INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) has public health relevance (WHO, 2016) challenging the health care systems on one hand, because of its high prevalence, morbidity and mortality yet being preventable and treatable on the other (López – Campos et al., 2016). Unfortunately, the mis-diagnosis and/or under-diagnosis of COPD contribute to its under-treatment of COPD and the lesser likelihood of effective interventions (Nascimento et al., 2007; Moriera et al., 2014). Annually, the global mortality from COPD is 3 million placing it at the fourth rank (Salvi, 2011) which is predicted to rise to the third rank by 2030 (WHO, 2016). In India, annual 500000 mortality causes from COPD are four-folds high than in the USA and Europe (Salvi et al., 2012; Gupta et al., 2013). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD, 2016) which is a project initiative by the National Heart, Lung and Blood Institute (NHLBI) and the World Health Organization (WHO), chronic obstructive pulmonary disease is characterized by persistent air-flow limitation that is usually progressive and is associated with exposure to noxious particles or gases resulting in an enhanced chronic inflammatory response in the airways and the lungs. The COPD condition includes chronic bronchitis and emphysema; in the former air-flow is impeded in the bronchi and trachea causing decreased expiratory flow rates while emphysema results from the proteolytic destruction of the lung parenchyma with the loss of lung elastic recoil (Sandford et al., 2001). The Indian study on Epidemiology of Asthma, Respiratory Symptoms and Chronic Bronchitis in Adults (INSEARCH) reported an overall prevalence of chronic bronchitis in adults (>35years) as 3.49% ranging from 1.10% in Mumbai to 10.00% in Thiruvananthapuram (Jindal et al., 2012) with bidi smokers at higher risk for developing it (8.20%) than their cigarette-smoking (5.90%) counterparts (Jindal et al., 2006a).

The disease-etioloxy is multifactorial and complex, manifesting from an interplay of host genetic-susceptibility and environmental exposures (Tonello and Poli, 2011). The genetic contribution of COPD in terms of heritability is 40-75% (El-Zein et al., 2012). Candidate and Genome Wide Association Studies (GWAS) have shown the association of as many as 125 genes and 16 loci with COPD (Probert et al., 2015). Among the risk-factors increasing the risk for development and pathogenesis of COPD, are included
tobacco smoke, increasing age, gender, occupational irritants, biomass fuels, low education level, environmental air pollution, low body mass index (BMI), childhood history of chest diseases and history of physician-diagnosed chronic chest diseases (Bakr and Elmahallawy, 2012; Varraso et al., 2015; Denguezli et al., 2016). In fact 3-10% of COPD cases are only attributable to occupational exposure (Hnizdo et al., 2002; Melville et al., 2010). The inflammation of lungs, evoked by particulate matter (PM) and components of tobacco smoke, air-pollution and occupational exposure, initiates the pathogenesis of COPD (Yoshida et al., 2006) although the pathophysiological mechanisms linking the inflammatory response to compromised lung function are complex and not well understood; also involved are with genetic predisposition, immune-regulation and cellular repair (Mac Nee and Tuder, 2009).

Observations on increased oxidative stress in lungs have documented damage to pulmonary cells, hypersecretion of the mucus, inactivation of anti-protease and exacerbation of lung inflammation (Maluf et al., 2007; GOLD, 2011) as well as the decreased total antioxidant capacity in COPD cases (Nadeem et al., 2005; Cristovao et al., 2013). However not all studies have reported oxidant and antioxidant imbalance in COPD. Crisovao et al. (2013) had observed increased malondialdehyde (MDA) levels while Moussa et al. (2014) have reported decreased MDA levels in COPD cases. Polyunsaturated fatty acids (PUFA) are present in the cell membranes and their oxidation to lipid hydroperoxides (Repetto et al., 2012; Ayala et al., 2014) causes lipid peroxidation, primarily forming the dialdehyde, MDA. Several studies have in fact shown the presence of increased MDA levels in the exhaled breath-condensate (Bartoli et al., 2011), blood-sera (Patil et al., 2011; Arja et al., 2013; Cristovao et al., 2013) and sputum (Antus et al., 2014) of COPD cases. For other oxidants including SOD (Gavali et al., 2013), vitamin C, vitamin E and GSH (Pawar et al., 2014); increased concentrations have been observed. Other pathophysiological changes reported in COPD cases are hyperinflation, diminished exercise capacity, malnourishment, decreased muscle strength, dyspnea (Wilson et al. 1995), functional impairment in daily life (Bowling, 2001) and reduced quality of life (Taillefer et al., 2003). These observations highlight the clinical heterogeneity of COPD.

A reliable pulmonary function test is the non-invasive procedure of spirometric measurements of forced expiratory volume in the first second (FEV$_1$) and the less than
0.7 ratio of \( \text{FEV}_1 \) to forced vital capacity (\( \text{FEV}_1/\text{FVC} \)); these indicators are useful in the diagnosis and staging of chronic obstructive pulmonary disease (Rabe et al., 2007; Falcon-Rodriguez et al., 2016). The GOLD classification for stratification with increasing severity of air-flow limitation assists in identifying mild, moderate, severe and very severe COPD cases (GOLD, 2016).

Paradoxically there is both, under-diagnosis and over- or mis-diagnosis of COPD, with more under-diagnosis at population level (Johns et al., 2014). The PLATINO (American Pulmonary Obstruction Investigation Project) study has revealed 83.30% undiagnosed COPD cases in the US (Moreira et al., 2014) and an almost similarly undiagnosed (80.00%) cases in Brazil (Santos et al., 2014). Spirometry programmes at primary care centres, as in Greece (Minas et al., 2010) and at population-level in high risk occupational settings (Omland et al., 2014), can assist in identifying undiagnosed COPD patients in early stages of disease, thereby offering better prognosis on treatment (Csikesz and Gartman, 2014). In India, scope for such a possibly exists as there are unexplained substantial geographical variations for the prevalence of COPD (Bose et al., 2015). This is probably because of limited use of spirometric measurements to diagnose COPD with dependency only on the questionnaire based data for prevalence-estimates (Gupta et al., 2014).

In the industrial sector, the construction industry is a source of continuous fugitive emissions which can induce COPD (Hnizdo et al., 2002). Stone-crushing, on which the construction industry depends, is intensive and laborious involving mining, crushing, grinding and sand-blasting, thereby generating fine dust clouds at the workplace, and therefore these are considered as high-risk activities with regard to particulate matter (PM)-exposure (Demircigil et al., 2010). PMs are a mixture of particles and droplets in the air, consisting of metals, acids, soil, and dust (U.S. Environmental Protection Agency, 2006; Rai and Panda, 2014). Measurements of the PM in ambient air are reported as the mass of particles with an aerodynamic diameter that is less than 2.5 \( \mu \text{m} \) (\( \text{PM}_{2.5} \)) or 10 \( \mu \text{m} \) (\( \text{PM}_{10} \)) (Zhu et al., 2006). Suspended particulate matter, dust exposure and heavy metals from mined rocks also induce an inflammatory response and increase cytokine production with concurrent increase in reactive oxygen radicals which can cause oxidation of macromolecules (Donaldson and Borm, 1998).
The stone-crushing and related activities in fact cause specific exposure to silica-containing dust which manifest primarily as silicosis (Golbabaei et al., 2004; Sivacoumar et al., 2006), COPD (Iftikhar et al., 2009), emphysema and chronic bronchitis (Jhoncy et al. 2011), lymph node bronchitis and rheumatoid arthritis (Stolt et al., 2005), renal an immune dysfunction (Gottesfeld et al., 2008), arthritis (Ugbogu et al., 2009), and cancer in severe cases (Jhoncy et al., 2011).

The development of the cancer of lung (Raviv et al., 2011), larynx (Mohamed and El maghraby, 2014), colon, rectum, larynx, prostate and urinary bladder cancer (van de Schans et al., 2007) in COPD cases is often an inadvertent manifestation from exposure to dust and dust constituents at the workplace (Mashammer and Neuberger, 2004) and as in all cancers, initiating from genetic damage (Hasty, 2005). However there is sparse literature on genetic damage assessment in those with such an occupational exposure. In peripheral blood lymphocytes (PBL) and in the nasal epithelium cells (NEC), micronuclei-induction was significantly higher in stone-crushing workers (Demircigil et al., 2010) and in PBL, Sobti and Bhardwaj (1991) had also reported increased chromosomal aberrations and sister-chromatid -exchanges (SCEs). The presence of particulate matter at stone-crushing units containing dust, smoke, silica, talc, heavy and the transient metals including lead, chromium, cadmium, selenium and nickel (Kusaka et al., 2001), which have inflammatory capacities (Upadhyay et al., 2014) and also mutagenic and/or carcinogenic potential (Valverda et al., 2001; Hengstler et al., 2003), may actually be the subtle causes for these effects. In fact, Increased oxidative stress and genomic damage from occupational exposures at the stone-crushing, construction, and demolition have in fact been documented (Halder and De, 2012, Demircigil et al., 2010; Ilahi et al., 2012).

DNA damage and repair are however normal cellular functions maintaining genomic integrity. This may be compromised on account of genetic variability causing increased susceptibility to the disease-state and/or genetic damage. Studies have revealed that genetic polymorphisms of DNA damage and repair genes viz. of Excision Repair Cross-Complementation Group 2 - ERCC2 (Savina et al., 2016), human 8-oxoguanine DNA N-glycosylase 1- hOGG1, X-ray repair cross-complementing protein 1- XRCC1 and the tumor protein 53- p53 (Nishank, 2015) have shown association with various diseases, including respiratory. The metabolic genetic polymorphisms of Cytochrome P450
oxidases (Chen et al., 2001; Li et al., 2006), glutathione-S-transferases (da Silva et al., 2008; Villarini et al., 2008; Wlodarczyk and Novicka, 2012), N-acetyltransferases (NAT) (Liu et al., 2007) and of XRCC1 (da Silva, 2014) also influence levels of genetic damage on account of differential free-radical scavenging activities of these enzymes. Among 125 candidate COPD genes, the SERPINA1 (Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1) encoding alpha-1-antitrypsin (AAT) is a likely COPD candidate gene, as AAT deficiency can cause COPD (da Silva, 2014). An association of the SERPINA1 S and Z genotypes has already been documented with COPD in some studies (Lieberman et al., 1972; Hersh et al., 2004). The SERPINA1 is a highly polymorphic (>125 SNPs) locus (earlier name Proteinase Inhibitor, Pi, 14q31-32.3; 12.2kb) comprising seven exons (Li et al., 1988) encoding a 52-kDa protein which normally inhibits the activity of the lung neutrophil elastase. The AAT deficiency therefore results in the destruction of the major structural proteins of the alveolar wall. The common alleles of SERPINA1 gene comprise the normal M alleles and its sub-types (PiM1 Ala, PiM1 Val, PiM3) and the deficient alleles are PiS and PiZ (Crystal, 1990). Among 95% of severe AAT deficiency cases, are homozygous (ZZ) for PiZ (ATS/ERS, 2003) and the PiSZ heterozygotes which are highly prone to COPD as AAT serum levels are one-thirds of the normal (Dahl et al., 2005). The PiMZ heterozygotes on meta-analysis indicated a slight increased risk for COPD (but without lung-function impairment) compared to the normal PiMM (Hersh et al., 2004). However, as per the current nomenclature (www.genenames.org/cgi-bin/gene_symbol_report?hgnc_id=8941) uses the SERPINA1 M1, M2, M3, S, Z symbols are used.

Underpinning and consolidating this background information, the present study was planned to cover a number of aspects viz. spirometric measurements for underdiagnosed COPD (occupational disease) cases against an occupational set-up (stone-crushing sites), top-soil analysis, assessment of inflammatory response manifesting as oxidative stress and in the oxidation of DNA and lipids, multiple tissue bio-samplings for genetic damage, molecular genotyping for disease-risk association, and ethnic-specificity for genetic predisposition to disease and genetic damage to avoid population-stratification bias.

A cross-sectional case-control study design was developed which entailed in situ spirometric identification of undiagnosed COPD cases from those employed at stone-
crushing units and self-reported as belonging to the same population sub-group (scheduled caste) from the same geographical areas of Bihar, adding to the robustness of the study. The occupational hazards at stone-crushing units from dust-constituting particulate matter, heavy metals and other noxious agents may induce genetic damage. Therefore it was thought appropriate to evaluate genetic damage in three tissues (PBL, buccal epithelial and urothelial exfoliated cells) encompassing different exposure routes and evaluating repairable (DNA damage) and unrepairable (chromosomal) damage besides DNA oxidation (oxidized purines and pyrimidines). DNA damage (PBL) and chromosomal (buccal and urothelial) damage are biomarkers of a biologically-effective dose (Mussali-Galante et al., 2005) and of early biological effects (Bonassi et al., 2007) of genetic alterations. The peripheral blood sera samples were analyzed for oxidative stress biomarkers of total oxidative status (TOS), total antioxidant capacity (TAC) and for the lipid-peroxidation marker of malondialdehyde (MDA). Another biochemical biomarker assessed from sera samples was alpha-1-antitrypsin (AAT) activity. As an inhibitor of serine proteinase (belonging to the SERPIN superfamily), AAT provides protection to tissues by preventing proteolytic damage as it acts when inflammatory cells release neutrophil elastase (Cox, 1995). Although AAT is synthesized in hepatocytes and macrophages, it protects the lower respiratory tract from proteolytic degradation.

The Single Cell Gel Electrophoresis (SCGE/comet) assay provides qualitative and quantitative (Cortés-Gutierrez et al., 2014) assessment of DNA damage at single cell level and has the sensitivity to detect single- and double-strand DNA breaks, alkali-labile and incomplete excision repair sites, and genomic structural discontinuities (Singh et al., 1988; Collins, 2004). Oxidized pyrimidines and purines as oxidative DNA damage levels, can be assessed by the modified comet assay (Collins et al., 2008). Tissue-specific genetic damage can be assessed as micronuclei in epithelial cells from toxicant(s) exposure via inhalation and ingestion (Kashyap and Reddy, 2012). The buccal and urothelial cells provide comprehensive cytogenetic assessment, not only of genome damage but also of cytotoxic and cytostatic effects (Fenech and Crott, 2002; Martinez et al., 2005; Speit and Schmid, 2006).

Moreover the determination of genetic polymorphisms along with biomarkers of genetic damage has the potential to increase sensitivity and specificity of the assays by
identifying effects and sensitive sub-groups (Franco et al., 2008). In view of this, genotyping was performed for seven SNPs of Glutathione-S-transferases (GSTT1, M1, P1313A>G) and Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1 (SERPINA1M1 (710T>C), M2(374 G>A), S (863A>T), Z (1096 G>T)). Among the metabolic phases I and II-pathways for exo- and endo-genous substances, the Glutathione-S-transferases (GSTs) are phase II enzymes which scavenge oxygen reactive species (Hayes and Strange, 2000). The GST SNPs however have reduced detoxification potential and increased susceptibility to diseases (Santovito et al., 2008).

The GSTP1 has more expression in the respiratory epithelium and therefore the P1 variants can affect respiratory outcomes (Gilliland et al., 2002). The GSTT1 and GSTM1 exhibit deletion-polymorphisms and the homozygous null (deletion) variants lack enzyme activity. The GSTT1, M1 and GSTP1 variants have shown associations with COPD (Stankovic et al., 2015) though not consistently (Žuntar et al., 2014; Stankovic et al., 2015). However the GST variants have also been documented to modulate of genetic damage (Moretti et al., 2007; da Silva et al., 2008; Cho et al., 2015) on account of diminished/depleted enzyme activities. The SERPINA1 variants have shown association with COPD in some studies (Kwok et al., 2004; Gupta et al., 2007) and not in others (Shim, 2001; Kim et al., 2005). Hence the SNPs of these genes have been studied.

As predisposition to disease and modulation of genetic damage may vary for population-groups (Ngo et al., 2014) because of genetic variability from SNPs, the biological significance of SNPs needs understanding which can initially can be studied from their distribution in different ethnic groups. Hence, variants of GST and SERPINA1 were genotyped in COPD cases belonging to Bihar. Furthermore as within a population, inter-individual variability may have differential health outcomes with some more vulnerable even within a sub-group, therefore sub-groups of a population vulnerable to disease-occurrence and/or genetic damage needs to be identified. In the present study hence, scheduled caste migrants from the same geographical area in the state of Bihar working at stone-crushing units in Punjab, comprised the cases of the present study with those working in jobs without exposures comprising the healthy controls.
With this purpose and in view of limited studies on genetic polymorphisms and genetic damage in COPD patients, the present study was planned coupling bio-monitoring at workplace to evaluate risks to health and genetic integrity. As genetic diversity is inherent in different Indian sub-populations, the present study was carried out on COPD cases (n=200) identified in situ at stone-crushing units, and in an ethnicity-, sex-, age-, and socio-economic status-matched healthy control group (n=200), where the cases and controls are economic migrants, working in Punjab but belonging to the state of Bihar.

**Rationale**

The permeative nature of dust-emissions arising from stone-crushing activities may increase risk for COPD and induce oxidative stress and genetic damage in an occupational-setting, more so in those with genetic pre-disposition.

**Hypothesis**

The activities in the stone-crushing industry generate dust and particulate matter, the constituents of which depend on soil geochemistry (e.g. heavy metals) of the region. Therefore for those employed in these activities with no protective gear, there is persistent and continuous exposure to dust from inhalation, contact and ingestion. This has the potential to compromise the cellular components of the air-lung barrier system and initiate local inflammatory responses and induction of respiratory distress (wheezing, coughing and phlegm production). In the absence of timely treatment and management, the early symptoms of chronic obstructive pulmonary disease (COPD) may develop from airflow-limitation such as mucus hypersecretion, broncho-constriction and reduced lung-elastic recoil force. Such an hazardous effect on the respiratory system can be assessed as a pulmonary function test from spirometric measurements (the gold standard for lung function) for identification of early COPD.

On exposure, the PM, respirable dust and its constituents can elicit an inflammatory response causing damage to cellular components via enhanced generation of ROS contributing to oxidative stress, which has the potential to oxidize cellular macromolecules comprising lipids, proteins and DNA. Hence detection of genomic damage and oxidative stress biomarkers of exposure and predispositional genotypes can assist to identify those who are at risk of developing COPD and its severity at an early stage for timely interventions.
In the present study therefore, besides in situ spirometric assessment of pulmonary function in those engaged in stone-crushing activities to even-out the underdiagnosis of COPD, the bio-monitoring for oxidative stress and genetic damage in multiple tissues (PBL, buccal and urothelial cells) and molecular analysis was also carried out. The study outcomes can be valuable for early identification of those with the at-risk genotypes for disease-etiology and/or genetic damage for preventive interventions.

**Objectives**

A cross-sectional case-control association study, as a first of its kind on a specific population sub-group, (scheduled caste from Bihar), was hence carried to completion on the following objectives:

1. to study some single nucleotide polymorphisms (SNPs) of the SERPINA1 (Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 1) and the Glutathione-S-transferases (GSTT1, GSTM1, GSTP1) genes,

2. to assess primary DNA damage and oxidative DNA damage using the alkaline Single Cell Gel Electrophoresis (SCGE) and the enzymatically-modified SCGE/comet assays in peripheral blood leukocytes,

3. to ascertain chromosomal damage using the Buccal and Urothelial Micronucleus assays,

4. to study the association of the studied SNPs stratified for COPD-severity and genetic damage levels,

5. to estimate levels of some oxidative stress biomarkers by spectrophotometric analysis in blood serum samples.