Sputum analysis is an indicator of deteriorating pulmonary health: Study on rural Indian women chronically exposed to biomass smoke during cooking

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Household air pollution (HAP) is a major health concern in the developing nations of Asia like India. Nearly 74% households in India use biomass for daily household cooking.
Rural women use crop residues, dried leaves, dung cake (biomass) as fuel for cooking
• Absence of separate kitchen

• Kitchens are poorly ventilated

• High levels of indoor air pollution as **biomass smoke** contains wide spectrum of **potentially health-damaging pollutants**: coarse, fine and ultrafine particles, carbon monoxide, oxides of nitrogen and sulfur, transitional metals, polycyclic aromatic hydrocarbons, volatile organic compounds, bioaerosols and black carbon
Air pollution exposure affect the structural integrity of the respiratory system; sloughing off of airway cells

↓

Cytological changes in respiratory epithelium; PM phagocytosis by the alveolar macrophages (AM)

↓

Particle-laden AMs generate ROS and TNF-α in airways

↓

TNF-α induces IL-8 production by airway epithelial cells

↓

Influx of activated inflammatory cells to the lung leading to pulmonary inflammation

↓

Cytokines produced by neutrophils, macrophages mediate inflammatory process

↓

Continuous insult by airborne pollutants leads to cellular damage that may lead to development of airway diseases
Why and What

• Microscopic examination of the exfoliated cells in spontaneously expectorated sputum provides important information regarding the pathophysiological changes in the lung tissue and development of lung disease including malignancy.

• Since most of the airway diseases, including cancer, take a long latent period to develop, the cellular changes are immensely helpful to identify persons at risk so that medical intervention can be initiated at an early stage for better therapeutic response.

• In view of this, an attempt was made to find out the cytological changes in sputum and examine the inflammation- and oxidative stress-induced changes in airway cells.

• Associate outcomes with levels of particulate matter (PM) and benzene - representatives of air pollutants in biomass smoke.
**Study period:** Summer months (April and May) of 2009 and 2010

**Study design:** Cross-sectional

**Ethics:** Study protocol was approved by the Ethical Committee of Chittaranjan National Cancer Institute, Kolkata, India
Participant recruitment and sample size
Selection criteria of sites:

- Location at least 5 km away from the national or state highways to minimize the impact of vehicular pollution

- No air-polluting industry like coal-based thermal power plant, brick kiln, sponge iron factory and rice mill within 5 km radius in order to control the impact of industrial pollution

- Both LPG and biomass are used by the villagers for domestic cooking
Total number of participants: 345 women

Exposed group: **196** biomass-using women
(median age: 34 years, range: 22-41)

Control group: **149** LPG-using women
(median age: 33 years, range: 23-40)

Inclusion criteria
✓ apparently healthy women
✓ non-smokers and non-chewers of tobacco
✓ cook regularly with either biomass or LPG for at least 2 h/day, 5 days/week for ≥5 years

Exclusion criteria
✗ mixed fuel users (biomass plus LPG/kerosene)
✗ pregnant or lactating
✗ currently under medication
✗ extreme body mass index (BMI <19 and >30 kg/m²)

Prior informed consent was taken from the participants of this study
Questionnaire survey

Information on demographics (age, education, habits, occupation of the participants, average family income, cooking hours per day, cooking-years, kitchen and fuel type, family), occupation of the spouse and environmental tobacco smoke (ETS) was collected through personal interview using structured questionnaire. Smoking habit of any member of the family was taken as exposure to ETS.
Sample collection for staining

- Sputum cytology is a cost-effective, non-invasive way of airway change detection, and is free of investigating complications

- **Early morning spontaneously expectorated sputum** samples were collected in sterile plastic sputum containers

- **Participants rinsed their mouth** with saline water and coughed vigorously to expectorate sputum

- **Four smears** were made on clean glass slides from the non-transparent high viscosity part of each sample

- Two samples for cytological analysis (qualitative and quantitative evaluation) using **Papanicolaou’s (Pap)** procedure and the other 2 slides were for **Perl’s Prussian blue method** (for evaluating siderophages, which are iron-containing alveolar macrophages)

- Each specimen was **labelled, fixed** (30 minutes in ethyl alcohol for Papanicolaou staining, and 10 min in 10% formalin for Perl’s Prussian blue reaction) and taken back to the laboratory for further examination
Indoor Air Quality

Aerosol monitor was placed in the breathing zones of the women cooking with biomass fuel or liquefied petroleum gas (LPG)
<table>
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<th>Parameters</th>
<th>Methods</th>
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<tr>
<td>Urinary trans, trans-muconic acid (t,t-MA)</td>
<td>High performance liquid chromatography with ultraviolet detector (HPLC-UV)</td>
</tr>
<tr>
<td>Sputum cytology</td>
<td>i. Papanicolaou staining, ii. Perl’s Prussian Blue method</td>
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<tr>
<td>IL-6, IL-8, TNF-α</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Flow cytometry</td>
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<tr>
<td>SOD</td>
<td>Spectrophotometry</td>
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</table>
Statistical analysis

- The results were statistically analyzed using **SPSS** statistical software (Statistical Package for Social Sciences for windows, release 10.0, SPSS Inc., Chicago, IL, USA)

- Any measured parameter was treated as a variable, either continuous (when computing univariately for correlation) or dichotomous (when examining association)

- Univariate analysis was carried out using Spearman's rank correlation test to find out the relation between two measurable parameters as continuous variables, and the result was expressed as rho value

- Multivariate logistic regression analysis was done

- Statistical **significance** was assigned at **p<0.05**
## Socio-demography of participants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LPG user (n=149)</th>
<th>Biomass user (n=196)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in year, median (range)</td>
<td>33 (23-40)</td>
<td>34 (22-41)</td>
</tr>
<tr>
<td>Body mass index (kg/m²), median (range)</td>
<td>23.7 (22.6-24.8)</td>
<td>23.4 (22.2-24.1)</td>
</tr>
<tr>
<td>Years of cooking, median (range)</td>
<td>15 (5-18)</td>
<td>16 (5-20)</td>
</tr>
<tr>
<td>Cooking hours per day, median (range)</td>
<td>3.0 (3-5)</td>
<td>3.5 (3-6)</td>
</tr>
<tr>
<td>Homes with separate kitchen (%)</td>
<td>89.4</td>
<td>59.4*</td>
</tr>
<tr>
<td>Smokers in the family (%)</td>
<td>51.3</td>
<td>53.7</td>
</tr>
<tr>
<td>Years of schooling, median (range)</td>
<td>9 (0-15)</td>
<td>4 (0-10)#</td>
</tr>
<tr>
<td>Food habit, mixed (%)</td>
<td>98.3</td>
<td>98.8</td>
</tr>
<tr>
<td>Occupation of the participants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Household works only (%)</td>
<td>88.6</td>
<td>86.9</td>
</tr>
<tr>
<td>Household + agricultural work (%)</td>
<td>11.4</td>
<td>13.1</td>
</tr>
<tr>
<td>Members in family, median (range)</td>
<td>4 (3-6)</td>
<td>5 (3-8)</td>
</tr>
<tr>
<td>Average family income per month (in US $)</td>
<td>85</td>
<td>43**</td>
</tr>
</tbody>
</table>

Significant (p<0.05) compared with control in *Chi-square test, #Mann-Whitney U test, and **Student’s t-test
Particulate pollution in indoor air

Box-whisker plots showing concentrations of PM$_{10}$ during cooking hours inside kitchens using exclusively liquefied petroleum gas or biomass fuel for cooking purpose. The lines across each box plot represent the median value. The lines that extend from the top and the bottom of each box represent the lowest and highest observations still inside the lower and upper limit of confidence.

$276 \pm 108 \mu g/m^3$

$97 \pm 36 \mu g/m^3$

2.9-times higher
Benzene exposure (urinary t,t-MA level)

LPG users: 1.1 ± 0.7 mg/l
Biomass users: 6.4 ± 4.3 mg/l

5.8-times higher
Quality Control of sputum samples

- Processed whole sputum samples were stained with Pap to obtain cell differentials by counting at least 1000 non-squamous cells.

- Sputum was not used if the percentage of squamous cells was greater than 80% or if the total number of non-squamous cells was less than 1000.

- Sputum of 14 (7.1%) biomass users and 10 (6.7%) LPG-using women were discarded as they contained > 80% squamous epithelial cells and saliva but not alveolar macrophages (AM) and therefore, not representative samples from the lower airways.

- The remaining samples from 182 biomass-users and 139 LPG-users were further examined.
**Differential distribution of cells in expectorated sputa of participants**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>LPG users (n=139)</th>
<th>Biomass users (n=182)</th>
<th>95% Confidence Interval for the difference in mean change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell/hpf</td>
<td>64.1 ± 11.2</td>
<td>88.2 ± 14.5*</td>
<td>21.3-26.9</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>74.2</td>
<td>69.9</td>
<td></td>
</tr>
<tr>
<td>Neutrophil/hpf</td>
<td>47.6 ± 8.3</td>
<td>61.7 ± 10.1*</td>
<td>12.1-16.1</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>2.8</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Eosinophil/hpf</td>
<td>1.8 ± 0.3</td>
<td>4.7 ± 0.8*</td>
<td>2.8-3.0</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>4.5</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte/hpf</td>
<td>2.9 ± 0.5</td>
<td>5.6 ± 0.9*</td>
<td>2.6-2.9</td>
</tr>
<tr>
<td>Alveolar macrophage (%)</td>
<td>5.9</td>
<td>12.8**</td>
<td></td>
</tr>
<tr>
<td>Alveolar macrophage/hpf</td>
<td>3.8 ± 0.7</td>
<td>11.3 ± 1.9*</td>
<td>7.2-7.8</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD and percentages; hpf, high power field of microscope (x400); p<0.05 compared with control in Student’s t-test (*) and Chi-square test (**)
Neutrophilia

Absolute number of neutrophils in sputum was 1.3-times higher in biomass users

Marked increase in neutrophil number in sputum of biomass users (b) compared with control (a)

Papanicolaou-stained

Sputum neutrophilia (neutrophil ≥75%) was found in 23.6% of biomass users compared to 12.9% of control (p < 0.05)
Eosinophilia

Marked increase in eosinophil number in sputum of biomass users (b) compared with control (a)
Papanicolaou-stained

Sputum eosinophilia (eosinophil ≥3%) that represent airway hypersensitivity to cumulative smoke exposures was found in 10.4% of biomass users against 4.3% of control women (p < 0.05)
Lymphocytosis

Absolute number of lymphocytes was 40% higher in sputum of biomass-using women

Marked increase in lymphocyte number in sputum of biomass users (b) compared with control (a) Papanicolaou-stained
Alveolar Macrophage (AM)

Biomass users had 3-times more AM number in sputum than the controls

Abundance of particle-laden alveolar macrophages in expectorated sputum of biomass users (b) compared with control (a)

Papanicolaou-stained
Siderophages (iron-containing AM)

Presence of siderophages detects **covert pulmonary hemorrhage** as deposition of iron causes oxidative stress, inflammation, and lung injury.

Mean number of siderophages per hpf was 5.1 (±2.0) in biomass users and 0.6 (±0.2)/hpf in the controls.

Also, 7.7% biomass users had Golde score more than 100 compared to 0.7% in the controls.

Blue-stained siderophages in sputum of a control woman (a) and a biomass-using rural woman (b). Note increased number of siderophages and heavy iron deposition in these cells in **b**. Perl’s Prussian blue reaction

- An average of 100 AM were graded for hemosiderin on a scale of 0-4, 0 being the minimum and 4 the maximum score for 100 macrophages.

- Hemosiderin resorption as per **Golde score** : 0-20 normal; 20-70 medium; >70 high.
Ciliocytophthoria

Presence of disintegrated distal tufts of cytoplasm with intact cilia from columnar epithelial cells

Present in 9.3% of sputum samples from biomass users compared with 1.4% of LPG users

Ciliocytophthoria in sputum of biomass-using rural women, suggesting respiratory viral infections in the airways. Papanicolaou-stained
Charcot-Leyden crystals

Cell membrane of eosinophils contains several enzymes including lysolecithinase. Together with phospholipase A and D, lysolecithinase spontaneously forms these rhomboid-shaped structures.

Present in 1.1% sputum samples of biomass users but none in LPG users.

Presence of Charcot-Leyden crystals in sputum of a village woman who used to cook exclusively with biomass fuel, suggesting airway allergy. Papanicolaou-stained.
Curschmann’s spiral

Present in sputum of 2.7% of biomass users against 0.7% of LPG users

Presence of Curschmann’s spiral in biomass users. Papanicolaou-stained
Air Pollution induces generation of Oxidative Stress

**2-fold increase in ROS generation in biomass-using women**

- **MFI of ROS generation**
  - Biomass: 338.3 ± 39.4
  - LPG: 706.4 ± 67.9
  - *P < 0.001*

**Erythrocyte SOD level declined by 31% in biomass-using women**

- **SOD (U/ml)**
  - Biomass: 775.4 ± 85.7
  - LPG: 535.3 ± 114.3
  - *P < 0.001*
Markedly elevated levels of pro-inflammatory mediators in sputum of biomass users implying pulmonary inflammation

- IL-6: 16.4 ± 4.8 (LPG), 59.5 ± 11.6 (Biomass) - 3.6-times higher in Biomass, \( P < 0.001 \)
- IL-8: 10.1 ± 3.3 (LPG), 26.7 ± 7.4 (Biomass) - 2.6-times higher in Biomass, \* difference
- TNF-alpha: 12.6 ± 6.2 (LPG), 36.9 ± 28.1 (Biomass) - 6.9-times higher in Biomass, \* difference
Some other cytological observations

Metaplasia (a) and Dysplasia (b) of bronchial epithelial cells - early morphological abnormalities towards the pathway of epithelial carcinogenesis

Goblet cell hyperplasia (c) - suggesting excess mucus production presumably to combat inhaled particulate pollutants

Clumps of ciliated and non-ciliated columnar epithelial cells (d) - suggesting injury to the airway wall following cumulative exposures to airborne pollutants
Associations of HAP with the outcomes
Logistic regression analysis of the association between particulate air pollution and sputum counts after adjusting for potential confounders

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Odds ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{PM}_{10}$</td>
</tr>
<tr>
<td>Cell/hpf</td>
<td>1.46 (1.12-3.38)</td>
</tr>
<tr>
<td>Neutrophil/hpf</td>
<td>1.38 (1.10-3.25)</td>
</tr>
<tr>
<td>Eosinophil/hpf</td>
<td>1.84 (1.38-4.49)</td>
</tr>
<tr>
<td>Lymphocyte/hpf</td>
<td>1.51 (1.23-3.58)</td>
</tr>
<tr>
<td>Alveolar macrophage/hpf</td>
<td>1.57 (1.14-3.27)</td>
</tr>
<tr>
<td>ROS</td>
<td>1.35 (1.13-1.78)</td>
</tr>
<tr>
<td>SOD</td>
<td>1.24 (1.13-1.34)</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.31 (1.12-1.46)</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.43 (1.28-1.89)</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>1.13 (1.04-1.21)</td>
</tr>
</tbody>
</table>

Potential confounders: education, family income and kitchen location
Correlation between years of exposure to air pollution and sputum cytological changes

Spearman’s rank correlation (rho value) between lifetime exposure and sputum cells

<table>
<thead>
<tr>
<th>Sputum cell parameters</th>
<th>Exposure to indoor air pollution from biomass burning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rho value</td>
</tr>
<tr>
<td>Cell/hpf</td>
<td>0.67</td>
</tr>
<tr>
<td>Neutrophil/hpf</td>
<td>0.32</td>
</tr>
<tr>
<td>Eosinophil/hpf</td>
<td>0.26</td>
</tr>
<tr>
<td>Lymphocyte/hpf</td>
<td>0.23</td>
</tr>
<tr>
<td>Alveolar macrophage/hpf</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Potential confounders: education, family income and kitchen location

Positive association between air pollution exposure and sputum cell number
Some points

- Assessing airway cytology using spontaneously expectorated sputum as the sample is a **safe, non-invasive and cost-effective technique** for the collection of samples from the airways.

- Examining sputum in the participants of this study revealed elevated numbers of alveolar macrophages and airway inflammatory cells, ciliocytophthoria suggesting hypersensitivity, inflammation and oxidative stress amongst the HAP-exposed groups of this study.

- Findings suggest that air pollution exposures through respiration can alter the function of airway cells and lung defence.

- Increased cell count in sputum observed in HAP-exposed women of this study may develop from influx of circulating leukocytes into the lungs to combat inhaled pollutants including bioaerosols.

- Abundance of iron-containing macrophages (siderophages) in sputum of the HAP-exposed women was also notably high suggesting covert pulmonary hemorrhage.
Conclusion

- In essence, this study has shown that women chronically exposed to pollution from biomass smoke have altered sputum cytology, increased pulmonary inflammation and airway oxidative stress.

- These may be the underlying key players involved in causing deterioration of the health of biomass-using women.

- Hence, intervention steps must be implemented by the regulatory authorities and policy makers to ensure good health and living conditions to the poor underprivileged women of rural India.
Publication from this study

Changes in sputum cytology, airway inflammation and oxidative stress due to chronic inhalation of biomass smoke during cooking in premenopausal rural Indian women

Anindita Dutta, Sanghita Roychoudhury, Saswati Chowdhury, Manas Ranjan Ray
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- Participants of the study
- My family
- ISEE 2014 Committee
Let us join hands to curb air pollution for a greener tomorrow

THANK YOU