Biomarker: Potential lead in cookstove-health studies

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According to WHO, of 56 million global deaths in 2012, 38 million, or 68%, were due to non-communicable diseases (NCDs).

NCDs constitute the cardiovascular diseases, cancers, diabetes, chronic lung diseases, etc.

Biomass smoke exposure plays a crucial role in NCD development as it contains a wide spectrum of potentially health-damaging pollutants.

Ultrafine particles (UFPs; diameter < less than 0.1 μm) represent a substantial proportion of particulate pollutants in biomass smoke.

Inhaled UFPs can readily cross the alveolar-capillary barrier reaching blood stream.

In circulation, UFPs adsorb onto the surface of erythrocytes and can travel to all vital organs of the body including the brain.

Therefore, exposure to biomass smoke can mediate systemic alterations besides its adverse effects on the lung, airways, heart, blood vessels, vital organs of the body.
Biomarkers from the field

- Biomarkers used in biomass smoke exposure studies

- **Inflammation** and **oxidative stress** are the main pathways leading to disease development

- To study development of cardiovascular diseases (CVDs) and chronic lung/airway diseases

  1. Alveolar macrophages (AMs): in sputum
  2. Tumor necrosis factor (TNF)-alpha: in sputum and blood
  3. Interleukin-8: in sputum and blood
  4. Interleukin-6: in sputum and blood
  5. C-reactive protein (CRP): in blood
  6. Platelet P-selectin (CD62P): in blood
  7. Soluble P-selectin (sP-sel): in blood
  8. Platelet aggregate: in blood
  9. Oxidized low density lipoprotein (oxLDL): in blood
  10. Circulating anticardiolipin antibodies (aCL): in blood
  11. Reactive oxygen species (ROS): in sputum and blood
Mechanism

Air pollution exposure, deposition of PM in airways

PM phagocytosis by the alveolar macrophages (AM)

Particle-laden AMs generate ROS and TNF-α

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

Upregulation of adhesion molecules in vascular endothelium and circulating leucocytes

Leukocyte immigration from blood to airways, ROS generation

UFPs migrate to blood, induce production of C-reactive protein and fibrinogen in liver

Platelets activation, coagulation cascade, oxLDL

Generation of foam cells, plaque formation in arteries, atherosclerosis

Rupture of plaques leading to angina and myocardial infarction
Alveolar Macrophages (AMs)

First line of cellular defense in the lungs
**Alveolar Macrophage (AM)**

- Macrophages are the principal defence cells in the airways and alveoli
- Include AMs, airway macrophages and interstitial macrophages
- AMs are the dominant phagocytic cells
- AMs play a pivotal role in lung defence through their activities like particle clearance from the inner airways by endocytosis and phagocytosis, followed by killing of invading pathogens via generation of oxygen radicals and release of degrading enzymes
- AMs actively participate in inflammation, wound healing and tissue repair through their vast array of secretory products
- In adult human lungs, there are approximately 480 million alveoli and each alveolus is defended by about 73 macrophages. Therefore, human lungs contain around 35 billion AMs for its defence
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 \textit{covert pulmonary hemorrhage} as deposition of iron causes oxidative stress, inflammation, and lung injury.

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.

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.

Perl’s Prussian blue reaction
Mediators of inflammation:

Pro-inflammatory cytokines and acute phase proteins as markers

1. Tumor necrosis factor-alpha (TNF-α)
2. Interleukin-8 (IL-8)
3. Interleukin-6 (IL-6)
4. C-reactive protein (CRP)
Tumor necrosis factor-alpha (TNF-α): in sputum and in blood

- A pleiotropic pro-inflammatory cytokine, causally involved in inflammatory bowel disease, rheumatoid arthritis, diabetes, multiple sclerosis and various pulmonary diseases

- Increases cardiovascular risk via different mechanisms, mostly taking place in endothelium, like inhibition of insulin signaling leading to nitric oxide synthesis in the endothelial cells, stimulation of adhesion molecules expression, etc.

- TNF-α stimulates IL-8 synthesis and release by different cell types

- TNF-α might have an influence on atherogenic process through its local action in adipose tissue, not only by inducing insulin resistance, but also by modulating adipocyte endocrine and paracrine function, for instance by increasing IL-8 synthesis and secretion

- Can be easily measured by enzyme-linked immunosorbsent assay (ELISA)
Interleukin – 8:
in sputum and in blood

- Pro-inflammatory cytokine belonging to a family of small proteins of 8–10 kDa
- Produced by various cell types involved in atherosclerosis, including peripheral blood monocytes, neutrophilic granulocytes, epithelial cells of various origins, T cells, fibroblasts, endothelial cells and vascular smooth muscle cells
- Chemotactic for neutrophils and T cells and recruits them to sub-endothelial space; involved in the immigration of monocytes into the subendothelial space
- IL-8 mediates downregulation of tissue inhibitors of metalloproteinases (TIMP) by oxLDL that ultimately results in the increased release of matrix-degrading metalloproteinases and in consequence the instability of atherosclerotic plaque
- OxLDL can stimulate production and secretion of IL-8 by macrophages from human atherosclerotic plaques
- Can be easily measured by enzyme-linked immunosorbent assay (ELISA)
Interleukin – 6:
in sputum and in blood

✓ A 26-kD cytokine with pleiotropic activities in both the immune and hematopoietic systems

✓ Produced in response to inflammatory stress by different cell types that include T cells, macrophages, and fibroblasts; one of the major regulators of the acute-phase response

✓ Elevated blood levels of IL-6 have been implicated in the pathogenesis of sepsis, acute respiratory distress syndrome, multi-organ failure, chronic inflammatory conditions such as rheumatoid arthritis, and it reduces FEV₁

✓ Can be easily measured by enzyme-linked immunosorbent assay (ELISA)
Markedly elevated levels of pro-inflammatory mediators in sputum of biomass users implying pulmonary inflammation

<table>
<thead>
<tr>
<th></th>
<th>Concentrations (pg/ml)</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>16.4 ± 4.8</td>
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<tr>
<td>IL-8</td>
<td>10.1 ± 3.3</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>12.6 ± 6.2</td>
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<td>LPG</td>
<td></td>
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<tr>
<td>Biomass</td>
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- IL-6: 59.5 ± 11.6
- IL-8: 26.7 ± 7.4
- TNF-alpha: 86.9 ± 28.1

P < 0.001

3.6-times for IL-6
2.6-times for IL-8
6.9-times for TNF-alpha

* indicates statistical significance.
Markedly elevated levels of pro-inflammatory mediators in blood of biomass users implying systemic inflammation

- IL-6: 1.6 times
- IL-8: 2.4 times
- TNF-alpha: 3.5 times

P < 0.001
C-reactive protein: in blood

- Acute phase protein; increases during systemic inflammation; made by the liver and secreted into the bloodstream

- Production regulated, principally, by IL-6 and TNF-α

- Promotes all stages of atherosclerosis, including plaque rupture

- Actively participates in atherosclerotic processes like: it induces blood monocytes to produce tissue factor, which, in turn, initiates coagulation; mediates LDL uptake by macrophages with the formation of foam cells; promotes complement activation, that, in turn, activates coagulation

- Can be easily measured by enzyme-linked immunosorbent assay (ELISA)
## C Reactive Protein and cardiovascular risk

<table>
<thead>
<tr>
<th>Cut-off value (mg/l CRP)</th>
<th>LPG users (%)</th>
<th>Biomass users (%)</th>
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</thead>
<tbody>
<tr>
<td>High CVD risk &gt;3.0</td>
<td>12.7</td>
<td>91.4</td>
</tr>
<tr>
<td>Average CVD risk 1.0-3.0</td>
<td>87.3</td>
<td>8.6</td>
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</tbody>
</table>
Platelets as markers

1. Platelet P-selectin (CD62P) – Flow cytometry
2. Soluble P-selectin (sP-sel) – ELISA
3. Platelet aggregation - Aggregometry
Activated blood platelets - I

- Platelet P-selectin (CD62P) is the surface marker of activated platelets
- Can be easily measured by flow cytometry

Overexpression of platelet P-selectin (CD62P)

% of 62P+ platelets

LPG

Biomass

4-times higher

* 9.1

2.4
Activated blood platelets - II

✓ In addition to being a platelet granule protein, P-selectin is also present in blood plasma as soluble P-selectin (sP-sel)

✓ Elevated level of sP-sel has been recorded in patients with hypertension, hyperlipidemia, atherosclerosis, etc.

✓ Can be easily measured by enzyme-linked immunosorbent assay (ELISA)

Elevated level of soluble P-selectin in plasma

Effect: Increased platelet activity, CVD
Platelet aggregometry

- Platelet hyperactivity is important for early phases of atherosclerosis, and platelet aggregation and thrombus formation are important underlying mechanisms of myocardial infarction, stroke and peripheral arterial disease.

- Platelets are also involved in inflammatory lung diseases and play a crucial role in the pathogenesis of acute lung injury.

- Can be measured in platelet aggregometer.
Histograms showing collagen-induced platelet aggregation in vitro. A higher rate of impedance was recorded in platelets collected from biomass-using women, suggesting increased aggregability. Bars indicate standard deviation of data; *P < 0.05 compared with liquefied petroleum gas–using control in Students t-test.
Oxidized Low Density Lipoprotein (oxLDL)

- Oxidative stress, oxidation of low density lipoprotein (LDL), generation of oxidized LDL (oxLDL), and transport of native and oxLDL across the endothelium into the artery wall are important initiating events for atherosclerosis.

- Circulating and especially tissue-retained oxLDL increases the inflammatory state of the endothelium.

- OxLDL induces foam cell formation, alteration of nitric oxide signalling, initiation of endothelial activation, and expression of adhesion molecules that accelerate leukocyte homing to the site of atherosclerosis.

- Can be easily measured by enzyme-linked immunosorbent assay (ELISA).
OxLDL in blood, increasing risk of CVD

3.7-times

Lowest oxLDL value observed among biomass-using women was more than the highest value recorded in the control group

Box-whisker plots showing concentrations of oxidized low-density lipoprotein in plasma of women who used to cook exclusively with liquefied petroleum gas or biomass fuel. 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. The circles outside the box represent outliers. The difference between these two groups was statistically significant (P < 0.05) in Mann–Whitney U test.

Dutta, A. et al./Indoor Air 21 (2011) 165–176
Anticardiolipin antibodies (aCL)

Cardiolipin (CL, bisphosphatidyl glycerol) is an important component of the inner mitochondrial membrane, typically present in metabolically active cells of the heart and skeletal muscles.

Serves as an insulator and stabilizes the activity of protein complexes important to the electron transport chain.

Only negligible amount of autoantibodies against CL (aCL) is present in blood of general population, but elevated levels of aCL have been found in association with risk of atherosclerosis and blood clots in veins or arteries.

Follow up studies have confirmed serum autoantibody against cardiolipin as a risk factor for developing CVD.

Can be easily measured by enzyme-linked immunosorbent assay (ELISA).
Anti-cardiolipin antibodies (aCL IgG, aCL IgM) in serum

% women above cut-off value of 10 GPL

- aCL IgG
  - LPG
  - Biomass

% women above cut-off value of 15 MPL

- aCL IgM
  - Biomass

- Values with an asterisk (*) indicate statistical significance.
Oxidative stress marker

Reactive oxygen species (ROS)

- An important component of oxidative stress are the reactive oxygen species (ROS) that include superoxide anion radical (O2–) and hydrogen peroxide (H2O2)

- Mitochondria are the largest producer of ROS

- Generation of ROS does not necessarily mean oxidative stress; it is the imbalance between levels of ROS and antioxidants that result in oxidative stress

- Can be easily measured by flow cytometry
ROS in sputum and in blood

2-fold increase in ROS generation in sputum of biomass-using women

44% increase in ROS generation in blood of biomass-using women
Conclusion

- Biomarkers may be used as good indicators to study development of NCDs
- Biomarkers should be widely used in cookstove intervention studies
- There are a lot of other biomarkers that need to be explored and implemented
- Biomarkers provide easier tools for identification of diseases
Acknowledgement

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Let us join hands for a greener today and tomorrow

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