MATERIAL AND METHODS

Disease-manifestation in an occupational setting can culminate from the involvement of environmental factors and genetic pre-disposition in concert with genomic instability from oxidative stress modulated by inherent metabolic genetic polymorphism(s). In the present study, therefore, initially COPD was identified from among stone-crushing workers initially using the spirometry analysis for pulmonary function. Genomic instability was ascertained in multiple tissues of COPD cases. The peripheral blood samples were also assessed for oxidative stress, alpha antitrypsin activity and lipid levels, and for extraction of whole genomic DNA. Multiplex and Polymerase Chain Reaction (PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP) were performed for single nucleotide polymorphism (SNP) analysis of some xenobiotic and COPD candidate genes. The study was conducted on COPD cases (n=200) and healthy controls (n=200) and the methodologies adopted are detailed below.

3.1 Institutional Ethics Clearance

The study protocol was cleared from all ethical angles by the Institutional Ethics Committee (IEC) of Guru Nanak Dev University, Amritsar in accordance with the Declaration of Helsinki as stated in the guidelines of the Indian Council of Medical Research (ICMR, 2006).

3.2 Informed Consent

The study was initiated only after voluntary written informed consent from the study participants (Appendix Ia, Ib). The consent form was also orally translated into the Hindi vernacular understood by the study participants who are migrants from the state of Bihar. Participation was completely voluntary and the participants were explained about the nature of the study, the experimental design, samples requirements, research outcomes and freedom of withdrawal at any time during the course of the study.

3.3 Site-Identification

The sites from where cases were to be identified entailed a field survey of stone-crushing units located in Gurdaspur and Pathankot districts. The unit heads were explained about the study and pertinent information about the stone-crushing units was recorded and compared with pre-existing requisitions available in guidelines issued by
the Punjab State Pollution Control Board (PPCB, 2009) in consonance with those of the Central Pollution Control Board (CPCB, 2009). Details on work activities, working schedules, work duration and the workforce were also maintained for each unit on a specific proforma (Appendix II). As many as 32 stone-crushing units (12 from Gurdaspur and 20 from Pathankot districts) were surveyed and individual contact was made with a total of 289 workers.

3.4 Site-Selection

The survey observations formed the basis for the selection of sites (n=22) for the main study from where cases were identified. The criteria for site-selection included those units which were continuously-operational, where there was no objection from unit heads, workforce belonged to same population sub-group and participants voluntarily provided written consent.

3.5 Effective Sample-Size and Power of the Study

The effective sample-size of the study was calculated on the basis of baseline averages of the assessed parameters of genetic damage in peripheral blood leukocytes (per cent DNA in tail), buccal mucosal cells and urothelial cells (micronucleated cell frequency) and the oxidative stress parameters in blood-sera obtained from a preliminary study on the same ethnic-specific group, and considering statistically significant increase at 80% power and a primary (alpha) error of 5% (Albertini et al., 2000). The calculated sample-size ranged between 80-120 and after taking into account, the minor allele frequencies (MAFs) of the seven SNPs (as available in literature and on consultation from SNPedia (http:www.snpedia.com), the sample-size increased to 132-168 (CATS Power calculator, www.cats-power-calculator.software.informer.com). In order to compensate for contingencies, an increase of 10% was compounded and rounded-off and therefore the study group (n=400) comprised 200 cases and 200 controls. On completion of the study, the retrospective power is 82-90% for various parameters (Appendix III).

3.6 Proforma-Designing and Validation

A proforma was specially-designed (Appendix II) after perusal of literature so that important occupational-specific information could be gathered and appropriately recorded from the study participants. Initially, the proforma was validated in a pilot study (Kaur and Gandhi, 2011a) and was subsequently modified to fill the lacunae for
language and clarity. Records on family, medical, genetic and exposure histories and dietary and life-style patterns and general information were maintained on the proforma recommended for human population biomonitoring (Carrano and Natarajan, 1988).

3.7 Study Design

In accordance with the objectives of the research protocol, a case-control, cross-sectional study design was thought appropriate comprising 200 cases and 200 controls. The cases were identified at stone-crushing units on the basis of pulmonary function test and the health symptoms. Healthy controls contacted from the general population, were matched for the ethnic population sub-group, age, gender, socio-economic status, smoking habits and alcohol drinking with no past/present history of exposure and any illnesses in the past six months.

3.7.1 Study Participants

The study group comprised unrelated adult males (≥25y) with the capability to independently undertake the pulmonary function test for spirometric measurements and not on any prescribed/regular medication with any recent illnesses or hospital admissions.

Inclusion Criteria

Cases - Sub-group from Bihar with ratio of forced expiratory volume in one second to forced vital capacity (FEV₁/FVC) meeting the criteria of <0.7 as per the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2010, 2016), working in the stone-crushing occupation for at least five years.

Controls - Healthy males from the general population with FEV₁/FVC >0.7, without any occupational/incidental or accidental exposure(s), with no history of stone-crushing or related activities; of similar ethnic background and geographic area.

Exclusion Criteria-Minors, adolescents, those unable to perform spirometry, relatives, those on medication or with any disease, those not willing to participate, those in other/related occupations, no other population sub-groups.

3.8 Sensitization and Counseling on Health Issues

Periodic occupational health-surveillance is mandatory in the developed world as per the guidelines by Occupational Safety and Health Administration (OSHA, 2009) and
Science and Technology Facilities Council (STFC, 2014). However the situation is rather dismissal even at present times in India as health surveillance is not offered at the workplace. The workers at the stone-crushing units visited for the present study, complained of respiratory distress and self-reported various ill-health symptoms and yet had not consulted a physician and were not taking any treatment. In the course of contact with the participants, they were sensitized about the occupational hazardous contaminants and proffered advice on effective protective and management measures. Unit heads were also cautioned about adherence to recommendations (CPCB, 2009; PPCB, 2009) for setting-up of stone-crushing units, providing protective gear (gloves, masks and overalls to the employees) and provision of health care and treatment facilities for the workforce in the light of known hazardous contaminants in the occupational setting.

3.9 Study Population

Unskilled labour from Uttar Pradesh (UP), Madhya Pradesh (MP) and Bihar has been periodically migrating to Punjab since independence (Gill, 1984; Singh, 1995). The industrial sector of Punjab attracts workers from UP and Bihar because of better employment opportunities, higher wages, lower economic- and social-exploitation and absence of caste-oppression (Sethi et al., 2010). Though mainly employed in industry and agriculture, the migrants are also engaged in building- and road construction, quarrying, brick-making and rickshaw-pulling (Sethi et al., 2010). The voluntary out-migration from poor house-holds of Bihar is because of the better employment and income opportunities despite at wage-rates lower relative to that acceptable by the local workers in Punjab (Ansari, 2001; Ghosh, 2007). Generally such migrants to other states have more representation of scheduled castes, scheduled tribes and other backward classes (Rajan, 2011). This was also the case in the present study.

The study participants of the present study comprised workers (cases) and controls belonging to various villages (Appendix IV), for 12 of the 28 districts of the state of Bihar and comprised the Musahar, Dusadh, Chaupal, Nat, Bhuiya, Dhobi, Munda, Mochi and Pasi scheduled castes. Of the Indo-Aryan origin, the people of Bihar comprise the five castes of Brahman, Bhumihar, Rajput, Bania, and Kayastha (http://www.indianmirror.com/culture/states-culture/bihar.html). The population of
scheduled castes in Bihar stands at 15.70% with 90% living in villages and two-thirds are poor; 53.50% of the families are landless and 71% illiterate (Government of Bihar, 2013; www.finance.bih.nic.in/Documents/Reports/Economic-Survey-2013 accessed on 26 March 2016).

**Population studies** - Some studies in biology on population groups from Bihar include those on blood-group typing, phenyl thio carbamide (PTC) tasting ability and on red-green colour blindness (Pandey et al., 2013). Molecular genetic studies have included assessment of genetic variation using autosomal microsatellite markers in some groups from Bihar among others (Kashyap et al., 2006) whereby the Baniya of Bihar revealed similarity with the South Indian groups and the Brahmins from Bihar and Bihar Kurmi along with other groups belonged to multiple clusters sharing a common genetic structure. The eNOS (exon 7 Glu298Asp) variant in tea-garden workers (with high prevalence of hypertension) migrants in Assam belonging to Madhya Pradesh, Bihar, Orissa and Andhra Pradesh, who did not vary significantly for sub-groups and for gender-wise distribution of genotypes (Shankarishan et al., 2011).

The results of the present study hence provide information on scheduled caste groups from Bihar who have been comprehensively evaluated for COPD.

**3.10 Information Gathering**

A face-to-face interview method was followed for each study participant for their general information, demographic details and for other inputs on lifestyle patterns and occupations; for workers at stone crushing units, the occupational exposure details on duration of working in stone-crushing activities, daily work-schedule and the type of activity performed were recorded. Health symptoms associated with exposure were also documented. Smoking history was recorded as ‘pack years’ viz. the number of bidis (Indian hand-rolled cigarettes) smoked per day divided by 20 (20 bidis/pack) and subsequently multiplied by the number of years since smoking (Premanand et al., 2007). Brinkman index (BI) was also calculated as the number of bidis’ smoked per day multiplied by the number of years since smoking (Kojima et al., 2007). Also as socio-economic status (SES) is an important determinant of health, nutritional status, mortality and morbidity (Oberoi, 2015), therefore, SES of the study participants was also determined using the Kuppuswamy scale (Oberoi, 2015). The SES score of <5-29;
(lower class-upper class) is based on education (1-7; ranging from illiterate to professional, honours), occupation (1-7; unemployed-professional) and monthly income (1-12; ≤Rs.100 to ≥Rs. 2000). Pedigrees (up to three generations) were also prepared using recommended pedigree nomenclature (Bennett et al., 2008) and analyzed for reproductive performance, birth defects and hereditary/familial conditions as well as to confirm endogamy and exclude those with inter-caste marriages in their families.

**Soil Analysis** - Top layer soil sediments from 22 stone-crushing units in duplicate were subjected to analysis using Energy-dispersive X-ray spectroscopy with Scanning Electron Microscope (EDX-SEM). The Sole gel method (Hench and West, 1990) was used for preparation of glass system. Vigorous stirring of the soil samples in sole gel was carried out for 1h and a transparent solution was obtained. The gel was freshly prepared by heating at 60 °C for 12 h, and left at 120 °C for 12 h. The moisture samples was dried by keeping samples at 60 °C. Platinum coating was used for making the sample conductive. The soil samples were crushed in agar and mortar for one hour. The X-ray diffraction (XRD) method was carried out using BRUKER D8 FOCUS XRD machine. The Energy-dispersive X-ray spectroscopy identifies the particular elements and their relative proportions. The relative proportion of the element was provided as atomic per cent (atomic %). Particulate matter sizes on SEM were determined using the SEM 4 software available with the equipment (Plate I).

### 3.11 In Situ Monitoring/Assessment

At the workplace itself against the occupational setting at stone-crushing units, the worker-participants were administered the questionnaire and counseled regarding their recorded information. Blood pressure (BP) and anthropometric measurements were taken using standard procedures. Spirometry was performed to identify the cases. Healthy controls from the general population without any exposure history were similarly assessed. The biological samples were also collected at the sites and then transported to the laboratory on ice.

#### 3.11.1 Blood Pressure Measurements

The recommended procedure (Chobanian et al., 2003; IGH III, 2013; James et al., 2014) was adopted for taking blood pressure manually using a mercury sphygmomanometer after the participant had rested for 15min and was sitting upright
and relaxed on a chair with the left arm supported comfortably at the vertical level. The pressure cuff was applied close to the upper arm and the cuff was rapidly inflated. The stethoscope was placed lightly over the brachial artery and the mercury column was allowed to fall. The Korotkoff’s sound indicated the systolic blood pressure (SBP). The mercury column was then allowed to fall till the sound ceased to be tapping, and then disappeared. This level was taken as the diastolic blood pressure (DBP). Three consecutive readings were taken at intervals of five minutes each and the calculated averages were recorded. Consideration of the SBP and DBP readings as per Indian Hypertension Guidelines (IGH-III,2013) and as per the Joint National Committee (JNC 7; Chobanian et al., 2003; JNC 8 James et al., 2014) assisted in categorizing the study participants as normal (SBP <120 mmHg and/or DBP 80 mmHg), pre-hypertensive (SBP 120-129 mmHg and/or DBP 80-84 mmHg), hypertensive (SBP ≥140 mmHg and/or DBP 90 mmHg), stage I hypertensive (SBP 140-159 mmHg and/or DBP 90-99 mmHg), stage II hypertensive (SBP 160-179 mmHg and/or DBP 100-109 mmHg) and stage III hypertensive (SBP ≥180 mmHg and/or DBP 110 mmHg). Mean arterial blood pressure (MAP) was calculated according to Perusse et al. (1989) using the formula of MBP= DBP + (SBP - DBP)/3. The difference between SBP and DBP is taken as Pulse pressure (PP).

3.11.2 Anthropometric Variables

Anthropometric measurements of height, weight, waist circumference and hip circumference and the derived indices of Body Mass Index (BMI), waist-hip ratio (WHR) and waist-height- ratio (WHtR) are indicators of health-status and disease (Khanana et al., 2011). Body Mass Index (BMI) is the most established indicator for nutritional status (Adak et al., 2006; Banik et al., 2009). BMI is a measure of excess weight relative to height, whereas waist circumference (WC) and the waist-hip- ratio (WHR) are indicators of body fatness (Gupta and Kapoor, 2014). WC is an aggregate measurement of total abdominal fat accumulation, indicating the abdominal adiposity (Okosuna et al., 2000). Waist-height-ratio is considered very important for obesity-assessment (Hsieh and Muto, 2005), and high WHtR indicates clustering of coronary risk factors among non-obese individuals. The standard methodology (Weiner and Lourie, 1981) was used for taking measurements of height, weight, waist and the hip circumferences, viz.
**Height:** Height was measured using a non-stretchable steel tape to the nearest 0.10 cm with the participant standing erect with the head in the ear-eye plane and barefoot along a wall with arms hanging.

**Weight:** As the participants stood barefoot without making any movement on a weighing scale, weight in kilograms was recorded to the nearest 0.50 kg.

**Waist circumference (WC):** At a level midway between the lower rib margin and iliac crest, waist circumference was measured using a non-stretchable steel tape around the body in horizontal position to the nearest 0.10 cm. Participants with WC < 85 cm are considered non-obese while those with > 85 cm are obese considering the male cut-off value (Snehalatha et al., 2003).

**Hip Circumference (HC):** Hip circumference was measured to the nearest 0.10 cm on placing a non-stretchable steel tape at the point where there is maximum extension of the buttocks.

**Derived Indices** - Body Mass Index (BMI), Waist-Hip-Ratio (WHR) and Waist-Height-Ratio (WHtR) were derived from the measured anthropometric variables.

**Body Mass Index (BMI):** BMI was calculated as weight in kilograms divided by height in meters squared (kg/m²) using the BMI classification given by Misra et al. (2009) which almost matches that of WHO (2004) for Asian Indians. Participants were considered underweight with BMI < 18.00 kg/m² and being normal (18.00-22.90 kg/m²), overweight (23.00-24.90 kg/m²) or obese (> 25.00 kg/m²).

**Waist-Hip-Ratio (WHR):** The waist-to-hip circumference ratio of < 0.88 in males indicates no obesity but if WHR ≥ 0.88, central obesity is indicated (Snehalatha et al., 2003).

**Waist-Height-Ratio (WHtR) or Waist-Stature-Ratio (WSR):** The ratio of waist circumference (cm) to height (cm) according to Hsieh and Muto (2005) on exceeding 0.5 (> 0.5), indicates obesity.

**3.11.3 Pulmonary Function Test**

Lung function assessment using a spirometer is recommended as essential for diagnosis of Chronic Obstructive Pulmonary Disease (COPD), for disease-severity and for monitoring of the disease-progression (Barnes and Fromer, 2011; GOLD, 2013). The
standard spirometry examines the maximal forced exhalation (forced vital capacity) after maximum deep inspiration (forced expiratory volume). Forced vital capacity (FVC) is the total volume of air that the patient can forcibly exhale in one breath. The forced expiratory volume in one second (FEV₁) is the volume of air that the individual is able to exhale in the first second of forced expiration. The FEV₁/FVC ratio (expressed as a fraction) is used to assess the lung obstruction. A FEV₁/FVC ratio of <0.7 indicates air-flow limitation in lungs with an obstructive pattern. FEV₁ and FVC are measured in liters (l) and are also expressed as percentages of the predicted values of FEV₁, FVC and FEV₁/FVC based on the variables of age, height and weight, being calculated by the software itself. The per cent predicted values are defined as the measured values of FEV₁, FVC and FEV₁/FVC divided by the actual FEV₁, FVC and FEV₁/FVC values an individual should have on the basis of age, height and weight.

The pulmonary function test (PFT) was conducted using a Spiro Excel Spirometer (Version 1.3) after the participants had familiarized themselves with the procedure of using the spirometer. The lung function assessment is performed while standing as per the instructions which are available with the spirometer. The best readings of FVC, FEV₁ and FEV₁/FVC are recorded correspondingly with their predicted and per cent predicted values. The Body Surface Area (BSA, m²) is also available for assessing the metabolic mass. On the basis of the Global Initiative for Chronic Obstructive Lung Disease guidelines (GOLD, 2013; 2016), participants were identified as mild, moderate, severe or very severe COPD cases taking into consideration the FEV₁/FVC ratio (<0.70) and the FEV₁ measurements. Stage I is mild COPD with FEV₁ ≤ 80% predicted, stage II is moderate with 50% ≤ FEV₁ < 80% predicted; stage III is severe with 30% ≤ FEV₁ < 50% predicted and stage IV is very severe with < 30% predicted or FEV₁ < 50% predicted.

Post-bronchodilator, rather than pre-bronchodilator lung function testing is an effective method for COPD identification (Johannessen et al., 2005), the pre-bronchodilator lung function assessment is also used for the outcomes of mortality and hospitalizations (Mannino et al., 2011). The COPD-severity categorization with a pre- bronchodilator <0.70 FEV₁/FVC ratio was adopted by Tilert et al. (2013) as both, the pre- and post-bronchodilator lung functions, have predicted mortality with similar accuracy (Mannino et al., 2011), thereby validating this approach and finds usefulness in those situations.
where only pre-bronchodilator lung function test facility is available and this has been also performed in a number of long-term studies for COPD identification (Miller et al., 2005). Accordingly for this study, the pre-bronchodilator lung function testing was conducted.

3.12 Collection of Samples

From each participant, three different tissue-samples were collected at the same time and comprised venous peripheral blood, buccal epithelial cells and urine specimens. Random non-fasting sampling was only feasible and it was performed in situ. The samples were transported to the laboratory on ice and processed for genetic damage assessment and serum-preparation within 4-5 h of collection. For molecular work, the blood samples were stored at -20°C till analysis. The serum was separated in the laboratory after centrifugation for 15 min at 1500 x g and stored at -80°C until analysis for biochemical biomarkers. All the samples were assigned codes.

**Peripheral Blood Samples:** Under aseptic conditions, cubital venous blood sample (6-7 ml) was drawn by a medical laboratory technician and dispensed carefully into vials with and without the anticoagulants [heparin, ethylenediaminetetraacetic acid (EDTA)] as required for assaying DNA damage and molecular genetic analysis, respectively.

**Buccal Epithelial Cells** - The participants, after thorough mouth-rinsing, provided buccal epithelial cells (from left and right cheeks separately) using a wet wooden spatula. Separate slide preparations (2/participant) were made for epithelial cells obtained from the inner left and right buccal mucosal membranes.

**Urine Samples** - Mid-stream urine (~200 ml) samples were also obtained on request from all participants in clean polypropylene vials.

3.13 Laboratory Work

This entailed use of aliquoted blood and serum samples for genetic damage investigations, oxidative stress assessment and molecular genetic analysis. The buccal mucosal samples and urothelial cells were processed for chromosomal damage assessment. The details on the apparatus, equipment and chemicals used, and the preparation of solutions and reagents for carrying out the analyses are presented in Appendices V.
**Genetic Damage assessment**- Standardized assays were carried out for assessment of DNA damage, oxidative DNA damage and chromosomal damage. Heparinized blood samples were investigated for both, DNA damage using the Single Cell Gel Electrophoresis (SCGE) assay, and oxidative DNA damage with the enzymatically modified SCGE/comet assay. The Buccal Micronucleus Cytome (BMNCyt) assay was performed on buccal smear preparations for assessment of DNA and chromosomal damage, cell proliferation and cytotoxicity/cell death. The urothelial micronucleus (MN) test was used to score chromosomal damage and atypical (cell-death events) in exfoliated epithelial urothelial cells.

**Biochemical Analysis**- This comprised spectrophotometric analysis of blood serum samples for lipid- and lipoprotein-profiling, lipid peroxidation, total oxidant status (TOS), total antioxidant capacity (TAC) and Alpha-1- antitrypsin (AAT) activity. Lipid profiling in sera samples comprised the use of standard kits for analysis on a semi-automatic blood analyzer. Malondialdehyde (MDA) levels for lipid peroxidation assessment, TOS and TAC levels and alpha-1-antitrypsin activity were estimated spectrophotometrically using standard protocols.

**Molecular Genetic Analysis**- Whole genomic DNA isolated from EDTA-peripheral blood samples was amplified by polymerase chain reaction (PCR) and on restriction analysis, genotyped for seven single nucleotide polymorphisms (SNPs) using methods available in literature.

**DNA Damage Assessment- The Single Cell Gel Electrophoresis (SCGE) Assay**- The alkaline SCGE (comet) assay is an established, sensitive method which measures standard DNA damage and in combination with specific enzymes viz. formamido pyrimidine DNA glycosylase (Fpg) and endonuclease III (Endo III), permits the assessment of specific regions of DNA damage (oxidized DNA damage). Cell viability is initially assessed before performing the assays.

**The Trypan-Blue Dye-Exclusion Test** - Blood samples are subjected to a ‘viability check’ which results in dead cells taking up the dye and appear blue while viable cells remain unstained.

**Principle**- The intact cell membranes of live cells exclude the Trypan Blue dye (Strober, 2001) which however is selectively capable of penetrating the cytoplasm of dead cells, staining them blue (Avelar-Freitas *et al.*, 2014).
Procedure- A cell suspension constituting 850 µl of phosphate-buffered saline (PBS) and 50µl of blood was prepared and to it was added 100 µl of the Trypan Blue Dye. After mixing, the contents were allowed to stand for ~40 min following which 15 µl of the suspension was pipetted on an hemocytometer; a clean coverslip was then placed gently on the slide avoiding bubble- formation, and under a binocular microscope a separate count of stained and unstained cells was made and the percentage of cell viability (unstained cells) was calculated. Only the samples with cell viability more than 90% were processed for the SCGE assays.

Principle of the SCGE Assay- The normal supercoiled DNA molecule even with a single nick, under electrophoresis relaxes the DNA loops permitting the migration of negatively charged DNA towards the anode. As only the DNA loops containing the breaks migrate out of the nucleoid towards the anode, presenting it a comet-like appearance (Singh, 1996; Tice et al., 2000; Collins, 2004). The damaged DNA appears as a ‘tail’ while the coiled DNA in the nucleoid, forms the comet ‘head’ (Ostling and Johanson, 1984; Tice et al., 2000). The standard SCGE protocol assesses single-strand and double-strand DNA breaks, alkali-labile sites and DNA-DNA and DNA-protein crosslinks (Collins et al., 2008).

Oxidative DNA damage- Digestion of DNA with a lesion-specific endonuclease after lysis of agarose-embedded cells in the standard SCGE assay, permits the assessment of oxidized bases as a measure of oxidative DNA damage (Collins et al., 1993; Dusinska and Collins, 1996). Use of formamidopyrimidine DNA glycosylase (Fpg) and Endonuclease III (Endo III) enzymes enables the recognition of enzyme-sensitive sites (Moller, 2006) with the removal of the damaged bases by glycosylase activity generating an AP-site, and the AP-lyase cleaves the phosphodiester backbone at AP-site creating a nick in the DNA (Collins et al., 2008). The oxidized purine 8-oxoGua, and also ring-opened purines, or formamidopyrimidines (Fapy) are recognized by Fpg while the oxidized pyrimidines are converted to DNA-strand breaks by Endo III (Collins et al., 2008). The buffer treatment reveals any additional breaks that occurred during incubation and so differs from the standard SCGE assay where this step is not required. The oxidized purines are detected on Fpg-treatment and oxidized pyrimidines by the EndoIII-treatment. Therefore the difference between the value of percent DNA in tail obtained after the enzymatic digestion and buffer treatment determines the net enzyme-sensitive sites (Azqueta et al., 2009).
Cell population under study- For the SCGE assay, the peripheral blood leukocytes (PBL) were used as these are easy to obtain and are available in large numbers (Collins et al., 2008). The PBL circulate throughout the body and come in contact with various physiological processes in all body organs, and so are exposed to numerous toxicants and stress-inducers. Therefore, PBL are considered as representative of genetic damage and oxidative stress from endogenous and exogenous substances in the body (Valavanidis et al., 2013). The assay in leukocytes provides better results in comparison to the lymphocytes as lymphocytes with more damage are prone to get lost during the isolation procedures (Dhawan and Anderson, 2009).

Experimental details- The alkaline SCGE/comet assay was performed adhering to the basic protocol of Singh et al. (1988), in accordance with general guidelines for use of the comet assay (Tice et al., 2000, Hartmann et al., 2003) though with some modifications comprising usage of agarose-coated slides in lieu of frosted glass slides and silver staining of nucleoids (Nadin et al., 2001; Garcia et al., 2007) rather than with ethidium bromide. The main methodological steps comprised preparation of gel-coated slides, embedding cell suspensions in agarose gel, lysis of the cell and nuclear membranes and of proteins, enzyme treatment with formamidopyrimidine glycosylase (FPG) and Endonuclease III enzymes, alkaline treatment (pH≥13), DNA unwinding, electrophoresis, staining and scoring.

Slides per sample- For each individual, eight slides (Stoyanova et al., 2010) were prepared: two slides were kept in the lysis solution until electrophoresis (standard SCGE protocol); two slides were placed in enzyme buffer (50µl; 40mM HEPES, 0.1M KCl, 0.5mMEDTA, 0.2 mg/ml BSA, adjusted to pH8 with KOH) comprising buffer treatment; and of the remaining four slides, two each were treated with 50µl of the enzymes Endo III (1:100) and FPG (1:1000) diluted with the enzyme buffer.

Agarose-coated slides- Washed and dried glass slides were pre-coated with normal melting agarose gel (NMPA, 1.00%) prepared in phosphate-buffered saline (PBS). The gel was allowed to set and the slides were dried and stored until use.

Standard SCGE method- Whole blood (30µl) mixed with 100 µl of low melting point agarose (LMPA, 0.5%) was poured on the pre-coated slides and a coverslip was gently
placed over it. The layer was allowed to set at 4 °C for 10min. A third layer (100 µl of 0.5% LMPA) was applied on the slides after removing the coverslips and was allowed to set similarly (4 °C, 10min). After removal of the coverslip, the slide was immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10-10.5 with addition of 1% Triton X-100 and 10% DMSO prior to use) and then again was kept at 4°C for at least 3-4 h to enable lysis of cell membranes and proteins, leaving DNA as ‘nucleoids’.

**Enzyme Digestion**- For the modified comet assay, the nucleoid DNA was digested with the lesion-specific Endo III, which detects oxidized pyrimidines and FPG which detects oxidized purines (Collins *et al*., 1993; Dusinska and Collins, 1996). These slides in the buffer and those to be treated with Endo III and Fpg were each given three washings with the enzyme buffer for 5min each. After placing coverslips on each, the slides were kept in a moist tray and were incubated at 37°C for 30 min (for buffer and FPG treatments and for 45 min for the Endo III-treatment.

**Electrophoretic Run**- After removal of the coverslips, the slides were placed in an alkaline solution (300 mM NaOH and 1 mM EDTA, pH 13) for 20min, followed by electrophoresis (25 min, 300 mA, 25 V) with the power supply adjusted so as to give a voltage gradient of 1 V/cm across the platform.

**Neutralization and Fixation**- The slide preparations were subjected to neutralization (0.4 M Tris, pH 7.5) so as to lower the pH at which DNA can no more unwind. This was followed by treatment with a fixative solution (15% trichloroacetic acid, 5% zinc sulfate, 5% glycerol), for 10 min, following three washings with distilled water.

**Staining**- After air-drying, the slide preparations were stained (under subdued light) in 16 ml of solution A (5% sodium carbonate) and 34 ml of solution B (0.10% ammonium nitrate, 0.10% silver nitrate, 0.10% silicotungstic acid, 0.15% formaldehyde, freshly prepared and kept in the dark) under constant shaking until greyish in appearance. This was followed by washing twice with distilled water and immersion of the slides in a stopping solution (1% acetic acid) for 5 min which was then followed by washing with distilled water and air-drying at room temperature.

**Image Capture**- The stained preparations were examined for comets under a binocular transmission microscope at 10X. Images were captured using a digital camera E-420
mounted on a binocular microscope (Plate II). An image-analysis programme, the Cell Profiler 2.0 (revision: 10997) software package for Windows which is freely available (http://www.cellprofiler.org/) and like other open-source softwares, measures biological phenotypes quantitatively (Kamentsky et al., 2011). Gonzalez et al. (2012) have provided details on using the Cell Profiler imaging-system for comet analysis. The use of the cell profiler enables fully-automated analysis of captured images with minimal user- intervention and exports images with comet outlines along with the spreadsheet with records of per cent DNA in tail, facilitating other analysis.

**Scoring of slides**- The per cent DNA in tail was recorded for 100 nucleoids per sample (50/slide) for the standard SCGE. The per cent DNA in tail is regarded as an optimal parameter of DNA damage assessment as the amount of DNA in tail represents the frequency of strand-breaks (Collins et al., 1997). The intensity of DNA and the extent of DNA migration are considered in per cent DNA in tail (García et al., 2011).

**Quantification of DNA Damage**- A good linearity has been observed between visual score (Damage Index) and per cent DNA in tail (Moller, 2006) and therefore for this study, the nucleoids were also visually classified on the basis of per cent DNA in tail as per the method of García et al. (2007). Each nucleoid was assigned a score on a 5-point scale (ranges 0–4) according to the amount of DNA in the tail of the comet. The category of damage may fall in any of the five non-overlapping types viz. category 0 (no damage) with DNA in tail < 5%; category 1 (low damage), with percent DNA in tail between 5–25%; category 2 (medium damage) where per cent DNA in tail is between 25–45%; category 3 (high damage) with 45–70% DNA in tail and category 4 (very high damage where > 70% of DNA is in tail (García et al., 2007; 2011).

On the basis of nucleoid categories Damage Index and Damage Frequency were calculated (da Silva et al., 2008).

Damage index (DI) is the quantitative measure of genetic damage (Franke et al., 2005).

**Damage Index** = [(nucleoids in Category 0 x 0) + (nucleoids in Category 1x 1) + (nucleoids in Category 2x2) + (nucleoids in Category 3x 3) + (nucleoids in Category 4 x 4)].

Damage frequency (DF) is the total number of cells (nucleoids) with damage (da Silva et al., 2008).
**Damage Frequency** = [(nucleoids in Category 1) + (nucleoids in Category 2) + (nucleoids in Category 3) + (nucleoids in Category 4)].

**Oxidative DNA Damage**- For oxidative DNA damage assessment, 400 nucleoids per sample (50 per slide) were scored for per cent DNA in tail and comprised the replicates of the buffer-treated, Endo III-treated and Fpg-treated samples. The buffer-treated slides represent controls and therefore per cent DNA in tail was subtracted from values obtained after enzyme treatment to obtain the measure of ‘net enzyme-sensitive sites’ (Collins *et al.*, 2008). Total oxidative DNA damage was obtained by (Fpg ± Endo III induced % DNA in tail) adding the damage to pyrimidines and purines (Collins *et al.*, 2008). The endonuclease III-sensitive sites and the formamidopyrimidine glycosylase (Fpg)-sensitive sites are markers for amount of oxidized pyrimidines and oxidized purines, respectively (Collins *et al.*, 1997).

### 3.13.2 The Buccal Cytome

Chromosomal damage was scored as micronucleated cells. The MN result from aneugenic and/or clastogenic events and provide a measure of chromosomal damage (Luzhna *et al.*, 2013). The other cytome indices (Thomas *et al.*, 2009) includes nuclear buds (DNA amplification), basal cells (measure of cell proliferation), binucleated cells (from cytokinesis-failure or cytokinesis-arrest) and among cell-death parameters are the pyknotic cells, the karyorrhectic and condensed chromatin cells (apoptotic events) and karyolytic cells (necrotic event).

**Cell Populations**- For this assay as many as eight cell types present in the buccal epithelium were examined. These include the basal cells, binucleated cells, karyorrhectic, karyolytic, pyknotic and condensed chromatin cells. The buccal mucosa is a stratified squamous epithelium consisting of four distinct layers (Holland *et al.*, 2008; Thomas *et al.*, 2009). The stratum corneum or the keratinized layer, which lines the oral cavity, comprises cells that are constantly lost due to everyday mastication; other layers are the stratum granulosum (or the granular layer) and the stratum spinosum (or the prickle cell layer) which lie beneath the stratum corneum and comprise differentiated, apoptotic and necrotic cells. The stratum germinativum (rete pegs) comprise the dividing basal cells and these basal cells get differentiated. The loss of the upper layer occurs from wear and tear and from apoptotic and necrotic events, and the lower layers replace it in this manner. Epithelial tissue exfoliated cells migrate
towards the surface within 7 to 21 days (Stich et al., 1983; 1984; Bonassi et al., 2011). These epithelial cells are used for the Buccal MN cyt assay as they may exhibit nuclear damage that occurred in the basal layer of epithelial tissue where cells undergo mitosis. A rapid turnover of epithelial tissues brings the cells to the surface where they exfoliate. As a consequence, the maximal rate of micronucleus (MN) formation in exfoliated cells is observed within weeks (1-3 weeks) after exposure to any genotoxic agent (Thomas et al., 2009; Bonassi et al., 2011).

The epithelial cells were collected using a minimally-invasive sampling method. Buccal mucosal cells were obtained by scraping the right and the left cheeks using a clean wooden spatula (Stich et al., 1982; Sarto et al., 1987).

The Buccal Micronucleus Cytome (BMNCyt) Assay- The assessment of genetic damage in epithelial buccal cells, obtained in a minimally-invasive method, is useful for monitoring populations for exposure to genotoxic agents (Bonassi et al., 2009). This assay is also advantageous because of its specificity for detecting the effects of exposure to inhaled or ingested genotoxic agents since buccal cells form the first barrier on the inhalation- or ingestion- routes.

Principle- Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments (clastogenicity) or intact whole chromosomes, lagging (aneugenicity) behind during the anaphase stage of cell division. Micronuclei hence represent the rejection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle et al., 1991; Fenech et al., 1999; Norppa and Falck, 2003). The other nuclear abnormalities are indicative of apoptosis (karyolysis, condensed chromatin and pyknosis) and necrosis (karyorrhexis, condensed chromatin) which may be consequences of genotoxic and cytotoxic effects on the epithelial cells of the oral mucosa from a disease and/or exposure. The BMnCyt is therefore a minimally invasive means of investigating events that are reflective of DNA damage (MNi and/or nuclear buds), cellular proliferation potential (basal and/or binucleated cells), and of cell-death (condensed chromatin, karyorrhectic, pyknotic and karyolytic cells) parameters (Thomas and Fenech, 2011).

Experimental details- Air-dried buccal smear slide preparations (2/participant) transported on ice to the laboratory, were fixed in methanol: acetic acid (v/v:3:1) for 15
min and then given hypotonic treatment (1N HCl ~8 min). As the Feulgen-Fast Green stains have specific and versatile DNA-specific staining properties (Thomas et al., 2009), the air-dried preparations were first stained with the Fuelgen reagent (stain) for 15 min followed by counterstaining for 2-5 min in 1% Fast Green. Feulgen is a DNA-specific stain and staining proceeds on detachment of the deoxyribose ring to form an aldehyde group that reacts with Shiff’s reagent and results in dark pink or red colouration (Torres-Bugarín et al., 2014). Fast green imparts green colouration to the cytoplasm and hence the main nucleus and any MN or nuclear buds appear purple- or pink- coloured against the green cytoplasmic background. The slides were coded and scored blind.

**Scoring methodology** - Criteria for identifying cells for inclusion into the MN frequency count were adopted as developed by Tolbert et al. (1991; 1992). The criteria considered are (a) intact cytoplasm and relatively flat cell position on the slide; (b) little or no overlap with adjacent cells; (c) little or no debris; and (d) nucleus normal and intact with the nuclear perimeter, smooth and distinct. The suggested criteria for identifying MN are: (a) rounded smooth perimeter suggestive of a membrane; (b) less than one-third the diameter of the associated nucleus, but large enough to discern shape and colour; (c) Feulgen positive i.e. pink in the bright-field illumination; (d) staining intensity similar to that of the nucleus; (e) texture similar to that of nucleus; (f) same focal plane as the nucleus; and (g) absence of overlap with, or bridge to, the nucleus.

**Scoring Scheme** - Typically, this comprises, screening 1000 cells per sample for cytokinetic defects (binucleated cells), proliferative potential (basal cell frequency) and cell-death (condensed chromatin, karyorrhectic, pyknotic and karyolytic cells) while a total of 2000 cells screened for DNA damage (MN and nuclear buds) in basal cells as per Thomas et al. (2009). For the present study 2000 cells (1000/slide) were however scored at a magnification of 40X under a binocular transmission microscope for micronuclei and nuclear buds and other cell types as currently documented in literature (Thomas et al., 2009). Confirmation of MN and NBuD was carried out under 100X (Plate II). Repair index (RI) which is an indicator of degree of genotoxicity (Celik et al., 2010), was also calculated. Random scoring was performed by a colleague for confirmation of identified MNi and NBuDs.
**Chromosomal Damage**- Micronuclei are acentric fragments or whole chromosomes which are indicative of chromosomal damage resulting from non-disjunction (Sgura *et al.*, 1997) or chromosome breakage (Sarto *et al.*, 1987) and are considered important biomarkers of cancer (Bonassi *et al.*, 2009; Fenech *et al.*, 2011).

**Micronucleated Cells**- These cells are characterized by the presence of one or more smaller nuclei called micronuclei in the cytoplasm in addition to the main nucleus with which there is similarity for stain-intensity and texture.

**DNA Amplification**- Nuclear buds (NBUDs) represent the regions of DNA amplification resulting from segregation of distinct regions of the nuclei (Fenech *et al.*, 2011). Cells with nuclear buds were earlier termed as broken eggs (Tolbert *et al.*, 1992). These cells are characterized by the presence of a small constriction, indicating budding in the main nucleus. Being attached to the main nucleus, the nuclear bud has similar morphology and staining properties as that of the main nucleus.

**Other Cell Populations**- Apoptosis is stimulated by exposure to mutagenic agents and acts as a protective mechanism for eliminating damaged cells; therefore increased apoptosis may be evidence of genotoxic damage (Tolbert *et al.*, 1992). Pyknosis, condensed chromatin and karyorrhexis accompany keratinization, which takes place as an adaptive response to cell damage (Pindborg *et al.*, 1980). In addition to these alterations, karyolysis can occur in cells undergoing necrosis (cell death) (Wyllie, 1981; Galluzzi *et al.*, 2012). The characteristic features of the buccal cytome cells are discussed below

- **Basal cells**- oval-shaped cells, uniformly stained nucleus, larger nucleus-to-cytoplasm ratio; scored for MN and NBuDs.
- **Differentiated cells**- oval- or round-shaped, uniformly stained nucleus, larger size than basal cells, smaller nucleus-to-cytoplasm ratio. These cells are scored for MN and NBUDS
- **Binucleated cells**- presence of two main nuclei of similar size and staining intensity
- **Condensed Chromatin cells**- the nucleus has a striated nuclear pattern, intensely stained aggregated chromatin
- **Karyorrhectic cells**- the nucleus shows extensive aggregation of chromatin (more than that of the condensed chromatin cells)
- **Pyknotic cells**- a small shrunken nucleus (~ 1/3rd - 2/3rd of the diameter of the main nucleus), intense and uniformly staining
- **Karyolytic cells**- these lack DNA and so have no nucleus
The numerated cells can be computed and compared to find out if the inherent DNA repair capacity is altered. The Repair Index has been given by Ramirez and Saldanha (2002) and is calculated as the sum of karyolytic and aryorrhectic cells divided by the total number of cells with MN and NBuDs.

3.13.3 The Urothelial Micronucleus Assay

Urinary bladder cells may serve as an appropriate index for monitoring the genotoxicity because the epithelial lining of the bladder is in direct contact with toxicants as the human body releases the toxicants maximally through urine (Buchet and Lauwerys 1985; Kurttio et al., 1998; Paul et al., 2013).

**Principle**- Contaminants may enter the body through dermal penetration, ingestion and/or inhalation thereby epithelial cells become the first barrier to absorption of exogenous factors (Esteban and Castano, 2009). Urinary bladder epithelial cells can encounter those toxicants which are not metabolized) as well as their derivatives which are formed after metabolic transformation (Blaszczyk and Mielzynska-Svach, 2014). MN in urothelial cells reflect damage to the bladder epithelial tissue, which has occurred approximately 1–3 weeks prior to the exfoliated cells appearing in urine (Stich et al., 1983). The samples from the urogenital tract were obtained on centrifugation of the mid- stream early morning urine (Warner et al., 1994).

**Experimental Details**- The standard protocol of Lehucher-Michel et al. (1996) was followed for assessment of genetic damage in urothelial cells. Urine sample collections in sterile plastic containers were stored at 4°C (~2-2.5h). The cells were concentrated by 10 min-centrifugation (400-500 X g) and the cell pellet was fixed in 5 ml of freshly prepared chilled fixative (methanol/acetic acid, 3: 1; 4°C). After 20 min at 4°C, centrifugation at 400 X g was carried out and again fixation was carried out for 20min at 4°C in 5 ml fresh fixative after discarding the supernatant. Again after 20 min at 4°C, centrifugation was again carried out and the pellet was re-suspended in a minute amount of supernatant. The cell suspension was gently dropped at arm’s length on inclined pre-cleaned glass slides which were then kept for drying (24h) at room temperature. The air-dried preparations were stained for 10 min in 10% Giemsa in PBS (pH 6.8).
Scoring- For each sample, two slides per sample were similarly prepared and coded. Coded preparations were scored under a transmission binocular microscope at 40X (Plate III). For each sample, 1000 intact urothelial cells were examined for the presence of micronuclei (MN) according to the criteria proposed by Stich and Rosin (1983). The confirmation of micronuclei was carried out at 100X (oil-immersion) and also by a colleague for the purpose of validation. Cells with micronuclei are characterized by the presence of both a main nucleus and one or more smaller nuclear structures called micronuclei which are round or oval in shape and their diameter should range between 1/3\textsuperscript{rd} and 1/16\textsuperscript{th} of the main nucleus. The micronuclei have the same staining intensity and texture as the main nucleus (Tolbert \textit{et al}., 1991; 1992). The other atypical cells (Blaszczyk and Mielzynska-Svach, 2014; Neresesyan \textit{et al}.,2014) scored included the karyorrhectic cells (nucleus with extensively aggregated chromatin), condensed chromatin cells (nucleus with striated pattern), karyolytic cells (nucleus is disintegrated) and the pyknotic cells (nucleus is shrunken) representing cell-necrotic (Karyorrhectic and condensed chromatin cells) and cell apoptotic (karyolytic and pyknotic) events.

3.13.4 Biochemical Analysis

Separated sera samples were processed for screening some biomarkers of oxidative stress, levels of alpha-1-antitrypsin (indicator of decreased efficacy of lung) and for lipid profiling.

3.13.4.1 Total Antioxidant Status- In the course of the normal physiological functions, any oxidative challenges are counteracted by the cellular defense (antioxidants) mechanisms (Sies, 2007). Endogenous antioxidants comprise enzymatic antioxidants involving glutathione peroxidase, catalase and superoxide dismutase and the non-enzymatic antioxidants include vitamins E and C, thiol antioxidants (glutathione, thioredoxin, and lipoic acid), melatonin, carotenoids, natural flavonoids (Rahman, 2007). Their function is to maintain optimal cellular functions and thus determine the competence of cellular defense mechanism against oxidative insult (Mates \textit{et al}., 1999). However individual analysis of the various antioxidants is time-consuming and labour-intensive (Wang \textit{et al}., 2011), and therefore the total antioxidant status (TAS) is a preferred measure of the overall antioxidative status (Erel, 2004). Another advantage of
TAC measurement is that it may include even those antioxidants not yet recognized or easily measured (Erel 2004; c.f. Cumurcu et al., 2009). The major antioxidant components of serum (assessed as TAC) comprise that of proteins (52.90%) followed by uric acid (33.10%), vitamin C (4.70%) and vitamin E (1.70%) whereas 5.20% is contributed by other antioxidants while the reference range of TAC levels is 1.49–1.97 mmol Trolox equivalent per litre (Erel, 2004).

**Principle**- The ABTS (2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonate))- based method (Erel, 2004) relies on the principle that in acidic medium (acetate buffer), ABTS gets oxidized to a deep green-coloured radical cation (ABTS⁺) on addition of using hydrogen peroxide. On dilution at high pH, the colour of the radical cation is spontaneously and slowly bleached. The antioxidants (present in the sample) also accelerate the bleaching rate to a degree proportional to their concentrations, with the bleaching rate inversely proportional to the total antioxidant capacity of the sample.

**Experimental Details**- The standard spectrophotometric protocol of Erel (2004) with certain modifications according to Gupta et al. (2009) was followed for estimation of TAC. For this, 5μl of the serum sample was mixed with 200 μl of the reagent 1 (acetate buffer 0.4 mol/l pH 5.8). After 5 minutes of incubation at room temperature, 20 μl of reagent II (ABTS⁺ in acetate buffer 30mmol/l, pH 3.6) was added. The absorbance of ABTS⁺ was recorded at 660nm on an ELISA- microplate reader and expressed in mmol Trolox equivalent per litre.

**3.13.4.2 Total Oxidant Status**- The total oxidant status (TOS) determines the overall oxidation state (Erel, 2004). The main TOS components in the serum include hydrogen peroxide and lipid hydroperoxide (resulting from the attack of the free radicals as the pro-oxidant systems outbalance the anti-oxidants) which have the potential for producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids, that may ultimately cause cell-death (El-Aal, 2012). The reference range for TOS is 6.31–24.38 μmol H₂O₂ equivalent/litre (Erel, 2005).

**Principle**- The oxidation of the ferrous ion-o-dianisidine complexes results into ferric ions (enhanced by abundant glycerol molecules present in the reagent I) which form a coloured complex with xylenol orange in an acidic medium whose intensity is
measured spectrophotometrically (Erel, 2005). The colour intensity relates to the total number of oxidant molecules present in the sample. The results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H₂O₂ equivalent per litre) as the assay is calibrated with hydrogen peroxide.

**Experimental Details**- According to the protocol of Erel (2005), to each well of a microplate, 35 μl of the serum sample was added 225 μl Reagent I(xylenol orange 150 μM, NaCl 140 mM and glycerol 1.35 M in 25 mM H₂SO₄ solution) and kept for incubation at 37°C for 5 minutes. Then 11 μl of Reagent II (ferrous ion 5 mM and o-dianisidine 10 mM in 25 mM H₂SO₄ solution) was added to each well and the absorbance was recorded at 560nm on an ELISA microplate reader. On calibration with hydrogen peroxide (used as a standard) and the values were expressed as H₂O₂ equivalent/l. The concentration of TOS was calculated by dividing the difference in absorbance of test samples and the standard, and multiplying it with the concentration of standard (20 μl H₂O₂).

Oxidative Stress Index (OSI) is a derived index obtained by the ratio of TOS to TAS level (Aycicek et al., 2005; Harma et al., 2005) and is an indicator accurate index of oxidative stress in the body (Wang et al., 2011).

### 3.13.4.3 Lipid Peroxidation: Oxidative Stress Assessment

Lipid peroxidation occurs by the action of excessive free radicals. Besides causing inter-related derangements of cell metabolism which include DNA-strand breakages, rises in intracellular free calcium ions (Ca²⁺), damage to membrane ion transporters and/or other specific proteins, oxidative stress also causes peroxidation of lipids (Halliwell and Chirico, 1993). Malondialdehyde (MDA) is a useful marker of oxidative stress and the antioxidant status (Gawel et al., 2004) being among the final products of peroxidation of polyunsaturated fatty acids.

**Principle**- The thiobarbituric acid (TBA) method is the most widely used method for lipid peroxidation assessment. It is based on the principle that Malondialdehyde, an aldehydic product of lipid peroxidation, reacts with TBA. When heated at low pH, this reaction leads to the formation of a pink chromogen, the [TBA]₂ –malondialdehyde adduct whose absorbance can be read at 535 nm.
Experimental Details- The lipid peroxidation levels were estimated spectrophotometrically (Beuge and Aust, 1978). To 40µl serum was added to 80 µl TBA-TCA-HCl (0.375g TBA, 15g tricholoroacetic acid, 2.17ml HCl) solution. After mixing on a vortex mixer, the contents were placed in a water bath (100°C) for 15min. On cooling centrifugation was carried out at 3000rpm for 10min. The supernatant was discarded and the absorbance of the fluorescent precipitate was read at 535 nm against the blank (40 µl of 0.01M PBS instead of serum sample). MDA concentration (in µmol/l) was calculated with the extinction coefficient of 1.56X10^5 M^-1 cm^-1. The concentration of MDA in the serum samples was calculated by using the formula as given by Jyothi et al. (2008) viz. C=E/K*L, where E-extinction absorbance, K- Molar extinction coefficient (1.5*10^5), L-Length of cuvette (1cm) and C- concentration of MDA(µmol/l).

3.14 Serum Trypsin Inhibitory Capacity (STIC) Assay

Alpha-1-antitrypsin (AAT) is the major circulating protease inhibitor which inhibits the activity of various proteases; if left unchecked by the protease inhibitor, these cause destruction of normal tissues (Gupta et al., 2007). Deleterious genetic alterations can lead to inadequate circulating AAT levels and consequently its insufficiency enables the free neutrophil elastase to degrade the structural protein, elastin, present in the pulmonary alveolar matrix (Owen, 2005). Therefore individuals with AAT deficiency may often develop chronic obstructive lung disease (COPD). Levels of AAT activity in blood serum samples of COPD cases and controls were determined using the STIC assay.

Principle- Serum Trypsin Inhibitory capacity (STIC) is an indicator of adequate lung functioning (Ahmad et al., 2006) and its levels can be estimated spectrometrically (Gupta et al., 2007). It is based on the principle that the hydrolysis of α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) is inhibited by the antirypic proteins present in the serum by trypsin in Tris buffer. The most common Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1 (SERPINA1) genes encodes alpha-1-antitrypsin protein. The M1 and M2 alleles (Ala213Val –M1, Arg101His -M2) are associated with normal alpha-1-antitrypsin levels (0.90 to 2.00 g/l) while in the S
(Glu264Val), protein levels are 50-60% of normal levels and in Z (Glu342Lys) 10-15% of normal protein levels are observed (da Silva, 2014).

**Experimental Details** - The standard (spectrophotometric) protocol of Dietz *et al.* (1974) was followed with modifications suggested by (Gupta *et al.*, 2007) for estimation of levels of alpha-1-antitrypsin activity. For this, 15-0 µl serum was added to 3-0 ml of Tris-HCl (0·1 M) containing 200 µl of trypsin. The contents were incubated at 25°C for 15 min and again after addition of 3ml of BAPNA. Following an incubation period of 10min, 750 µl of acetic acid (30%) was added and the contents were mixed thoroughly. Absorbance of this solution was taken at 410 nm. The normal value of STIC in the control population has been reported in the range from 1·10 to 2·10 mg of trypsin inhibited/ml of serum (Lieberman *et al.*, 1972; Ahmad *et al.*, 1979)

### 3.15 Lipid Profile

Lipid Levels in blood are influenced by nutrition, body weight, physical activity, medications, age, sex, racial differences, dietary factors, socio-economic status, geographic conditions and genetic factors (Hunt *et al.*, 1989; Durgawale *et al.*, 2009; c.f. Shahnam *et al.*, 2010). Malnutrition (secondary to reduced dietary-intake and malabsorption) has the potential to impair essential polyunsaturated fatty acid (PUFA) and the composition of lipoprotein particles. Lipoproteins also contribute to the maintenance of cellular membrane structure and function. Any disturbances in lipoproteins resulting from peroxidative attack may affect their normal metabolism. Essential fatty acid deficiency and malnutrition can also negatively affect both, the intestinal absorption of fat and lipoprotein metabolism (Levy *et al.*, 2000). Therefore lipid profiling can provide insights about cardiovascular complications as high lipid and lipoprotein levels are implicated in heart disease (Begum *et al.*, 2010). The levels of total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were determined for all participants. However non-fasting samples were analyzed as cases (workers) showed non-compliance for providing fasting blood samples. Incidentally, literature documentations have revealed that non-fasting serum samples are more strongly related to risk of cardiovascular diseases than fasting lipid profiles because the non-fasting samples represent adequate capacity of an individual during postprandial metabolism (Mora, 2008 Kolovou *et al.*, 2011; Doran *et al.*, 2014).
Standard kits were used for estimation of TC and TG and HDL levels on a semi-automated analyzer. The levels of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were obtained using Friedewald’s equation (Friedewald et al., 1972). Various indices for prediction of risk to cardiovascular diseases were also calculated from the lipid profile panel.

**Total Cholesterol (TC)**- As per the instructions available with the kit, to 10µl of serum sample 500µl of the working reagent was added with the blank comprising 10µl of distilled water (in place of serum sample) and 500µl of the working reagent. A standard solution was prepared by adding 10µl of standard reagent to 500µl of the working reagent. After incubation at 37°C for 10 minutes the absorbance of standard and of each test (serum) sample against blank was recorded at 505nm (reference value on kit for normal TC levels is 140-250mg/dl).

**Triglycerides (TG)**– According to the instructions available with the kit, 500µl of the working reagent was added to 5µl of serum sample. The blank (500µl working reagent + 5µl water) and standard (500µl working reagent + 5µl standard) were also prepared and similarly incubated at 37°C for 10 minutes. Absorbance of the standard and sample were recorded at 505nm (Reference value on kit for normal TG is 65-180 mg/dl).

**High Density Lipoprotein Cholesterol (HDL-C)**- Following the kit’s protocol, to 250 µl of HDL-C precipitating reagent, 62.5 µl of serum sample was added; these were mixed well and left at room temperature for 10 minutes followed by centrifugation at 3000rpm for 10 min. To 25 µl of the supernatant, 500 µl of cholesterol reagent was added and contents were again incubated at 37°C but for 5 min. The absorbance was taken at 505nm (reference value on kit for normal HDL-C levels is 65-180 mg/dl).

**Other Lipoproteins** - Low Density Lipoproteins (LDL) and Very Low Density Lipoproteins (VLDL) were derived using the Friedewald equations (Friedewald et al., 1972), viz.

\[ \text{LDL Cholesterol} = [\text{TC}-\text{HDL} - (\text{TG}/5)] \] and \[ \text{VLDL cholesterol} = \text{Triglycerides}/50 \].

**Lipid Indices** - The lipid profile panels were used to calculate various CVD risk indices. Higher indices relate to higher risk of cardiovascular events.
As per SRL Diagnostics (www.srlworld.com) cut-offs for TC/HDL and LDL/HDL are as follows:

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC/HDL: Low risk</td>
<td>3.3-4.4</td>
</tr>
<tr>
<td>Average risk</td>
<td>4.5-7.0</td>
</tr>
<tr>
<td>Moderate risk</td>
<td>&gt;7.1-11.0</td>
</tr>
<tr>
<td>High risk</td>
<td>&gt;11.0</td>
</tr>
<tr>
<td>LDL/HDL: Desirable/Low Risk</td>
<td>0.5-3.0</td>
</tr>
<tr>
<td>Borderline/Moderate Risk</td>
<td>3.1-6.0</td>
</tr>
<tr>
<td>High Risk</td>
<td>≤30.0</td>
</tr>
</tbody>
</table>

Criteria for determining dyslipidemia- The study participants were categorized as dyslipidemic on the basis of the criteria given in the kit as well as that given by the ATPIII guidelines (Cleeman, 2001). Dyslipidemia is defined as increased total cholesterol and decreased low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels. The reference value for total cholesterol was 140-250mg/dl (kit) and <200mg/dl (ATPIII); for triglycerides it is 65-170mg/dl (kit) and <150mg/dl (ATPIII) and for HDL-C it is 45-65mg/dl (kit) and <40mg/dl (ATPIII)

3.16 Molecular Genetic Analysis

The molecular genetic analysis was carried out for some gene variants of Glutathione-S-transferase (GSTT1, GSTM1 and GSTP1) and Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1, SERPINA1 (T710C, G374A, A863T, G1096A) on whole blood isolated genomic DNA samples. The methodology involved for the isolation of genomic DNA, DNA quantification and steps in the amplification of DNA, restriction digestion, genotyping and sequencing are described below in detail.

DNA Isolation- The phenol-chloroform (organic) method (Gill et al., 1987) was adopted for genomic DNA extraction from whole blood. The organic method has advantages over the inorganic (Ammonium nitrate) method as proteins, lipids,
carbohydrates and cell debris are removed through extraction of the aqueous phase along with the organic mixture of phenol and chloroform (Sambrook and Russel, 2001; Chomczynski and Sacchi, 2006). Peripheral blood and red cell lysis buffer (3ml each) were mixed in a 15ml tube, kept on ice (~30min) and then shaken vigorously to lyse the red blood corpuscles (RBCs) followed by centrifugation of contents (3000rpm, 30min). The supernatant (containing lysed RBCs) was discarded and the pellet with white blood corpuscles (WBCs) was re-suspended in 3ml of red cell lysis buffer. Centrifugation was repeated until a clear white pellet was obtained to which was added WBC lysis buffer (1ml) and 10% SDS (56 µl; sodium dodecyl sulphate, pH 7.2). The contents were incubated overnight at 37°C. The next day, one ml of PCA (phenol 25 ml: chloroform 24 ml: isoamyl alcohol 1 ml) was gently mixed to the contents at room temperature and an emulsion was formed. On centrifugation (1500rpm, 15min), an upper aqueous (organic) and an inorganic phase (protein layer) were distinguished. The upper aqueous phase was pipetted into a fresh tube without disturbing the protein layer, and chilled ethanol (2.5ml) was gently added along the sides of tube to precipitate the DNA. After gentle spooling, precipitation of DNA occurred. The DNA was transferred into a 1.5ml tube and given two washings with 70% ethanol; the ethanol was then decanted and the pellet was air-dried at room temperature. Tris-EDTA buffer (100 µl, pH 8.0) was then added and the contents were kept overnight for incubation at 37°C for dissolving the DNA. The extracted DNA was stored at -20°C till further analysis.

Quantification of DNA- The extracted genomic DNA samples were quantified on 0.8% agarose gel electrophoresis (Sambrook et al., 1989). After the gel was cast and allowed to polymerize (30min), DNA samples (2 µl) mixed with 3µl of bromophenol blue dye were loaded in the wells of the gels with the standard (2 µl of (250 ng/µl) Lambda (λ) DNA loaded in the first well. Electrophoresis was carried out at 100V for 30min. The quantity as well quality of DNA (bright intensity of fluorescence and lack of smears) was compared to that of the lambda DNA under UV-transillumination. The
quantity of DNA in the samples of all participants ranged from 200-250 ng and therefore the samples were processed for DNA amplification (Plate IV).

**Genetic Polymorphisms**- The *GSTM1* and *GSTT1* genes show deletion polymorphisms (Nelson *et al.*, 1995 c.f. Cho *et al.*, 2005) and homozygotes for null mutants lack the enzyme. The *GSTP1* (rs 1965) gene variant in exon 5 results from A>G transition (A313G) causing isoleucine to valine substitution at codon 105 (Cho *et al.*, 2004; Dimov *et al.*, 2008) and the homozygotes (GG) have decreased activity. The *M1* allele of the *SERPIN A1* gene results from a T > C transition in exon 3 (T710C) and leads to valine to alanine substitution at amino acid 213 (Val 213 Ala). The *M2* allele of the *SERPIN A1* occurs due to G > A transition (G374A) in exon 2 and causes arginine to histidine substitution at 101 amino acid. The enzymatic activity of *M1* and *M2* homozygotes are at par to that of the wild type. An A > T transversion (A863T) in exon 3 (*S* allele) causes glutamic acid to valine substitution (Glu264Val) at amino acid 264 (Bartels *et al.*, 2009) by which the activity is decreased. In exon 5, a G > A transition (*Z* allele) causes glutamic acid to lysine (Glu 342Lys, G1096A) substitution (Bartels *et al.*, 2009) and the enzyme activity is absent.

**DNA Amplification**- For the seven SNPs, the primer sequences required for DNA amplification were obtained from BLAST and also as available in literature. Molecular genotyping of *GSTT1* and *GSTM1* genes was carried out by multiplex-PCR (Girisha *et al.*, 2004; Vedyakov and Toneskii, 2006) and for the genetic variants of *GSTP1* A313G (Vettriselvi *et al.*, 2006) and of *SERPIN A1* (*M1* and *M2*, Cha *et al.*, 2008), PCR-RFLP was performed. Genotyping of the *SERPIN A1* *S* and *Z* alleles was carried out by multiplex-PCR followed by restriction digestion (Cha *et al.*, 2008). The thermal cyclers supplied by Applied Biosystem and Eppendorf Systems were used for running the PCR. The details about the selected genetic variants alongwith the primer sequences are given in Table 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic Variant (SNP)</th>
<th>Chromosome Position</th>
<th>Location</th>
<th>Amino Acid Change</th>
<th>Nucleotide Change</th>
<th>Primer pair sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| GSTT1      |                       | 22q11.23            | -        |                  | Gene Deletion 16-kb | F 5' - TT CCT TAC TGG TCC TCA CAT CTC - 3'  
|            |                       |                     |          |                  |                  | R 5' - TCA CCG GAT CAT GGC CAG CA - 3'                  | Girisha et al. (2004);  
|            |                       |                     |          |                  |                  |                                                           | Vetrivelvi et al. (2006)      |
| GSTM1      |                       | 1p13.3              | -        |                  | Gene Deletion 54-kb | F 5' - GAA CTC CCT GAA AAG CTA AAG C-3'  
|            |                       |                     |          |                  |                  | R 5' - GTT GGG CTC AAA TAT ACG GTG G-3'                   |                               |
| Internal Control  |                       |                     |          |                  |                  | F 5' - TGCCAAGTGAGGCAACCAA-3'  
| (Intron 3 of  |                       |                     |          |                  |                  | R 5' - GCATCTTGCTGTCAGAT-3'                               |                               |
| HLA-DQB1)  |                       |                     |          |                  |                  |                                                           |                               |
| GSTP1      | rs1695                | 11q13               | Exon 5   | Ile105Val        | Transition ΔTC→GTC | F 5' - ACCCCCGGGCTTATGGGAA -3'  
|            |                       |                     |          |                  |                  | R 5' - TGAGGGCAAGAAAGGCCCT-3'                           | Cha et al. (2008)             |
| SERPIN A1  |                       |                     |          |                  |                  |                                                           |                               |
| S (rs17580)|                       | 14q32.13            | Exon 3   | Glu264Val        | Transversion GΔA→GTA | F5' - TGAGGGGAAACTACAGCACCTC-3'  
|            |                       |                     |          |                  |                  | R 5' - AGGCTTGACGGATCTTGGTCA-3'                         |                               |
| Z (rs28929474) |                    |                     | Exon 5   | Glu342Lys        | Transition GAG→AG  | F5' - ATAAAGCTGCTGACCATCGTC-3'  
|            |                       |                     |          |                  |                  | R 5' - TGAGGGATCTGACCACTTTGTC-3'                         |                               |
| M1 (rs6647)|                       |                     | Exon 3   | Ala213Val        | Transition GCG→GTG | F5' - CCCACCTTTCCTTCCACGGCAAATGGG-3'  
|            |                       |                     |          |                  |                  | R5' - GGGCTTCAGGCCACCATGGCTAGAGGAGTG-3'                 |                               |
| M2 (rs709932) |                    |                     | Exon 2   | Arg101His        | Transition CGT→CAF  | F5' - GCAGGACAATGCGCTTCTGCTTCTGCTC-3'  
|            |                       |                     |          |                  |                  | R 5' - CCACCATGCTACGAGCCCTGAGGAGGAG-3'                  |                               |
Using appropriate allele-specific primers, a 15 µl reaction for each gene variant was set-up. Genotypes of *GSTM1* and *GSTT1* were determined by multiplex PCR (co-amplification of *GSTM1* and *GSTT1*) using intron 3 of HLA class II histocompatibility antigen, *DRB1* beta chain (*HLA DRB1*) as internal control. Negative controls (without genomic DNA) were run with all reactions to rule-out DNA contamination. The details of the PCR cocktails and the cycling conditions are presented in Table 2.

Table 2. Constituents and Cycling Conditions used for PCR amplification of *GST* and *SERPIN A1* Gene Variants

<table>
<thead>
<tr>
<th>Constituents /Steps</th>
<th>GSTT1 and GSTM1 (Multiplex PCR)</th>
<th>GSTP1</th>
<th>SERPIN A1 S and Z (Multiplex PCR)</th>
<th>SERPIN A1 M1</th>
<th>SERPIN A1 M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constituents</td>
<td>PCR Amplification : 15 µl Reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR Water (µl)</td>
<td>8.4</td>
<td>10.9</td>
<td>11.3</td>
<td>11.9</td>
<td>11.2</td>
</tr>
<tr>
<td>10X Buffer (µl)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTPs (µl) (12.5mM)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Primers(µl)</td>
<td>Forward 0.4 (<em>GSTT1</em>)+ 0.4 (<em>GSTM1</em>) + 0.4 (Internal control )</td>
<td>0.5</td>
<td>0.3 (S) + 0.3 (Z)</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Reverse 0.4 (<em>GSTT1</em>)+ 0.4 (<em>GSTM1</em>) + 0.4 (Internal control )</td>
<td>0.5</td>
<td>0.3 (S) + 0.3 (Z)</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>DMSO(µl)</td>
<td>1.5 (10%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Taq Polymerase(µl)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Genomic DNA (µl) (50ng/µl)</td>
<td>0.6</td>
<td>0.8</td>
<td>0.4</td>
<td>0.4</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Steps</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C (5min)</td>
</tr>
<tr>
<td>35Cycles</td>
<td>95°C (5min)</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C (45sec)</td>
</tr>
<tr>
<td>35Cycles</td>
<td>95°C (45sec)</td>
</tr>
<tr>
<td>35Cycles</td>
<td>95°C (45sec)</td>
</tr>
<tr>
<td>Annealing</td>
<td>61°C (45sec)</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C (45sec)</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C (10min)</td>
</tr>
<tr>
<td>Infinity</td>
<td>22°C</td>
</tr>
</tbody>
</table>
Agarose Gel Electrophoresis for analyzing PCR products- The PCR products of different gene variants were checked on agarose gels (1.2%-2.3%) of different concentrations depending on the band sizes of the gene variants. The electrophoresis of the agarose gel was carried out at 100V for 30 min in the electrophoretic buffer (0.5mM EDTA, boric acid and TRIS) and after the run were viewed the agarose gels were then viewed under UV transilluminator. Gel pictures were captured on a gel documentation system (Plates V-VIII).

3.16.1 Molecular Genotyping

Glutathione S-transferase M1 and T1

The multiplex-PCR products of GSTT1 and GSTM1 gene variants were characterized by visualization resolved on 1.2% agarose gel electrophoresis. The genotype was determined by the presence or absence of 220bp PCR amplicon of GSTM1 gene and 450 bp of GSTT1 gene. The 796 bp-amplicon representing the internal control was present in all the samples. Accordingly genotyping was done as T1M1 (220bp, 450bp and 796bp), T1M0 (450bp and 796bp), T0M1 (220bp and 796bp) and T0M0 (796bp).

PCR Restriction Fragment Length Polymorphism- GSTP1 (rs1695; A313G) and variants of SERPIN A1 (S and Z, M1 and M2) were subjected to restriction digestion using specific restriction enzymes. The restriction digested products were resolved on agarose gels (2-3%) by electrophoresis at 75V for 80min.

Glutathione S-transferase P1 (A313G) - The GSTP1 PCR amplicon (176bp) was digested using the BsmA1 (Alw 26I, 55 °C) enzyme. The AA genotype resulted in an undigested band of 176bp, the AG allele resulted in three fragments of 176bp, 91bp and 85bp and the GG genotype was digested into two fragments of 85bp and 91bp.

Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1 (SERPIN A1)

S and Z alleles- The S and Z PCR amplicons (121bp and 179bp, respectively) were digested with Taq I polymerase (65°C). The expected genotypes were MM (100bp, 157bp,), MS (157 bp,121 bp,100 bp), MZ (179 bp,157 bp,100 bp), SZ(179 bp,157 bp,121 bp,100 bp), SS (157 bp,121 bp) and ZZ (179 bp,100 bp). However, all cases and controls recorded only the MM genotype.
**M1 (T710C)** - On restriction digestion of the M1 amplicon (360bp) with BstEII at 60°C, two fragments (311 and 49bp) imply the CC genotype, four fragments (311, 28, 83, 49 bp), the heterozygous (CT) and the three fragments (228, 83 and 49bp) the TT genotypes.

**M2 (G374A)** - The M2 PCR amplicon (462bp) was digested using RsaI (37°C) enzyme. The GG genotype results in two bands (383 and 79 bp), the GA allele resulted in three fragments (462 bp, 383bp, 79 bp) and homozygous recessive (AA) remains undigested (462bp).

To confirm the genotyping and check for any errors, 10% of the samples were rechecked for molecular genotypic analysis. The PCR amplicon-sizes, restriction digestion enzymes, gel concentrations used and the restriction digestion products with genotypes of the SNPs are presented in Table 3.

**Table 3. Details of PCR Amplification and Restriction Digestions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allelic variants</th>
<th>PCR Amplicon (bp)</th>
<th>Restriction enzyme</th>
<th>Gel Concentration</th>
<th>Restriction product size (bp)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GST</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GSTT1</em></td>
<td></td>
<td>220</td>
<td></td>
<td>1.2%</td>
<td>220,450 and 796</td>
<td>T1M1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>450 and 796</td>
<td>T1M0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>220 and 796</td>
<td>T0M1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>796</td>
<td>T0M0</td>
</tr>
<tr>
<td><em>GSTM1</em></td>
<td></td>
<td>450</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal Control</td>
<td></td>
<td>796</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intron 3 of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>HLADRB1</em>)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GSTP1</em> (A313G)</td>
<td></td>
<td>176</td>
<td>†BsmAI (Alw26I)</td>
<td>3%</td>
<td>176</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85,91,176</td>
<td>AG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>85,91</td>
<td>GG</td>
</tr>
<tr>
<td><strong>SERPIN A1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S</em> (A863T)</td>
<td></td>
<td>121</td>
<td>TaqI</td>
<td>3%</td>
<td>100,157</td>
<td>MM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100,121,157</td>
<td>MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100,157,179</td>
<td>MZ</td>
</tr>
<tr>
<td><em>Z</em> (G1096A)</td>
<td></td>
<td>179</td>
<td></td>
<td></td>
<td>100,121,157,179</td>
<td>SZ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>121,157</td>
<td>SS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100,179</td>
<td>ZZ</td>
</tr>
<tr>
<td><em>M1</em> (T710C)</td>
<td></td>
<td>360</td>
<td>BstEII</td>
<td>2%</td>
<td>49,311</td>
<td>TT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>49,83,228,311</td>
<td>TC</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>49,83,228</td>
<td>CC</td>
</tr>
<tr>
<td><em>M2</em> (G374A)</td>
<td></td>
<td>462</td>
<td>RsaI</td>
<td>2.5%</td>
<td>79,383</td>
<td>GG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>79,383,462</td>
<td>GA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>462</td>
<td>AA</td>
</tr>
</tbody>
</table>

†current nomenclature Alw26I
3.17 Statistical Analysis- Initially Excel (Microsoft, 2010, USA) software for Windows was used for data entry and database maintenance. For the statistical analyses the Statistical Package for Social Sciences (SPSS, Version 16) and the MedCalc softwares (https://www.medcalc.org) for Windows XP was used. To test the normality of the data, the Kolmogorov-Smirnov test was performed (Appendix VII). As the data were not normally distributed Mann-Whitney test was performed (Appendix VII, Table 2). However the parametric tests were also performed and significant levels were similar. Hence the results as the parametric test have been retained. Also as the sample size exceeded 30 and as explained by Garth (2008) and Fagerland (2012), the parametric statistical analyses can be performed if sample size exceeds >30. The general demographic and lifestyle characteristics, exposure history parameters as well as the in situ assessed parameters were summarized with means and standard error of mean (mean±S.E.M.) for the continuous variables and the categorical data were presented in numbers and percentages, wherever appropriate. The distribution of the categorical data in the study group was compared using the Chi-squared analysis. The equality of baseline data and of the assessed parameters (continuous variables) across the study group was evaluated using the Students’t-test for comparisons between two categories whereas Analysis of Variance (ANOVA) followed by Tukey’s test was done for multiple comparisons. Pearson correlation analysis and the Analysis of Variance (ANOVA) were carried out to assess the association (if any) of the confounding variables with the assessed parameters of genetic damage, oxidative stress and molecular genotyping. For finding the predictors of these assessed parameters, the linear regression (univariate, multivariate, combined multivariate and step-wise) analyses were performed. To assess whether the observed genotypic distributions were consistent with Hardy-Weinberg equilibrium, the Court Lab calculator (www.tufts.edu/~mcourt01/.../Court%20lab%20-%20HW% 20calculator) was used. The Principal Component Analysis (PCA) was used for assessment of both, disease-risk and for genetic damage by analyzing the inter-relationships among a large number of variables and to explain these variables in terms of a smaller number of variables (factor reduction) with a minimum loss of information. Distortions in the genotype frequencies were identified between cases and controls with two-by-two contingency tables using Chi-squared analysis.
Genotype frequencies for the cases and controls were examined separately and compared with the scientific literature using meta-analysis. The general process as proposed by Lee (2015) for meta-analysis of genetic association studies was performed to analyse the variance and presence of heterogeneity in studies; this comprises Hardy-Weinberg test to check study quality (already performed by authors) and the Cochran Q test for checking inter-study heterogeneity and construction of forest plots for checking population bias. The available molecular genotyping results in cases and controls for the GSTT1, M1 and P1 SNPs (not for SERPINA1 M1,M2, S and Z because of lack of appropriate data in documented literature) were compared to those available in different studies (in PubMed) carried out in different population groups. For this, the MedCalc (www.medcalc.org) software was used for calculation of odds ratios (ORs) and construction of forest plots. The steps included manually searching of the literature on PubMed using such keywords as ‘Genetic Polymorphism and COPD’, ‘GSTT1 and COPD’, ‘GSTM1 and COPD’ and ‘GSTP1 and COPD’. The studies from different populations, which met the criteria of genotypic data being available and use of case-control design, were only included. For the GSTT1 gene, of the 13 available studies only 11 studies were considered; for GSTM1 of the 15 studies only 12 studies had the genotypic data and of the 11 available studies for GSTP1, only eight published studies were included for meta-analysis. A manual search was also made for the cross-references of these studies for additional literature. Heterogeneity between studies based on $I^2$ based-Q (inconsistency based Chi-squared statistic) test was also recorded.

Associations between SNPs (individual and combinational) and risk of COPD were tested in genetic models by Odds ratios (ORs) at 95% confidence intervals (CIs) using the binary logistic regression analysis (considering the wild-type variant as referent). For the dominant model, the genotypes of minor homozygotes and heterozygotes were combined and compared with the homozygous wild genotypes; for the recessive model analysis homozygous wild genotypes and heterozygotes were combined and compared with the homozygous variant genotypes. The additive model comprised comparison of homozygous wild type genotype with homozygous variant genotype and in the co-dominant model, homozygous variants were combined and compared to the heterozygous variant. Bonferroni correction was applied to validate the significance values obtained on Odds ratio analysis. The Haploview software (version 4.2, USA)
was used to analyze the association between haplotypes and disease (COPD). Linkage disequilibrium (LD) analysis was performed using genotype data from all the participants. The pattern of LD was analyzed using two parameters, $r^2$ and $D'$. A receiver operating characteristic (ROC) curve was used to assess the validity of parameters on basis of sensitivity and specificity and for setting of the optimal cut-off values in order to discriminate cases from controls. The cut-offs obtained from the ROC curves were then used for categorization of continuous data (genetic damage, oxidative stress) for the Multifactor Dimensionality Reduction (MDR) analysis. The MDR analysis was carried out using the MDR (3.0.2 version) software for gene-gene and gene-environment interaction analyses. Graphical representation of the data was made on Microsoft Excel. Statistical significance was established when $p<0.05$.

3.18 Limitations and Shortcomings- The present study has some limitations. Firstly, being confined to a particular population sub-group, the study limits generalization of the results to other population groups. Therefore, identification of COPD among other occupational groups and ethnicities will encourage replication and provide for comparisons. Secondly, participants were volunteers from same population sub-group, so there may be some selection-bias. The non-inclusion of females is a limitation on one hand. On the other hand, the study is homogeneous as it excludes changes which can be caused by gender differences. Despite adequate sample size of the present study, a larger sample size would provide more insights, and more so for comparisons with hospital-based patients.