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Pro-Inflammatory Effects in ex vivo Human Lung Tissue of Respirable Smoke Extracts from Indoor Cooking in Nepal

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Author Contributions: B.KC, PS.M and SP.P conducted exposure monitoring and sample collection in field. B.KC, D.T, C.K.B collected tissue samples and performed all tissue cultured work. B.KC and A.P.H performed cytokine screening Luminex assay. B.KC, PS.M I.S, SP.P and I.P.H conceived the study, designed experiments, analysed data, performed statistical analyses and wrote the manuscript.

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Abstract

Rationale: Exposure to biomass smoke is believed to increase the risk of developing chronic obstructive pulmonary disease (COPD). However, little is known about the mechanisms underlying responses to biomass smoke in human lung.

Objectives: This study had 2 objectives: firstly to quantify 'real-life' exposures to particulate matter (PM_{2.5}) and carbon monoxide (CO) measured during cooking on stoves in rural areas of Nepal in different geographical settings, and secondly to assess the effect of biomass smoke extracts on inflammatory responses in ex vivo human lung tissue.

Methods: Personal exposures to PM_{2.5} and indoor near stove CO concentrations were measured during cooking on a range of stoves in 103 households in 4 different Nepalese villages situated at altitudes between ~100m to 4000m above sea level. Inflammatory profiles to smoke extracts collected in the field were assessed by incubating extracts with human lung tissue fragments and subsequent Luminex analysis.

Results: In households using traditional cooking stoves the overall mean personal exposure to PM_{2.5} during cooking was 276.1 µg/m³ (SD 265 µg/m³) and indoor CO concentration was 16.3 ppm (SD 19.65 ppm). The overall mean PM_{2.5} exposure was reduced by 51% (p=0.04) in households using biomass fuel in improved cook stoves (ICS) and 80% (p<0.0001) in households using LPG. Similarly, the indoor CO concentration was reduced by 72% (p<0.001) and 86% (p<0.0001) in households using ICS and LPG respectively. Significant increases occurred to 7 of the 17 analytes measured following biomass smoke extract stimulation of human lung tissue (IL8, IL6, TNFα, IL1β, CCL2, CCL3, and CCL13).

Conclusions: High levels of real life exposures to PM_{2.5} and CO occur during cooking events in rural Nepal. These exposures induce lung inflammation *ex vivo* which may partially explain the increased risk of COPD in these communities.

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Inhalation of particulate matter of $<2.5\mu\text{M}$ ($\text{PM}_{2.5}$) from sources such as tobacco smoke, vehicle exhausts and factory emissions is known to cause lung inflammation (1, 2). Extensive epidemiological evidence also shows that high levels of exposure to $\text{PM}_{2.5}$ are associated with increased risk of developing Chronic Obstructive Pulmonary Disease (COPD), a disease characterised by lung inflammation. Whilst COPD is a major cause of morbidity and mortality globally (3), COPD morbidity and mortality varies across regions. Over 90% deaths attributed to COPD occur in low and middle income countries (LMIC) (4) and in LMICs a significant contribution to disease burden is believed to come from exposure to poor air quality, either outdoors or indoors (5-7). In LMICs, one major source of indoor air pollution is cooking using biomass on traditional stoves in poorly ventilated houses (8).

Despite the epidemiological evidence that household air pollution (HAP) contributes to respiratory symptoms and increased risk for COPD (9, 10), little is known of the mechanisms underlying biological effects of inhaling products of biomass combustion (11). A few *in vitro* studies have shown increased markers of inflammation in different cell lines stimulated with biomass smoke extracts (12-14). However, the data on the effects of biomass smoke exposures are limited compared to the extensive data on the effects of exposure to tobacco smoke extracts (15-16) and no data are available in human lung tissue *ex vivo*.

Approximately half of the world's population, and more than 90% of households in rural parts of LMICs, use solid biomass fuel as a primary source of energy for cooking (17, 18). In Nepal, 80% of the 30M population live in rural settings, and $>80\%$ use locally sourced wood, crop residues or dried dung as a cooking source (19). Most cooking takes place on traditional cook stoves (TCS) which are 3 stone or 2 pot style open fires in rooms without a chimney or

proper ventilation and this would be predicted to cause high levels of exposure to PM_{2.5}, carbon monoxide(CO) and other health damaging pollutants (20). The emissions of fine particulate matter, in poorly ventilated dwellings using inefficient stoves can reach up to 100 fold higher than the air quality standard set by the World Health Organization (WHO) (21). Two recent studies using static sampling conducted in Sarlahi and Janpapur district of Nepal have found a daily average indoor PM_{2.5} concentration of 1376 µg/m³ (22) and a 48-hour average PM_{2.5} concentration of 417.6 µg/m³ (23). Consistently higher concentrations of indoor air pollutants in households using biomass fuel has been reported in similar studies in other countries (24-27). Following several national initiatives in Nepal, improved cooking stoves (ICS) have been trialled in some villages: these have an improved combustion system and/or vent fumes through a chimney (28). According to the Central Bureau of Statistics, Nepal, ~21% of households use Liquefied Petroleum Gas (LPG) stoves (29). Preliminary work performed in Nepal has shown reductions in levels of HAP with use of these improved cooking stoves and LPG stoves, though the reduction achieved varied between studies (30-32). Although in Nepal and other parts of South Asia studies have been done examining indoor exposures none of these studies have investigated lung inflammatory responses (33-35).

The aims of this study were therefore two fold, namely to define the range of personal exposures encountered by individuals cooking on both TCS and ICS in real life in Nepal, and to investigate the potential pro-inflammatory consequences of these exposures using *ex vivo* human lung explants as a model system.

Methods

Site Description

This study was conducted in four different rural or semi-rural settings representing different geographical regions in Nepal (Figure 1). The sites were Simreni village in Chitwan (~ 200 meter above sea level (masl)), Bhujel Gaun in Pokhara (~1200 masl), Salambu in Majhi Feda (~ 1800 masl) and Kyanzing Kharka in Langtang (~ 4000 masl) (see section E1 for a more detailed site description).

Exposure Monitoring

Real life exposure to PM_{2.5} and CO concentration was monitored in a total of 103 households from all four villages with different stove designs: details are presented in Table 1. The exposure monitoring was performed for a single cooking episode in each household throughout the cooking period; however, a short term non-cooking period exposure concentration was also measured in each household. The cooking period varied in each household in each monitoring site with the average (SD) of 78 (15.9) minutes, 79 (24.2) minutes, and 68 (15.2) minutes in Salambu, Pokhara and Chitwan respectively. As stoves in Kyanzing remained in use for 16-17 hours a day, the monitoring period was fixed to 120 minutes in each household to represent the cooking period. Non-cooking period exposure was monitored for a total of 40 minutes in each household with a continuous 20 minutes exposure before cooking commence and 20 minutes after cooking ended. Real time personal exposures to PM_{2.5} were measured using an Aerosol Mass Monitor (Aerocet831, Met One Instrument, Inc) (36, 37) which was attached

directly to the person involved in cooking. The cook was asked to carry an aerocet on their back, and its inlet connected by an adjustable tube placed to be near to the cook's breathing zone. An Indoor air quality (IAQ) meter (Advancedsense[®] Pro IAQ, Graywolf Sensing solutions, USA) was used to monitor real time indoor CO exposures. The monitoring was performed for cooking and non-cooking period by placing the IAQ meter approximately 1 meter from the stove and approximately 1.5 meters off the floor to capture direct real time CO exposures. Exhaled breath CO concentration of people involved in cooking was also measured as a biomarker of CO exposure. Personal exhaled breath CO concentrations were monitored using a micro CO meter (CareFusion, UK) (38). The concentration of CO in exhaled breathe for each individual was taken at three different time points in three monitoring sites except in Kyanzing. The first measurement was taken before cooking started, the second measurement was taken during cooking and the third was taken immediately after cooking. For each phase, measurements were taken three times and the mean of three readings were calculated. In Kyanzing, exhaled breath CO concentration was measured in individual using TCS and ICS. All equipment details and calibration studies can be found in the online data supplement section E2.

Smoke Extract Collection

A total of 52 smoke extract samples including 33 samples from biomass combustion in TCS, 10 samples from biomass combustion in ICS and 6 samples from LPG were collected from the four villages. From each monitoring site ambient air samples were also collected using the same methods. Full details can be found in online section E3.

Endotoxin Quantification

Endotoxin concentration present in the smoke sample collected from different locations was quantified using a Limulus Amebocyte lysate (LAL) assay (Pierce™ LAL Chromogenic Endotoxin Quantification Kit, ThermoFisher Scientific, US) (29,30). See section E4 in the online data supplement for more detail. Airborne endotoxin concentrations are known to vary depending on the type of fuel used, cow dung and agricultural residue being higher source of endotoxin than fuel wood (41). Households in this study in all monitoring sites primarily used fuel wood for cooking. Endotoxin concentrations in smoke extract samples collected in homes using biomass fuel and LPG stoves were also measured.

Human Lung Tissue Processing

Human parenchymal lung tissue was obtained with informed written consent from patients undergoing lung resection surgery from the Papworth Hospital Tissue Bank, UK. The majority of donors (11/14) were ex-smokers (quit smoking ≥ 5 years), two individuals were current smokers and two individuals were never smokers. There were 9 males and 5 females with a mean age of 62.6 ± 9.3 years. The methods for tissue sample processing and mediator assays are presented in section E5, and demographics of all donor patients are presented in the online data supplement Table E2.

Statistical Analysis

All data were normalised using wet tissue weights in individual experiments. Data are expressed as means +/- standard error of the mean (SEM). All data were analysed using

GraphPad Prism Software (Version 7, GraphPad Software Inc.). All statistical significance tests were performed using t-test for comparison of two variables and using ANOVA for comparison of three or more variables (a value of $p < 0.05$ being considered as significant).

Ethical Approval

Ethical approval for lung tissue studies was obtained from Papworth Hospital Research Tissue Bank with the ethics reference 08/H0304/56+5. Field based exposure monitoring work was conducted under the ethical approval of the research proposal entitled 'Epidemiological response to air pollution exposure in Nepal' granted by the Nepal Health Research Council (NHRC) (reg.no. 395/2016).

Results

Indoor Air Pollutant Exposures during Cooking in 'Real Life' Rural Nepal

Representative $PM_{2.5}$ and CO profiles showing the variation seen during cooking periods using biomass fuel in TCS, biomass fuel in ICS and LPG are shown in Figure 2 and Figure 3 respectively. There was considerable variability in exposure levels to both pollutants through the cooking period. Both $PM_{2.5}$ and CO levels registered high concentrations more than once during cooking, with the variation in exposure levels in individual households being dependent on the cooking practises followed. Greater variation in the peak concentrations were noted in households using TCS compared with households using ICS or LPG stoves, and also levels were higher in homes using TCS.

The overall mean PM_{2.5} exposures from all households using TCS from all sites showed that the population using biomass fuel in Nepal are exposed to high concentrations of indoor PM_{2.5} (overall mean 276.1 µg/m³). There was a significant reduction in mean PM_{2.5} exposure concentration in households using biomass fuel in ICS (51% reduction, p=0.04) and in households using LPG (80% reduction, p<0.0001). (Figure 2 (D)). Similarly, the population using biomass fuel in TCS were exposed to higher concentration of CO (overall mean 16.3 ppm). These CO concentrations were significantly reduced in households using biomass fuel in ICS (72% reduction, p=0.0002) and in households using LPG (86% reduction, p<0.0001) (Figure 3 (D)). However, even the reduced exposures for PM_{2.5} using either ICS or LPG remained higher than the WHO safe recommended concentration of 25 µg/m³ (21). The exposure levels for CO using both ICS and LPG remained below the WHO guidelines.

The mean PM_{2.5} and CO exposures calculated across the whole cooking period in households using TCS were significantly higher (~4.6 fold for PM_{2.5}, p<0.0001 and ~8 fold for CO, p<0.0001) than the non-cooking period exposures (Figure 4 (A) and (B)). The overall mean PM_{2.5} and CO exposures in the household using ICS was 2.8 fold (p<0.001) and 3.4 fold (p<0.0001) higher in cooking periods compared to the non-cooking periods. The overall mean PM_{2.5} exposures in households using LPG were not significantly different (p=0.84) between the cooking and non-cooking periods, however, the overall mean cooking period CO exposures were ~1.5 fold (p=0.02) higher than non-cooking period exposures.

Differences in mean PM_{2.5} and CO exposures during cooking and non-cooking periods in each monitoring site using different stoves, along with indoor temperature and relative humidity is presented in Table 2. The overall exposures to mean PM_{2.5} was significantly higher in

the higher altitude regions in Langtang ($746.1\mu\text{g}/\text{m}^3$) and Salambu ($500.6\mu\text{g}/\text{m}^3$) than in lower altitude regions in Pokhara ($211.9\mu\text{g}/\text{m}^3$) and Chitwan ($121.9\mu\text{g}/\text{m}^3$). The reductions in $\text{PM}_{2.5}$ exposures when ICS was used instead of TCS were 88% ($p<0.0001$) and 59% ($p<0.01$) in Kyanzing and Salambu respectively, whereas the percentage reductions in CO exposures were 78% ($p<0.001$) and 72% ($p<0.05$) respectively. Similarly, the reductions in $\text{PM}_{2.5}$ exposures in households using LPG in Pokhara and Chitwan were significant compared with TCS, 75%, $p<0.01$ in Pokhara and 55% $p<0.01$ in Chitwan. The percentage reductions in CO exposures in households using LPG in Pokhara and Chitwan were 85% ($p<0.05$) and 79% ($p<0.001$) respectively.

The concentration of CO in exhaled breath was measured to estimate changes in exhaled breath CO levels due to short term exposure to HAP. The pattern of exhaled breath CO variations between different cooking phases in households using TCS in three monitoring sites are presented in Figure 5 (A-C). For all subjects using TCS in all sites exhaled breath CO was elevated over baseline during cooking period observations. Post-cooking levels were lower than the cooking period level, however the levels were still higher than the levels noted in pre-cooking observations. The overall mean exhaled CO levels in individuals using ICS in Kyanzing showed a percentage reduction of 75% ($p<0.0001$) compared with individuals using TCS (Figure 5 (D)). Use of LPG in Chitwan showed that exhaled CO levels were not significantly elevated during cooking (Figure 5 (E)) as compared to non-cooking levels.

Human Lung Tissue Cytokine Responses

Ex vivo human lung tissue responded to LPS stimulation by producing 14 quantifiable signals out of the 17 inflammatory analytes assayed across all donors (data not shown) in keeping with our previously published data (42). Increased levels of 7 mediators were detected in the supernatants from lung tissue stimulated with biomass smoke extract samples (Figure 6). The relative magnitude of cytokine responses varied between LPS and biomass smoke extract stimulation (see Table 3)

The data shown in Figure 6 and Table 3 show overall means for all samples collected from all monitoring sites in households using TCS. The inflammatory profiles observed in lung tissue stimulated with site specific biomass smoke extracts were quantitatively similar across all sites (see online data supplement Figure E4). There was a trend towards a positive response from tissue treated with ambient air samples but no response was statistically significant.

Impact of Improved Stove Designs and Clean Fuel on Cytokine Responses

Having demonstrated that biomass smoke extracts collected from cooking with TCS in a real life setting induced a pro-inflammatory response in human lung tissue, and given that use of ICS and LPG reduced exposures by up to 51% and 80% respectively, we investigated smoke extracts from cooking with ICS and LPG on inflammatory responses in human lung tissue. The smoke samples derived from cooking with ICS still induced an inflammatory response in human lung tissue (Figure 7 (A) and (B)) for 6 of the 7 analytes. Interestingly, the smoke extracts derived from cooking with LPG also induced quantifiable responses although increases for only IL-8 and

TNF- α were statistically significant over the untreated basal condition (although a smaller number of samples was available for this comparison).

Endotoxin Concentration in Indoor Air Sample

The levels of endotoxin in the indoor smoke extract samples collected during cooking using different cooking fuels were analysed. Endotoxin was present in all samples. The highest levels of endotoxin was measured in air samples using biomass fuel (5.49 ± 2.86 EU/mL); levels in air samples using LPG were lower (2.14 ± 1.31 EU/ML). We also looked for potential correlations between the level of endotoxin present in samples and cytokine expression from stimulated *ex vivo* lung tissue. Expression of IL-8 ($r=0.46$, $p=0.01$) and CCL2 ($r=0.38$, $p=0.04$) were moderately correlated with endotoxin levels, but whilst r values remained positive no significant correlations were seen between the expression of IL-6 ($r=0.25$, $p=0.18$), IL-1 β ($r=0.35$, $p=0.06$), and CCL3 ($r=0.24$, $p=0.2$) and endotoxin levels.

Discussion

The main aims of the studies described in this paper were to measure real life personal exposure to PM_{2.5} and indoor near stove CO concentration during indoor cooking in rural Nepal and to gain insight into the potential effects of exposure of human lung tissue to respirable material generated from burning biomass. We studied 4 different sites in rural Nepal, chosen because they were in different altitude and climate regions. Whilst there were some differences in the absolute exposures observed between different households and in different

geographical regions, the main conclusion that can be drawn from this work is that generally high levels of PM_{2.5} exposures were seen whilst cooking on traditional stoves, in the range that is likely to be harmful to human health. The exposures observed in this study in different locations were between 5-29 fold higher than 24 hour WHO standards for indoor PM_{2.5} (25 µg/m³) (21). Very few previous studies performed in Nepal have tried to measure personal exposure to indoor PM_{2.5}, although previous studies using gravimetric sampling have also shown high concentrations of indoor PM_{2.5} in households using biomass fuel consistent with our findings (23, 31). Personal exposures to PM_{2.5} observed in this study were generally comparable to those observed elsewhere in studies in lowland South Asia (26, 43). Increased exposure to CO resulted in increased exhaled CO concentrations in individuals post cooking, demonstrating that exposure to higher levels of CO in the indoor atmosphere resulted in increased levels in the lung. The high levels of PM_{2.5} and CO seen in the cooking environment in the majority of rural households in Nepal are because cooking takes place indoors, typically using traditional stove designs with no flue, and in poorly ventilated rooms. Typical households would undertake this activity either twice or three times a day, with cooking periods being up to 2h each. Cooking is mainly done by women, although children are often also present in the room, and these exposures are likely to be to be an important cause of chronic respiratory disease especially in Nepalese women.

The use of improved stove designs (with a flue to vent smoke out of the room) has been found in other settings to potentially be effective in reducing indoor exposures (44, 45). The reduction in real life indoor exposure with ICS in this study was around 70% which is in line with reductions reported in a previous study conducted in Nepal using gravimetric sampling (30).

However, the exposures seen with ICS remain well above the threshold level recommended by WHO and other organisations.

We next examined the potential for respirable biomass to produce pro-inflammatory effects in human lung tissue using biomass smoke extracts collected in the real life setting. These extracts produced an inflammatory response in human lung tissue, although the magnitude of the response was less than that seen with previously investigated stimuli such as LPS which induces inflammation through TLR4 activation (42, 46, 47). Interestingly, both ICS and LPG samples also produced an inflammatory response. One possible explanation for this is that ambient air pollution may be contributing to these responses, but there was no significant response in human lung tissue *ex vivo* to ambient air sample extracts (data not shown). It seems more likely therefore that the extracts contain additional inflammatory stimuli such as VOCs which are contributing to these responses. These data also suggest that further reductions in exposure, perhaps through source control by improved venting of fumes or better ventilation, will be required to prevent lung inflammation in real life.

Whilst the data presented here show clearly that high levels of personal exposures occur in rural Nepal during cooking using traditional techniques and that these are likely to produce lung inflammation, there are some potential limitations of our work. First, it is difficult to accurately model true lung exposure even using the approach we have utilised of collecting samples in real life to use in human lung tissue experiments. Samples were stored at -20°C during transport, but it is still possible some activity may have been lost during sample transfer. In addition, estimating the dilution factors used in our experiments may have resulted in underestimating true inflammatory effects. Samples were collected using a pump set at 3 L/min

into a volume of 10mls of medium: the final concentration human lung tissue exposed to was a 10% dilution of this extract. In the human lung, the actual tissue exposure will depend upon the dynamic equilibrium between inhaled and exhaled material, ventilation rates, the amount of lung lining fluid, and the effective volume of distribution of inhaled material. The volume of the lung lining fluid is believed to be around 20-50mls (48) but the true volume of distribution of the active components of inhaled biomass smoke will vary depending upon the physicochemical properties of the constituent being considered. It is possible therefore that we may have underestimated the true local exposure in the lung using the experimental design we adopted. None the less we have tried to model real exposures as closely as possible.

As biomass smoke contains a wide range of potentially active compounds, including carbon monoxide, VOCs, PAHs, aldehydes, free radicals, sulphur and nitrogen oxides, benzene, and particulate matter (5, 20) inflammatory responses will be driven by a range of different mechanisms. We measured endotoxin levels in our samples, and as would be expected these were moderately elevated, and hence some of the responses could be driven directly through TLR activation. The observation that there were qualitative differences between LPS responses and biomass responses suggests a range of pathways are likely to be involved.

In summary, we have shown for the first time that biomass smoke samples collected in a real life environment from rural Nepal have pro-inflammatory effects in human lung tissue. These data support the need to reduce exposures in order to improve respiratory health in this setting, but suggest that additional methods other than those currently being trialled may be needed to reduce exposures to levels which will prevent lung inflammation from occurring in real life settings.

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Figure Legends:

Figure 1: Study location: (A) map of Nepal with elevation and location of all four monitoring site villages, (B) Kyanzing Kharka, Langtang (4000 m above sea level), (C) Salambu, MajhiFeda (1800 m above sea level), (D) Bhujel Gaun, Pokhara (1200 m above sea level), and (E) Simreni village, Chitwan (200 m above sea level)

Figure 2: Representative temporal variation in real life personal exposure to $PM_{2.5}$. A-C represents minute to minute variations in $PM_{2.5}$ exposure using (A) biomass fuel in TCS, (B) biomass fuel in ICS, and (C) LPG stove in one representative house. (D) mean $PM_{2.5}$ exposure during cooking using biomass in TCS and ICS, and using LPG stove. The data in (D) represents mean (\pm SEM) of all households mean data over total cooking period for TCS (n=76), ICS (n=17) and LPG (n=10). The mean exposure when using ICS and LPG was compared with mean exposure using TCS. (Significance test was carried out by Kruskal-Wallis test followed by Dunn's multiple comparison test with $*=p<0.05$, $****=p<0.0001$). TCS: Traditional Cook Stove, ICS: Improved cook stove, LPG: Liquefied petroleum gas.

Figure 3: Representative temporal variation in real life CO exposure. A-C represents minute to minute variations in CO exposure using (A) biomass fuel in TCS, (B) biomass fuel in ICS, and (C) LPG stove in one representative house. (D) mean CO concentration during cooking using biomass in TCS and ICS and using LPG. The data in (D) represents mean (\pm SEM) of all households mean data over total cooking period for TCS (n=76), ICS (n=17) and LPG (n=10). The mean

exposure when using ICS and LPG was compared with mean exposure using TCS. (Significance test was carried out by Kruskal-Wallis test followed by Dunn's multiple comparison test with ***= $p<0.001$, ****= $p<0.0001$). TCS: Traditional Cook Stove, ICS: Improved cook stove, LPG: Liquefied petroleum gas.

Figure 4: Cooking and non-cooking exposure variation among different cook stove use across all sites. (A) $PM_{2.5}$ exposure, and (B) CO exposure. The data shown are mean (\pm SEM) of all households from all sites with TCS (n=76), ICS (n=17) and LPG (n=10). The variation between two periods for each stove was assessed using Mann-Whitney t-test, *= $p<0.05$, ***= $p<0.001$, and ****= $p<0.0001$)

Figure 5: Exhaled breath CO concentrations at the start of cooking, during cooking and post-cooking using different cook stoves. (A) Salambu using TCS (n=17), (B) Pokhara using TCS (n=19), (C) Chitwan using TCS (n=33), (D) Kyanzing using TCS (n=11) and ICS (n=18), and (E) Chitwan using LPG (n=10). The variation in exhaled breath CO concentration for each monitoring phase was assessed using 1way ANOVA Friedman test followed by Dunn's multiple comparisons test with *= $p<0.05$, *8= $p<0.01$, **= $p<0.001$, and ****= $p<0.0001$. The data shown are mean (\pm SEM) of all individuals.

Figure 6: The inflammatory response of *ex vivo* human lung tissue following smoke extracts from TCS stimulation. Biomass smoke stimulation showed an effect on cultured human tissue explant by significantly inducing 7 analytes. The data represents mean fold increases in 5

independent donor experiments using biomass sample from all 4 monitoring sites (mean of 20 data points) and are shown as mean (\pm SEM). Raw data were normalized using wet tissue mass for each analyte before computing fold stimulation and elevated levels between smoke extract treated and untreated basal condition were checked in raw data using Wilcoxon matched paired test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

Figure 7: Comparison of concentration of analytes following stimulation with smoke extract from different cooking fuel sources. The inflammatory response seen following stimulation with the samples from (A) biomass combustion in ICS, and (B) LPG combustion. Data shown are the overall mean (\pm SEM) fold increase of 20 data points ($n=5$ donors X 4 different ICS samples) and 10 data points ($n=5$ donors X 2 different LPG samples). Raw data were normalized using wet tissue mass for each analyte before computing fold stimulation and elevated levels between smoke extract treated and untreated basal condition were checked in raw data using Wilcoxon matched paired test (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$).

Table 1: Number of households sampled in each monitoring site.

Monitoring site	Total Household sampled	Cooking methods		
		Biomass (TCS)	Biomass (ICS)	LPG
Kyanzing	14	5	9	N/A
Salambu	25	17	8	N/A
Pokhara	27	24	N/A	3
Chitwan	37	30	N/A	7
Total	103	76	17	10

N/A: Cooking method was not used in the respective site.

Table 2: Cooking and non-cooking period exposure concentration in (A) Kyanzing, (B) Salambu, (C) Pokhara and (D) Chitwan. Differences were assessed using Man-Whitney tests. The data shown are mean (\pm SD) of all households in each monitoring sites.

(A) Kyanzing						
	Cooking	TCS Non -cooking	p-value	Cooking	ICS Non -cooking	P -value
T ($^{\circ}$ C)	14.1 (2.3)	13.75 (6.18)	0.73	18.01 (6.23)	20.19 (8.2)	0.6
RH (%)	42.1 (3.4)	37.8 (11.6)	0.55	34.67 (10.8)	29.57 (13.8)	0.15
PM _{2.5} (μ g/m ³)	746.1 (318.9)	21.3 (16)	0.007	91.27 (70.11)	16.78 (15.3)	0.001
CO(ppm)	12.6 (4.7)	1.15 (0.69)	0.015	2.68 (1.47)	1.11 (0.58)	0.001
(B) Salambu						
	Cooking	TCS Non- cooking	p-value	Cooking	ICS Non- cooking	p- value
T ($^{\circ}$ C)	20.65 (4.68)	18.04 (4.09)	0.15	17.86 (2.3)	14.3 (3.18)	0.4
RH (%)	42.91 (9.92)	47.1 (9.51)	0.21	48.6 (7.72)	58.23 (7.9)	0.12
PM _{2.5} (μ g/m ³)	500.6 (314.6)	104.45 (101.15)	0.0001	203.25 (61.42)	84.8 (20.1)	0.0002
CO(ppm)	25.78 (30.4)	3.28 (3.38)	0.0001	7.06 (2.48)	2.81 (3.27)	0.01

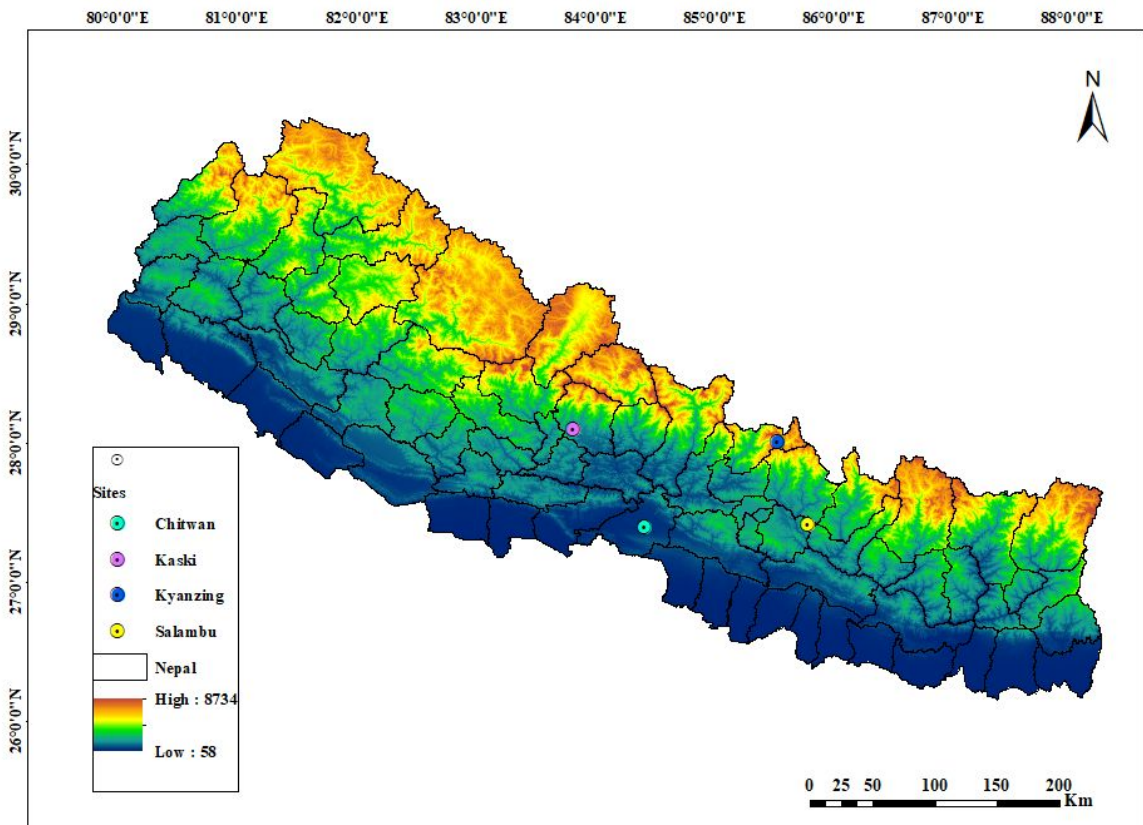
(C) Pokhara						
	TCS			LPG		
	Cooking	Non-cooking	p-value	Cooking	Non-cooking	p-value
T (°C)	16.6 (3.35)	16.3 (4.22)	0.33	13.8 (2.83)	11.7 (0.14)	0.71
RH (%)	40.05 (9.51)	35.77 (8.62)	0.20	48.76 (6.9)	44.55 (2.05)	0.52
PM _{2.5} (µg/m ³)	211.93 (133.44)	44 (15.85)	0.0001	52.4 (14.5)	33.4 (0.4)	0.1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

(D) Chitwan						
	TCS			LPG		
	Cooking	Non-cooking	p-value	Cooking	Non-cooking	p-value
T (°C)	27.4 (3.870)	27.98 (7.52)	0.97	31.09 (3.45)	30.8 (3.4)	0.81
RH (%)	59.3 (11.7)	59.39 (11.05)	0.94	55.06 (9.25)	56.6 (9.1)	0.87
PM _{2.5} (µg/m ³)	121.9 (57.8)	79.48 (48.9)	0.001	54.68 (31.02)	53.4 (20.3)	0.71
CO(ppm)	9.08 (6.98)	5.37 (7.91)	0.01	1.85 (0.94)	1.6 (0.81)	0.24

Table 3: LPS and biomass smoke extract responses in *ex vivo* human lung tissue. The data in the table shows mean (SEM) fold increases of each analytes following LPS and biomass stimulation (n=5 independent donors experiments).

Analytes	IL8	IL6	TNF α	IL1 β	CCL2	CCL3	CCL13
LPS stimulation fold change	7.56 (2.05)	5.86 (1.73)	196.8 (65.94)	49.45 (9.2)	6.8 (2.03)	12.93 (3.35)	4.62 (1.56)
Biomass stimulation fold change	1.89 (0.21)	2.15 (0.35)	2.97 (0.36)	1.88 (0.35)	2.9 (0.57)	1.23 (0.05)	1.47 (0.14)
% of LPS response	25	36.7	1.5	3.8	42.6	9.5	31.8

Figure 1



A



B



C



D



E

Figure 2

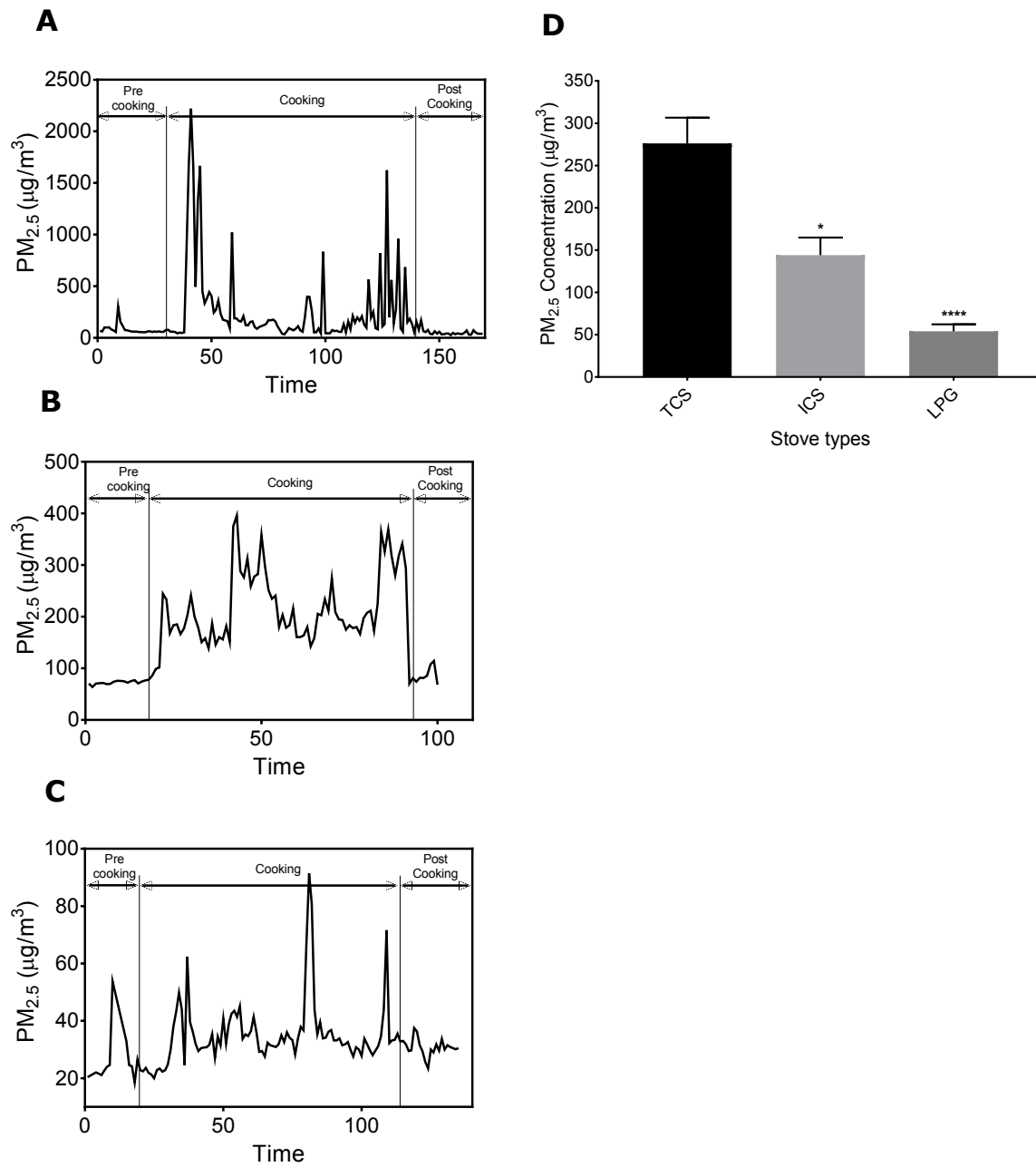


Figure 3

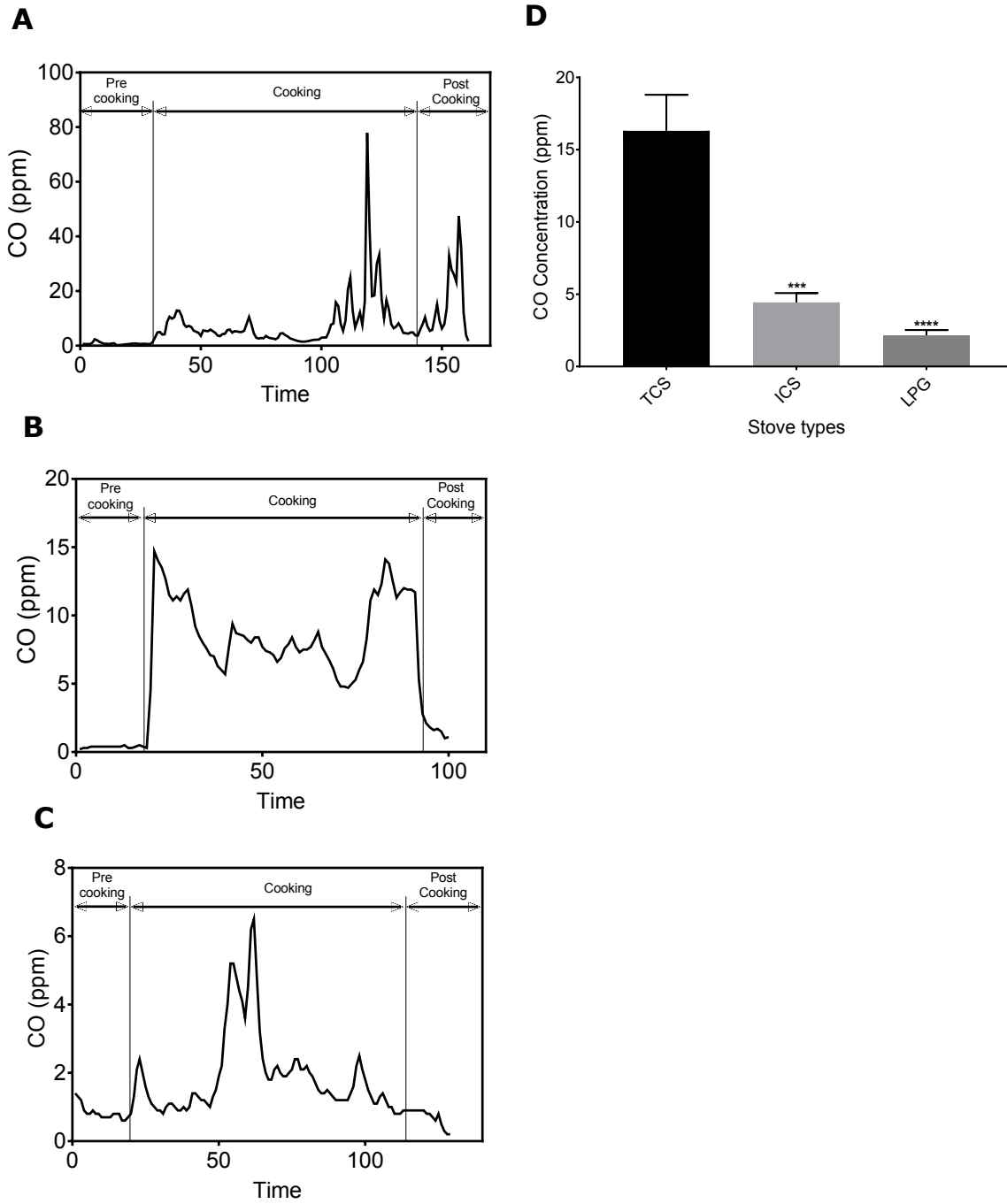
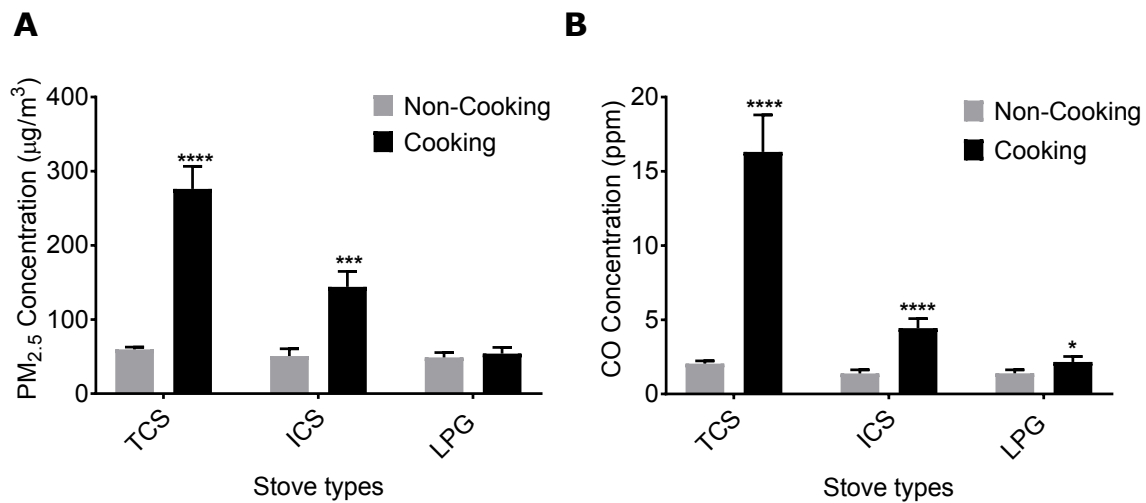


Figure 4



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Figure 5

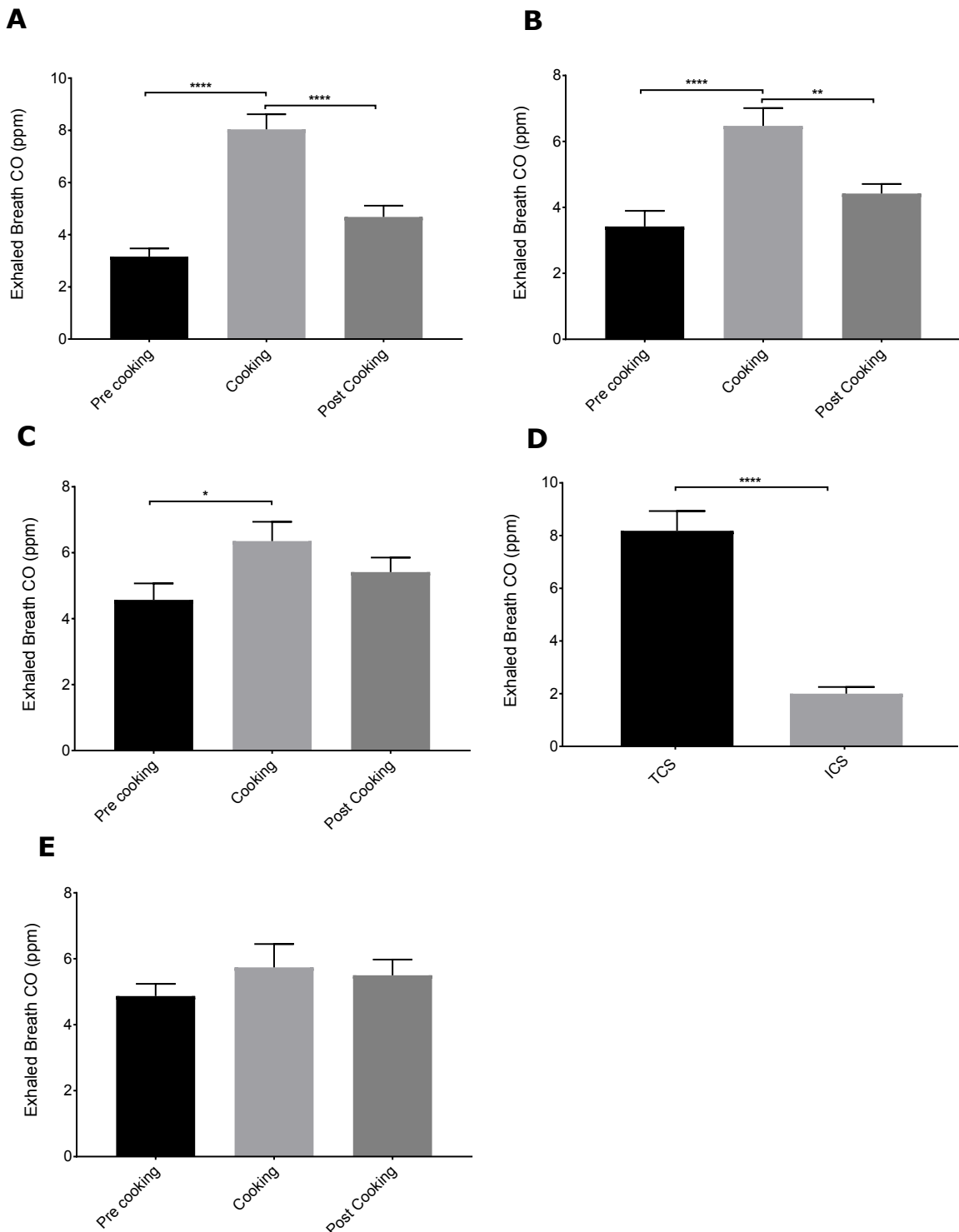


Figure 6

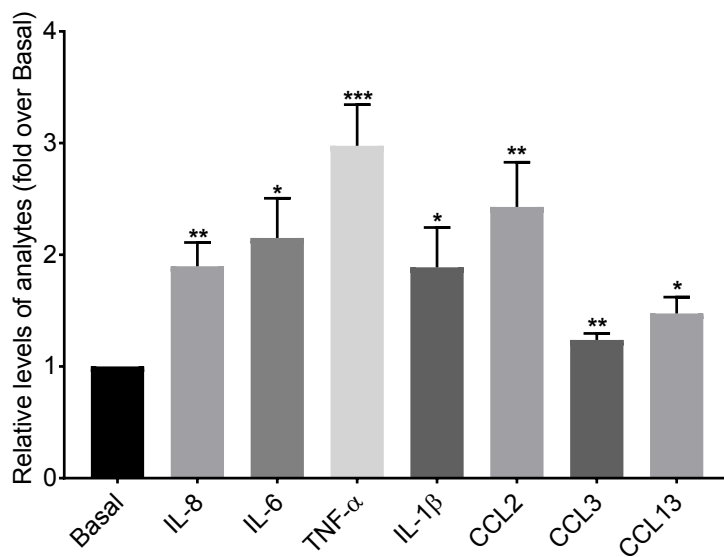
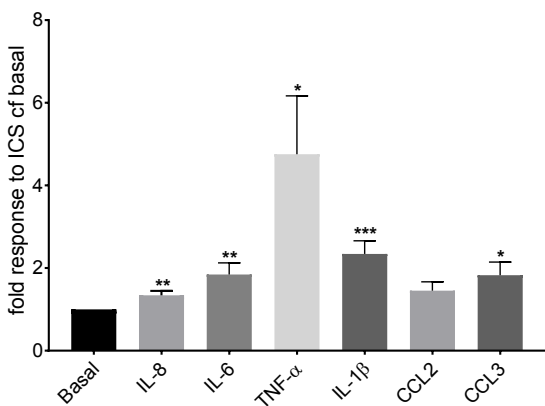
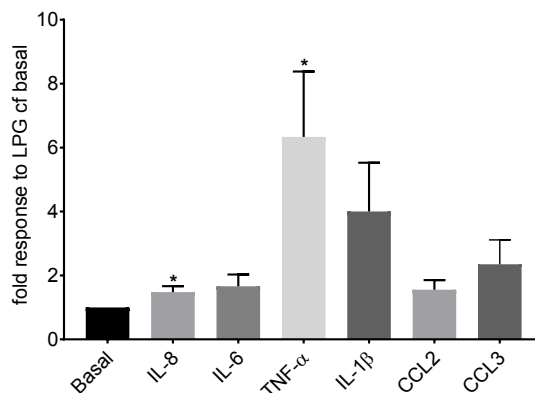


Figure 7

A



B



**Pro-Inflammatory Effects in ex vivo Human Lung Tissue of
Respirable Smoke Extracts from Indoor Cooking in Nepal**

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Charlotte K Billington, Ian Sayers, Siva Praveen Puppala, Ian P. Hall,

Online Data Supplement

For Review Only

Online data supplement Section E1: Study sites and stove use

Nepal is known for its rich biodiversity, culture and specifically the Himalayan ecosystem. It is a small country (total area of 147,181 sq. km.) located at latitudes between $26^{\circ} 22'$ and $30^{\circ} 27'$ north and longitudes between $80^{\circ} 04'$ and $88^{\circ} 12'$ east. Within this region, the country provides a unique geography with low lying plains at ~ 70 m above mean sea level (masl) and high elevation mountains at up to 8848 m above mean sea level. The country is topographically divided into three regions according to altitude variation; the Himalayan region (at 4000 m or above), the mid hill region (1000 to under 4000 m) and the plain region (70 m to under 1000 m).

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

Salambu is a small village in the MajhiFeda VDC of Kavrepalanchowk district located in the hill region of the country. The study site in Salambu had a total of 88 households at the time of the study, out of which 25 households were sampled for the exposure monitoring. Improved cook stoves were distributed among all households in this study site, but over time, most of

the households had tended to come back to the traditional mode of cooking on a three stone stove. A few households still cooked on ICS but also had a TCS in use. The exposure monitoring was performed in 17 households using TCS and 8 households using ICS.

The exposure monitoring in Pokhara was performed in a small semi-rural village called Bhujel Gaun which is located at a distance of 12 km west of the main Pokhara city. Bhujel Gaun had a total of 40 households at the time of the study, out of which 27 households were selected for the exposure monitoring. Neither of households had improved type of cook stove installed at the time of our study, however, few households used LPG as a primary source of cooking. The monitoring was performed in the household using biomass fuel in TCS (24 households) and households using LPG (3 households).

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

Combustion of biomass fuel was the primary source of energy for cooking and heating in all four sites. Cooking activities were undertaken indoors at

all sites. Households using TCS and LPG cooking stoves were monitored in Chitwan and Pokhara, whereas those using TCS and ICS were monitored in Langtang and MajhiFeda. Details of the cook stove design in each of the monitoring site is presented in Figure E1 (A-F).

Online data supplement Section E2: Equipment comparison and calibration study

The Aerocet is a small, lightweight, battery-operated, handheld aerosol mass monitor which simultaneously monitors levels of particulate matters with various sizes including PM_{1} , $PM_{2.5}$, PM_{4} and PM_{10} . The Aerocet monitors the level of particles every 1 minute with a fixed flow rate of 2.83 L/min. Aerocet uses the particle count to mass conversion principle and runs in a continuous mode with a resolution of $0.1 \mu\text{g}/\text{m}^3$. The Aerocet counts and sizes particles in five different size ranges then used a proprietary algorithm to convert count to mass concentrations.

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

range of CO concentration of a meter is 0-500 ppm with an accuracy of ± 2 ppm.

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

All instruments used in this study were factory calibrated prior to the field campaign. Pre-and post-field campaign comparison was also done with standalone instruments or with the calibration systems. In order to eliminate any measurement biases and to validate the measured concentrations from Aerocet, pre or post field collocation runs were conducted for all sets with an environmental dust monitor GRIMM-EDM-180D (GRIMM Aerocol Technik GmbH & Co. Kg. Dorfstrasse-9, Germany). GRIMM is a factory calibrated, US-EPA certified sophisticated real time aerosol mass monitor which optically measure every single particle in the sampled air. All sets were run over a period of 12 hours with GRIMM and

the PM concentrations from each Aerocet were compared with the data obtained from GRIMM and appropriate correction factor was derived. The correction factor was derived using a linear relationship between results from two types of instrument as shown in Figure E2. The correction factor of each Aerocet set used in each monitoring site is presented in Table E1.

The CO gas sensors were calibrated following the protocol in a lab at 0 ppm using a zero-air gas (Specialty Gases Ltd, UK), and at 81 ppm using CO calibration gas mixture (Alchemic Gases and Chemical Pvt. Ltd, India). Micro CO meter was calibrated according to the instruction provided in a Micro Co meter operation manual using 20 ppm CO calibration gas provided by CareFusion (Cat. No 36-MCG020).

Online data supplement Section E3: Smoke extract Collection

A randomly selected 16 TCS samples (4 each from each site), 5 ICS samples (3 from Kyanzing and 2 from Salambu), 4 LPG samples (2 each from Bhujel gaun and Simreni village) and 4 ambient air samples (1 each from each of four village) were used for experiments examining inflammatory responses. We collected biomass smoke samples directly into 10 ml of cell and tissue culture media (DMEM, Dulbecco's Modified Eagle's Medium, ThermoFisher Scientific) using an impinger system and vacuum pump at a constant flow rate of 3 L/m during the cooking duration. Once the collection process was completed the sample was immediately

transferred into a 15 ml conical centrifuge tube and stored in a temporary field freezer maintaining the temperature below -10°C and subsequently stored at -20°C at the laboratory in Kathmandu. These samples were then transferred to Nottingham in a vacuum thermos flask filled with ice. All samples used for the *ex vivo* experiments described in this paper remained frozen when checked upon arrival in Nottingham. The samples were then stored at -20°C freezer at University of Nottingham until use.

Online data supplement Section E4: Endotoxin quantification

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

Online data supplement Section E5: Sample processing and mediator assays

Analyses of >50 donor samples showed no significant differences in responses in individuals based on FEV₁/FVC values (Figure E3). The tissue sample was first washed in Tyrode's buffer solution, and whole tissue weight was taken. The tissue sample was then dissected into small pieces of 50-100 mg (wet weight) and incubated for 24h in 1 ml of RPMI 1640 media (with 2.05 mM L-glutamine and 25 mM HEPES) (Sigma, 51536C) containing antibiotics and antimycotics (penicillin, streptomycin and fungizone) (Sigma, A5955) (Tracy et al 2017) After resting overnight, the media was replaced, and explants were stimulated with media, LPS (1µg/ml) as a positive control or biomass smoke sample (10% v/v). Tissue samples were incubated for a further 48h, followed by the collection of supernatants for subsequent protein analyses. Experimental conditions for tissue work in this study were performed in duplicate for each donor.

We initially choose a panel of 17 analytes to be assayed based on our previous work (E1) Luminex screening Assays (with Human Premixed Multi-Analyte kit, R&D SystemsTMCatNo#LXSAHM) were used to analyse tissue culture supernatants as per the manufacturer's instruction and used elsewhere (E2, E3). In the Luminex assay plate, each supernatant sample was assayed in duplicate.

Online data Supplement Figure Legends

Supplemental Figure E1: **Types of stove in different monitoring sites.**

TCS: traditional cooking stove; ICS: improved cooking stove

Supplemental Figure E2: **Representative plot of comparison of GRIMM and two different sets of Aerocet derived PM_{2.5} measurements.**

Supplemental Figure E3: **Lack of correlation between induced cytokine levels in tissue culture supernatant following LPS (1 μ g/ml) stimulation and corresponding subjects lung function (FEV1/FVC).**

(a) IL-8 (pg/ml/mg) vs FEV1/FVC (%), (b) IL-6 (pg/ml/mg) vs FEV1/FVC (%), (c) TNF- α (pg/ml/mg) vs FEV1/FVC (%), and (d) IL-1 β (pg/ml/mg) vs FEV1/FVC (%). Data are from lung tissue donors where n=individual donor for each analyte.

Supplemental Figure E4: **Site specific secretory profile of *ex vivo* human lung tissue following stimulation with the sample collected from each site.** Data represents mean (\pm SEM) fold increase over basal from duplicate readings in 5 independent donor experiments for each site.

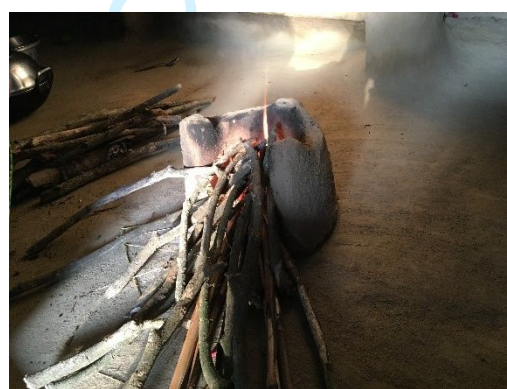
Figure E1**(A) ICS (Kyanzing, Langtang)****(B) TCS (Kyanzing)****(C) ICS (Salambu)****(D) TCS (Salambu)****(E) TCS (Pokhara)****(F) TCS (Chitwan)**

Figure E2

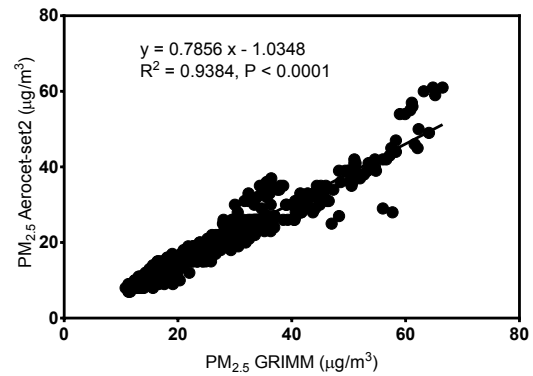
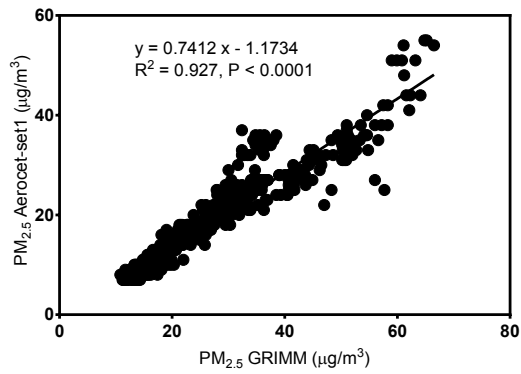


Figure E3

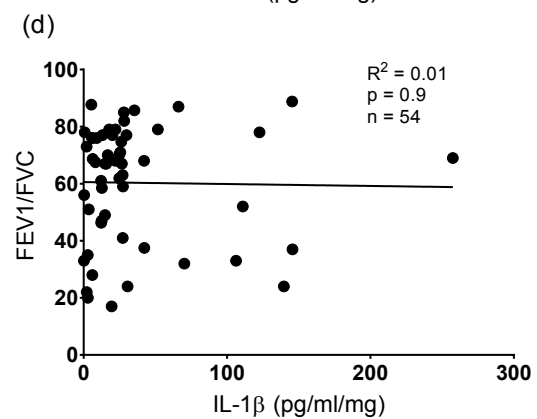
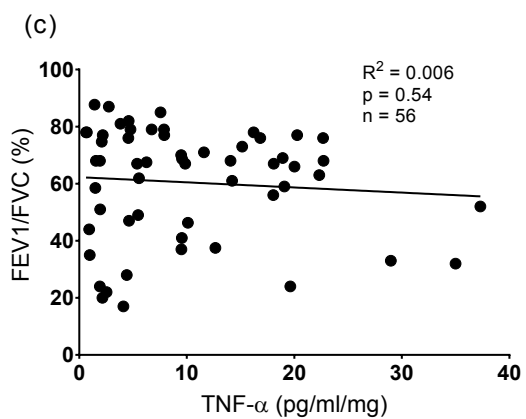
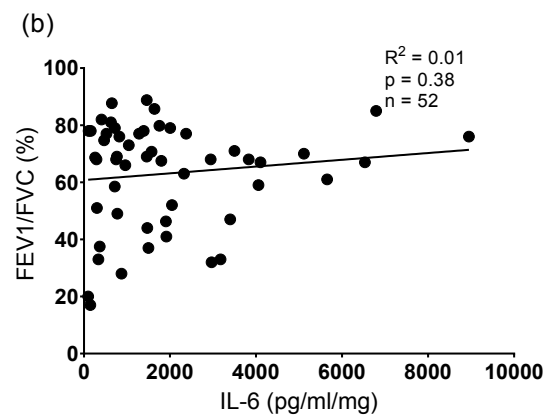
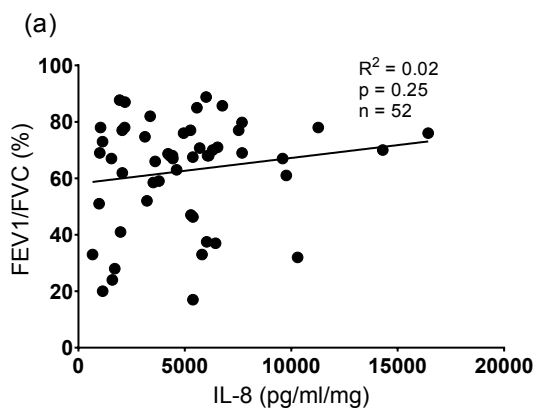


Figure E4

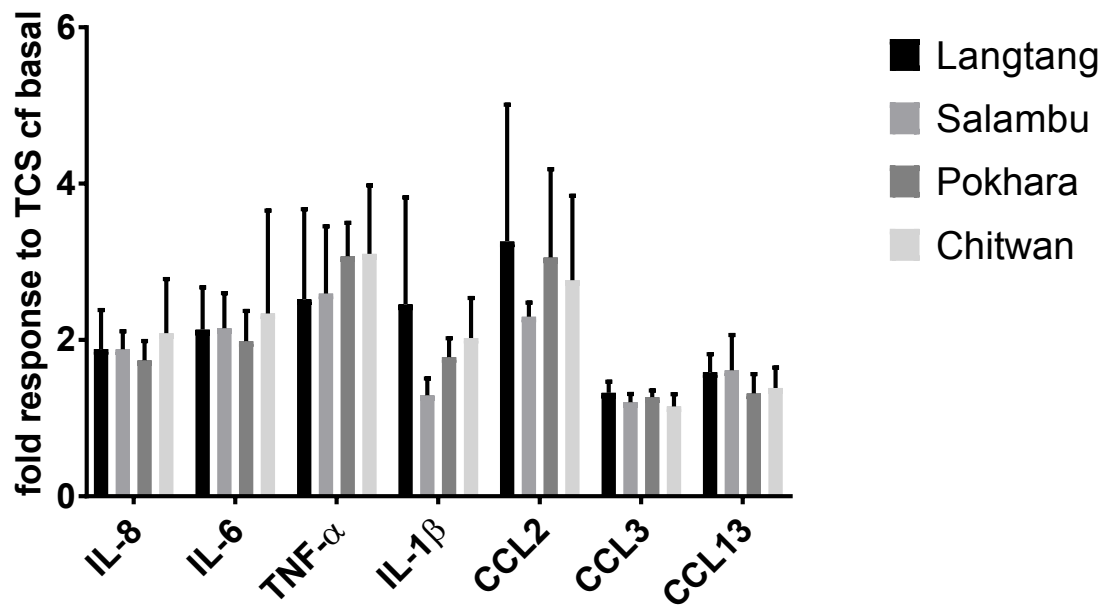


Table E1: Correction factor for all sets of Aerocet for PM_{2.5} measurement in all monitoring sites.

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

Table E2: Patient demographics of lung tissue donors

Donor ID	Age	Gender	FEV1 (% predicted)	FEV1/FVC (%)	Smoking Status	BMI
D1	68	M	26.6	37	Ex-smoker	20.83
D2	51	F	54	58.5	Ex-smoker	n/a
D3	49	M	55.4	74.7	Ex-smoker	30.6
D4	47	F	121.4	88.8	Ex-smoker	21.3
D5	71	M	58.4	37.5	Ex-smoker	34.83
D6	62	F	104.9	70	Ex-smoker	23.09
D7	71	M	86.1	68.7	Ex-smoker	31.46
D8	57	M	98.9	87.7	Ex-smoker	21.88
D9	64	M	42	85.7	Never Smoker	28
D10	65	M	95.7	79.8	Ex-smoker	27
D11	70	F	52.4	46.3	Current Smoker	23.93
D12	n/a	n/a	105	69	Ex-smoker	24.9
D13	72	F	107.8	70.7	Never Smoker	31.73
D14	55	M	80.2	67.5	Ex-smoker	32.92
D15	74	M	91.9	61.9	Current Smoker	23.15

n/a: data not available

References

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E2. Armstrong J, Sargent C, Singh D. Glucocorticoid sensitivity of lipopolysaccharide-stimulated chronic obstructive pulmonary disease alveolar macrophages. *Clin Exp Immunol* 2009; 158(1): 74-83.

E3. Gao J, Iwamoto H, Koskela J, Alenius H, Hattori N, Kohno N, Laitinen T, Mazur W, Pulkkinen V. Characterization of sputum biomarkers for asthma-COPD overlap syndrome. *Int J Chron Obstruct Pulmon Dis* 2016; 11: 2457-65.