do you cook?	- Wood - Charcoal - Wood & charcoal - Paraffin	- Electricity - Don't cook - Other - Don't know
Where do you cook?	- Kitchen inside main house - Kitchen away from house - Outside on veranda	- Inside main house - Don't know
Where do you keep the stove?	- Inside - Outside - Don't know	
How do you heat the house?	- Wood - Charcoal - Wood & Charcoal - Paraffin	- Electricity - Don't heat - Other - Don't know
How do you heat the bedroom?	- Wood - Charcoal - Wood & charcoal - Paraffin	- Electricity - Don't cook - Other - Don't know
How do you light the house?	- Paraffin tin lamp - Paraffin lamp with glass - Candle	- Electricity - Don't light - Flaming torch
Are there any other sources of smoke at home?	Yes /no (details)	
Are there any other sources of smoke at work?	Yes / no (details)	
Have you ever smoked cigarettes?	Yes / No If yes: Date started: .... Date stopped:	 Number per day: .... Did you ever smoke more? Estimated pack years: ....
Are there any cigarette smokers at home?	Yes /No	