Introduction

General Introduction

Cigarette smoking is the most significant cause of preventable death worldwide. About 50% of all smokers are likely to be killed by a form of tobacco related disease. About 1 in every 10 deaths is related to tobacco use. Annually, about six million deaths are attributed to tobacco out of which five million deaths are current or former users and about 600,000 are due to second hand smoke affecting non-smokers. The projected death toll may rise up to eight million annually by 2030 unless checked by stringent measures. An estimated one billion smokers are there in the world with 80% of them inhabiting in low and middle income countries. In 20th century, tobacco caused 100 million deaths and if trends remain unchecked, the toll may rise to one billion in 21st century (WHO fact sheet No 339). An estimate amongst US population suggested smoking caused 13.2 and 14.5 years of life for male and female smokers respectively (Centers for Disease Control and Prevention 2002).

The primary organ affected by cigarette smoking is undoubtedly the lung where it causes a series of pulmonary diseases and carcinoma of lung. About 70% of all deaths of lung, trachea and bronchus carcinoma are caused by tobacco use. The smoking effect spreads throughout the body affecting the normal functioning of various organs along with cancer of different tissues. Smoking causes cancer of Lip, Oral Cavity, Pharynx, Esophagus, Stomach, Pancreas, Larynx, Trachea, Lung, Bronchus, Cervix, Uteri, Kidney, Renal Pelvis, Urinary Bladder and Acute Myeloid Leukemia. Smoking also accounts for various forms of cardiovascular diseases namely Ischemic Heart Disease, Other Heart Disease, Cerebrovascular Disease, Atherosclerosis, Aortic Aneurysm and Other Arterial Disease. Amongst various forms of respiratory diseases, smoking causes Pneumonia, Influenza, Bronchitis, Emphysema and Chronic Airway Obstruction (Centers for Disease Control and Prevention 2008).

Several measures were adopted throughout the world to combat the epidemic. Steps taken include banning of advertisement and sponsorship of tobacco products, increase in tax and price of tobacco products, prohibition of smoking in all public places and work places, and display of graphics and pictures on the tobacco packaging about devastating health effects caused by
tobacco products. A complete ban on tobacco product advertisement resulted in 7% decline in average tobacco consumption with some countries even experiencing 19% decline. However, only 19 countries representing 6% of world population are covered under comprehensive advertisement ban. Increase in tax and costlier tobacco product affect tobacco consumption especially in young and poor peoples. Increase in tobacco cost of about 10% decreases consumption by up to 4% in high income countries and up to 8% in low income countries although only 27 countries representing less than 8% of world population applied tax rate greater than 75% of retail price. Nineteen countries representing 15% of world population adopted best pictorial warnings practice and forty two countries representing 42% of world population adopted mandate pictorial warnings (WHO fact sheet No 339). In spite of stringent measures, even 5.5 trillion cigarettes are still smoked each year.

An estimate by policy exchange reveals that the total cost of smoking to the society is about 13.74 billion pounds including 2.7 billion pounds by National Health Service for treating smoking related diseases, 2.9 billion pounds due to loss of productivity during smoking breaks and 2.5 billion pounds for absenteeism (Nash 2010). Another study indicated during 2000-2004, cigarette smoking and exposure to smoke caused 443,000 premature deaths, loss of 5.1 million years of potential life lost along with 96.8 billion dollars of productivity losses in United States (Centers for Disease Control and Prevention 2008).

Cigarette smoking harms almost each and every organ of the body. It causes almost 90% and 80% of lung cancers in men and women respectively. About 90% of deaths from COPD are also caused by cigarette smoking. According to estimates by WHO, COPD will become third leading cause of death worldwide by 2030 (World health statistics 2008). Compared to nonsmokers, smokers bear two to four times higher risk of coronary heart disease and stroke, 23 times and 13 times elevated risk of developing lung cancer in men and women respectively, 12 to 13 times increased risk of dying from COPD (USDHHS 2004). Cigarette smoking mortality is even more than combined deaths of human immunodeficiency virus (HIV), illegal drug use, alcohol use, motor vehicle injuries, suicides, and murders (Mokdad 2004; Centers for Disease Control and Prevention 2008). Undoubtedly, tobacco is the biggest enemy we face.
History of tobacco use

The origin of tobacco cultivation and evolution of the practice of smoking involves various continents and inhabitants of diversified culture and status. According to Huron Indian myth, "In ancient times, when the land was barren and the people were starving, the Great Spirit sent forth a woman to save humanity. As she traveled over the world everywhere her right hand touched the soil, there grew potatoes. And everywhere her left hand touched the soil, there grew corn. And in the place where she had sat, there grew tobacco". Paleontologists from Meyer-Honninger Paleontology Museum have discovered 2.5 million years old fossilized tobacco of Pleistocene Era in the Maranon river basin in Peru. Instead of these outstanding discoveries, no regular and persistent habit of tobacco use was known in ancient world.

The present day tobacco plants started growing c 6000 BC in America. It was around c 1 BC that Native Americans started using tobacco in the form of smoking and chewing. By c 1 AD, tobacco was abundant in America. However the first illustration of smoking appeared in pottery vessels dated before 11th century.

In the year 1492, the first discovery of tobacco appears by the description by Columbus as "certain dried leaves which gave off a distinct fragrance" brought by native Americans while stepping on the New World arriving on the beach of San Salvador Island. The first referral of tobacco by Columbus appeared in Christopher Columbus' Journal in 1492 as "We found a man in a canoe going from Santa Maria to Fernandia. He had with him some dried leaves which are in high value among them, for a quantity of it was brought to me at San Salvador". However the dried leaves were later thrown away. During the second voyage of Columbus, a monk named Ramon Pane elaborately described about inhalation of smoke by Indians. He is also considered to be the pioneer in introduction of tobacco in Europe.

Subsequently, emigration from Europe to America continued with more than 85,000 people leaving from Seville of Spain from 1506 to 1560. As a consequence of such migration of populations, tobacco use in the form of smoking grew very fast and authors visiting the new continent mentioned such trend in their writings. Smoking was first observed by Rodrigo de Jerez and Luis de Torres. Jerez became the first European smoker and carried the habit to Spain.
where eventually it became a trend although Jerez was initially jailed for 7 years for smoking (Borio Gene, Tobacco Timeline: The Twentieth Century 1950–1999—The Battle Is Joined).

During the course of time, tobacco spread over the world from Europe and Middle East to Asia and Africa up to Oceania. The mode of use of tobacco also changed during the past few centuries with snuff of 18th century changing to cigar of 19th century and 20th century using manufactured cigarettes. By the first decade of 21st century, smoking capsized a major population of world and started to show the deleterious effects of the habit in terms of mortality and morbidity.

**History of Cigarette smoking in India**

The introduction of tobacco in India dates back to 1600 (The history of tobacco smoking, WHO). Portuguese sailors in search of Indian spices and silk are credited with introducing tobacco in the Indian continent. By the time Portuguese arrival in India, tobacco use spreaded from American ‘Indians’ of new world to old world of Europe. Following the trading route of Portuguese, the capital city of Bijapur of the kingdom of Adil Shahi, presently Karnataka of south India, was first to be introduced with Tobacco. Initially, tobacco was even used in barter trade. Although in early days, tobacco use was limited to the upper income class of India, but gradually it reached to the more generalized population. During seventeenth century, tobacco use started to escalate amongst Indians (Report on Tobacco Control in India).

Following British colonial rule, tobacco yield and use changed drastically. American tobacco used to be traded for purchasing Indian goods. After independence of American colonies in 1776, the British East India Company started to cultivate tobacco as a “cash crop”. Tobacco became a valuable commodity for both “domestic consumption and foreign trade”. Previously, exported tobacco leaves were used to manufacture cigarettes in Britain. Cigarettes re-imported to India used to serve the growing cigarette market. With rise in Indian demand for cigarettes, the Imperial Tobacco Company started cigarette production in India itself.

The rise of beedi industry in India dates back to the end of nineteenth century and by first three decades of twentieth century, the custom spread all over India. In comparison to expensive cigarette consumption, cheaper beedis inevitably received favor from the general mass of India.
and beedis surpassed the demand of cigarette as a cheap form of tobacco use compared to cigarettes.

In independent India, tobacco flourished both “as a crop and as an industry”. The reason behind obviously being the revenues obtained from tobacco, as a valuable commodity of export and the employment opportunity it opened to Indian market.

**Smoking forms of Tobacco**

Tobacco smoking in Indian subcontinent changed its form from time to time. In the initial times of seventeenth century when the tobacco use was escalating amongst royals, the most usual form used to be **hookah**, sometimes referred to as water pipes. It is even considered as “the introduction of tobacco into India”. Tobacco and a smoking pipe were received as a gift by Akbar from Portuguese. Instead of resistance from Hakim Abul Fath, the chief justice and administrator of Akbar’s court, Akbar decided to circulate tobacco but with a modification of the smoking mode. To minimize possible deleterious effects, the smoke is first passed through water to purify it. The purification system generated the hookah which was prevalent in early days of smoking. Although such measures were taken, but that simply used to remove particles from the smoke and further cooled down the smoke without actually decreasing the harmful effects of smoking. In spite of smoke taken through water, it delivers almost same amount of nicotine and even may deliver 100-200 times more smoke than cigarette smoking. Hookah smokers are susceptible to the same kind of diseases as caused by cigarette smoking. In Mughal emperor, hookah became a part of Indian culture with the access mostly limited to the elite class of the society, particularly in northern India. Mughal paintings demonstrate the habit of smoking in both men and women. Later the manufacture of hookah spread across India.

Another form of tobacco smoking is **cigar** and is usually considered as the most primitive form of smoking in history. Dried and fermented leaves of tobacco are rolled and wrapped in a leaf of tobacco and the smoke from burning of tobacco was drawn into mouth. Around 1670, cigars were introduced in India. Use of cigars were well known till 1970s when a steep increase in excise duty on cigars and cigarettes lead to gradual decrease in cigar consumption. But still its use continues and it possesses similar health effects as cigarette smoking.
Undoubtedly, the most abundant form of tobacco smoking in India is beedi that can be considered as non-filtered cigarettes that account for three fourths of cigarette consumption in India. It is tobacco encased within a tendu or temburni leaf. It delivers a high yield of nicotine, carbon monoxide and tar and demonstrates most devastating effects of tobacco smoking. It is indeed more harmful than conventional cigarette smoking.

**Cigarette smoking**

A generalized description of cigarette is dried tobacco leaves cut finely and rolled in a thin paper. Undoubtedly, cigarette smoking is the prime most form of tobacco smoking in the world and also associated with generation of a huge amount of revenue. The cigarette smoking started from the new world to European countries. Around 1880, mechanical manufacture of cigarettes started. The first cigarette factory established was in St Petersburg in Russia named Ferme Cigarette Factory. In 1906, the first cigarette factory named Indian Tobacco Company or ITC (formerly Imperial Tobacco Company) was established in Munger, Bihar. “Scissors” was the first brand launched in 1912.

With time, the tobacco industry in India grew and use of tobacco also escalated in both smoking and smokeless ways. According to GATS India (Global Adult Tobacco Survey India), 35% of adults using tobacco in one form or other and 14% of adults smoke tobacco. A total 111.2 million peoples of India smoke tobacco with 24% males and 3% females are smokers of tobacco. Amongst tobacco smokers, almost 9% prefer bidis followed by cigarette smokers comprising 6% with hookah smoker being 1%. Cigarette smoking is more preferred in urban areas than rural areas. Estimates revealed that cigarette smokers smoke 6.2 sticks daily and for bidi smokers, the number is 11.6 sticks per day. About 25% of regular cigarette smokers smoke 10 cigarettes and more than 50% of bidi smokers smoke 10 bidis per day.

According to GYTS (Global Youth Tobacco Survey, 2000-2004), 8.3% amongst students are current smokers ranging from 2.2% in Himachal Pradesh to 34.5% in Mizoram. Current cigarette smoking prevalence ranges from 0.5% in Goa to 22.8% in Mizoram. As far as second hand smoke exposure is concerned, about 34.6% of students were affected within their homes ranging from 9.9% in Punjab to 79.0% in Meghalaya. About 48.7% of students were exposed to second hand smoke outside their homes ranging from 23.5% in Punjab to 84.4% in Meghalaya.
Cigarette smoking associated diseases

CS is a mixture of about 4000 compounds which includes several short lived and long lived persistent free radicals along with oxidants and toxic compounds. Apart from being an inducer of oxidative stress, it also induces carcinogenicity owing to the presence of several carcinogenic compounds. The complex composition of CS makes it particularly difficult to identify the key components and the mechanisms underneath the disease development.

Cigarette smoking has been associated with several life threatening diseases and is believed to be a significant contributor to mortality and morbidity worldwide. Apart from several degenerative diseases, it also causes cancer of lung and other organs. Exposure to CS components creates a positive feedback loop including the inflammatory cells and apoptosis that is exacerbated by infection with pathogens leading to elastin degradation and tissue destruction (Sussan 2011).

Cigarette smoking and cancer

Cigarette smoking has been suggested to cause carcinoma of lung for very long time (Doll 1950). Currently, cigarette smoking is considered as the most important risk factor for lung cancer incidence and the risk increases with both number and prolongation of smoking habit (Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012). Suggested proportion of lung cancer due to cigarette smoking has reached 90% (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). As the research continued to explore the capacity of CS to cause cancer, gradually prominent link between Cigarette smoking and cancer of different tissues evolved indicating carcinogenic compounds after entry into the body through lung gets distributed in diversified tissues and causes the induction of cancerous growth of tissues. The
cancers caused by cigarette smoking not only are confined to the respiratory passage and lung, but also includes several distant tissues probably by the distribution of disease causing compounds through circulation. Cancers of respiratory passage where maximum effect of cigarette smoking is exerted span from oropharynx, larynx and esophagus up to trachea, bronchus and lung parenchyma (USDHHS 2010). Cancer of oral cavity including lip and tongue and also cancer of nasal cavity, paranasal sinuses and nasopharynx are attributed to cigarette smoking as well (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans).

Although the incidence of lung cancer is declining in both males and females in US, but still maximum number of mortality from cancer is caused by lung cancer and cigarette smoking has been identified as the major cause behind the incidence of lung cancer in US (Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012). Globally, lung cancer surpasses all other cancer type in both incidence (16.5%) and mortality rate (22.5%) in men. Among women, lung cancer occupies fourth position in incidence rate (8.5%) and second in terms of mortality rate (12.8%) (Ferlay 2010; Bray 2012).

Symptoms indicating lung cancer include persisting cough with blood in the sputum. Symptoms also involve “chest pain, voice change, and recurrent pneumonia or bronchitis”. A broad classification of lung cancers for the sake of treatment is small cell (14%) and non-small cell lung cancer (Cancer Facts & Figures 2012. Atlanta: American Cancer Society; 2012). In small cell lung cancer type, the cancer cells are small and full of nucleus. This type of cancer spreads rapidly and associated with cigarette smoking. Non-small cell lung cancer includes several types’ namely squamous cell carcinoma, adenocarcinoma (bronchiolar and alveolar carcinoma) and large cell carcinoma (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). Cancer of other tissues by cigarette smoking includes stomach, liver, pancreas, kidney, cervix, bladder and acute myeloid leukemia (USDHHS 2010; IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). Apart from these, cancer of urinary tract particularly bladder, ureter and renal pelvis are associated to cigarette smoking (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans).

Several carcinogenic compounds have been identified in the cigarette smoke (CS) that includes polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP), N-nitrosamines including NNK (N-nitrosamine4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone)
and NNN (N'-nitrosonornicotine), aromatic amines, benzene, aldehyde, oxidants and ethylene oxide. Of these compounds, the most competent lung carcinogens are considered to be NNK and NNN. Using animal models and human samples, the role of these compounds in inducing lung cancer has been established (Hecht 1988; International Agency for Research on Cancer 2006).

**Cardiovascular diseases (CVD)**

Cigarette smoking is a major cause of CVD worldwide with 40% mortality due to cigarette smoking caused by CVD (Ezzati 2005). Ischaemic heart diseases have been projected to be the leading cause of mortality worldwide in 2030 causing 14.2% of all the deaths (World Health Statistics 2008). Epidemiological studies confirmed that cigarette smoking is a major cause behind CVDs including “coronary heart disease, stroke, aortic aneurysm, and peripheral vascular disease”. The risk of CVDs increase with number and duration of cigarette smoking with cessation of smoking leading to decreased risk of CVD development (Burns 2003).

Regarding the pathophysiology of CVD development by CS, no complete and clear mechanism has been proposed with contribution from several pathological processes seeming responsible for the same. CS mediates oxidation of low density lipoprotein (LDL) to the oxidized form which is taken up by macrophage cells resulting in the formation of foam cells (Shashkin 2005). Foam cell formation is associated with pro-inflammatory cytokine secretion that in turn elevates inflammatory responses (Bernhard 2011). Recruitment of inflammatory cells further enhances oxidative stress following “oxidative bursts” and initiates damaging body cells. Such phenomenon starts a cycle of oxidative damage and inflammation and subsequently such unregulated inflammation is suspected to be the development of autoimmune disease. Oxidative modification of biomolecules alters self epitopes into altered ones that are identified as foreign molecules and thereby inciting inflammatory response against these (Wick 2004).

Endothelial cell dysfunction is also associated with CVD. Smoking induced oxidation of microtubute system of endothelial cells leads to change in cellular shape. CS leads to contraction of endothelial cells that further results in loss of “cell-to-cell contacts” and subsequent detachment of endothelial cells (Bernhard 2011). Another mechanism proposed for CS induced endothelial cell dysfunction is related to cell death which is more necrotic than apoptotic. It was also shown that N-acetyl cysteine is the only antioxidant that prevents such phenomenon.
indicating the role of metals in such incidences (Bernhard 2003). Two other mechanism proposed to add to CS induced CVD development are thrombogenesis promotion and fibrinolysin alteration (Burke 2003).

Several components of CS have been suspected as contributor to CVD development including nicotine, carbon monoxide, Polycyclic aromatic hydrocarbons (PAHs), lipopolysaccharides, reactive oxygen species and metals (Bernhard 2011; USDHHS 2010).

Respiratory diseases

The primary effect of cigarette smoking in undoubtedly imposed upon the lungs and pulmonary passages which is the chief exposed surface of our body to the outer environment. Smoking cigarettes leads to the inhalation of smoke through the nasal passages up to lung where complex pathological processes results in different types of pulmonary diseases. Research of several decades proved a significant relationship between smoking and different pulmonary diseases. In terms of mortality and morbidity, the most significant respiratory diseases caused by smoking include lung cancer as discussed above, chronic obstructive pulmonary disease (COPD), pneumonia, tuberculosis and interstitial lung diseases (ILDs).

COPD is characterized by airflow obstruction with obvious interference with normal breathing with generally a progressive nature of disease advancement. COPD disease condition comprises of changes that are both reversible and irreversible. Reversible changes include airway fibrosis and resulting narrowing, loss of elastic recoil property of lungs due to alveolar destruction, degeneration of alveolar support affecting smaller airways structure. The reversible clauses include mucus, plasma exudates and inflammatory cell accumulation in bronchi, contraction of smooth muscle in airways and hyperinflation during exercise (Rabe 2007).

Pulmonary tuberculosis results from infection with Mycobacterium tuberculosis and is symptomized by cough with blood in cough, weight loss, fever, breathing difficulty, chest pain and wheezing (PubMed Health 2011). The association between smoking and tuberculosis was drawn well before around 1918 but only recently it gained attention (Slama 2007). Several studies and reviews concluded in favor of an association between smoking and tuberculosis with smokers identified to be more susceptible to tuberculosis (Kolappan 2002; Maurya 2002; Lin 2007; den Boon 2005; Gajalakshmi 2003).
Almost 300 million peoples worldwide suffer from **asthma** (Action plan of the Global Alliance against Chronic Respiratory diseases, 2008-2013). It is a chronic inflammatory disease characterized by airway hyper responsiveness with associated breathlessness, wheezing, coughing and chest tightness (GINA 2006). The morphological features include airway smooth muscle increase, epithelial basement membrane thickening, mucus hyper secretion and mucus plugs along with infiltration of inflammatory cells.

**Interstitial lung diseases** (ILDs) is a collective term representing several pulmonary abnormalities generally symptomized by “dyspnoea, dry cough, diffuse interstitial infiltrates, restrictive lung function pattern, and impaired gas exchange”. Among different types of ILDs, respiratory bronchiolitis-associated interstitial lung disease and pulmonary Langerhans’ cell histiocytosis in adults have been suggested to be resulting from smoking habits (Behr 2002).

**Fractures**

CS exerts detrimental effect on bone metabolism and risk of bone fracture has been shown to be related to smoking. A meta-analysis, it was found that postmenopausal bone loss is greater in case of smokers compared to nonsmokers. The risk of hip fracture in smokers increases with increasing age. The increased risk of hip fracture in smokers is attributed to prolonged bone loss over years (Law 1997). In another meta-analysis, it was observed that current smokers are under significantly increased relative risk of bone fracture particularly hip and spine fracture without wrist fracture risk. Smoking cessation resulted in somewhat decreasing the risk (Vestergaard 2003).

**Fertility**

The effect of smoking on fertility status of smokers has been demonstrated using meta-analysis. Clinical pregnancy rates using assisted reproductive technology (ART) showed significantly decreased of clinical pregnancy odds ratio per cycle in smoking patients compared to their nonsmoker counterparts (Waylen 2009).

**Ocular damage**

Age related macular degeneration has been linked with smoking status of individuals. A meta-analysis concluded an increased risk of ocular damage in smokers than nonsmokers.
(Neuner 2009). However detailed mechanisms of smoking mediated effects on eyes are unknown but protective effects of antioxidants and density of macular pigments are decreased by smoking.

**Neurological effects**

The risk of Parkinson’s disease has been shown to be decreased by about 50% by smoking although detailed mechanism and CS components responsible for such observation are unknown (Hernan 2002; Allam 2004)

**Rheumatoid arthritis**

Rheumatoid arthritis is an autoimmune disease condition in which T helper 1 cells mediated immune response is prevailed. A meta-analysis revealed increased risk of the disease (31% and 87% in women and men respectively) in smokers. Even cessation of smoking also yields no difference in risk of developing the disease. Another report identified association between maternal smoking during pregnancy and incidence of rheumatoid arthritis particularly juvenile rheumatoid arthritis within first seven years of birth in girl child (Sugiyama 2010; Jaakkola 2005).

**Prenatal and Postnatal effects**

Several studies indicated the evidence that prenatal and postnatal cigarette smoke exposure may cause several health abnormalities in children. Exposure to cigarette smoke affects adversely the growing fetus in utero particularly lung and immune system development. Components of CS also cross placenta affecting fetal growth and increasing preterm delivery risk. Environmental tobacco smoke exposure is associated with increased prevalence of lower respiratory tract infection in infancy and early childhood (Li 1999). Respiratory tract infection due to parental smoking increases occurrence of asthma in later childhood (Peat 2001). Maternal cigarette smoke exposure also has been found to be associated with issues like blood pressure (Brion 2008) and weight (Oken 2008) of child. Another study concluded that prenatal exposure to CS is more related to development of asthma in children than postnatal exposure (Cunningham 1996). When the effects of paternal smoking and maternal smoking were compared, maternal smoking was found to increase the risk of sudden infant death syndrome.
fourfold where paternal smoking affecting about 1.5 times elevated risk in absence of maternal smoking (Mitchell 2006). Apart from these, environmental cigarette smoke was found to pose increased risk of developing otitis media (Moritsugu 2007).

**Emphysematous lung damage**

Emphysema is defined as “abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls and without obvious fibrosis” (American Thoracic Society 1962). Emphysema of lung is characterized by “progressive and irreversible loss of pulmonary alveoli” (Kubo 2011). The two major components defining the disease condition of emphysema are progressive airspace enlargement and destruction of alveolar walls. Several different types of emphysema are considered including centriacinar emphysema, panacinar emphysema, distal acinar emphysema and irregular emphysema with centriacinar type being most common and caused primarily by smoking (Figure 2).

The earliest description of emphysema dates back to 1834 that was based on the observations of lung postmortem cut surfaces (Laënnec 1834). Previously, it was hypothesized that compression of capillaries due to overinflation of lung with subsequent degeneration of lung tissue (McCallum 1940).

**Centriacinar** or **centrilobular** emphysema is characterized by destruction and associated expansion of respiratory bronchiole (proximal and central part of acinus) however distal acinus and alveolar structure remain unchanged. This type is more regularly encountered in the upper lobes particularly the posterior parts than the anterior parts and chiefly caused by cigarette smoking. It is accompanied by septal loss around respiratory bronchioles that progresses distally involving adjacent area. At the initial stages, the distal structures like alveolar ducts, sacs and alveolar structures remain unaltered indicating unaffected peripheral lobules (Pipavath 2009).

Figure 2 Different types of pulmonary emphysema. TB- tertiary bronchiole, RB- respiratory bronchiole, AD- alveolar duct and AS- alveolar sac (Pipavath 2009).

In **panacinar** or **panlobular**
emphysema, entire respiratory acinus is affected with damage mainly localized in the lower lung lobules including basal segments and anterior margins of the lung. Individuals with α1-antitrypsin (AAT) deficiency exhibit such emphysematous damage. In this type of emphysema the alveolar septal loss is observed in both primary and secondary lobules together with alveolar bronchioles, alveolar sacs and ducts. The main function of AAT is to bind to neutrophil elastase and its inactivation. In case of nonsmokers, neutrophil accumulation in lung is unlikely but in case of smokers, neutrophils accumulate in the lung due to inflammation. Individuals with normal AAT levels neutralize neutrophil elastases but with low levels or even absence of neutrophil elastases in the lung, the elastase activity remained unchecked leading the way to proteolytic degradation. Compared to centrilobular type, symptoms are observed pretty earlier in case of panacinar emphysema (Pipavath 2009).

**Distal acinar or paraseptal** (or superficial or mantle or linear) emphysema is considered by the presence of airspace enlargement at the periphery of acini. As this type of emphysema affects the most distal parts of acinus including alveolar sacs and ducts leaving the respiratory bronchioles, hence these are termed as distal acinar emphysema. The cause of this type of emphysematous lesions is not well known. Usually these lesions occur at a subpleural location in the anterior and posterior upper lobes, occasionally involving posterior lower lobes (Wright 2005).

Another type of emphysema that is not confined to a particular space affecting any part of the acinus is **irregular or paracicatricial** emphysema that is “secondary to airspace distortion” and develops around scars caused by silicosis, tuberculosis, bronchoalveolar carcinoma, sarcoidosis etc (Pipavath 2009).

**Cigarette smoking and protein modification**

Oxidative modification of proteins due to exposure to cigarette smoking has been suggested by several studies. Carbonyl formation, the hallmark of oxidative protein modification has been observed by many studies indicating a major effect of CS exposure being the carbonyl formation and associated alterations of protein structure and function. Oxidative modification of plasma proteins was observed in smokers blood indicating the potential role of cigarette smoking in oxidative modification of proteins (Pignatelli 2001)When isolated plasma was incubated with
gas phase of CS, formation of carbonyl and loss of protein sulfhydryl group was observed confirming potential oxidative damage by CS (Reznick 1992). It was also reported that gas phase oxidants resulted in lipid peroxidation and atherogenic lipoprotein alterations (Frie 1991). In an in vivo study using rabbit as the model animal and injecting CS extract in the ear vein, it was shown that CS not only oxidized low density lipoproteins, but also caused an elevated lipid peroxide levels with decreased vitamin E level indicating a role in atherosclerosis development (Yamaguchi 2001). The carbonyl formation by CS solution to microsomal proteins and blood plasma was observed to be increasing in a time dependent manner with oxidative modification also confirmed by tryptophan loss and bityrosine formation (Panda 1999). It was furthermore suspected that metals in CS play a significant role in oxidative modification (Bernhard 2005). In another study, it was observed that aldehydes present in CS decreased human plasma protein sulfhydryl and increased protein carbonyls (O'Neill 1994).

Protein oxidation itself results in several modifications of protein that alters structure and function of proteins. It was shown using in vitro systems that oxidation of proteins lead to aggregation, fragmentation, amino acid modification as well as proteolytic degradation susceptibility. Using BSA as the model protein, the modification of amino acids was observed that directly indicated alteration of protein primary structure. Amongst the amino acids, tryptophan, tyrosine, histidine, and cysteine were most sensitive. Alteration of primary structure in turn leads to gross alteration of secondary and tertiary structure. Using BSA, it was observed that oxidation of proteins denatures or increases hydrophobicity, generating covalently cross linked dimmers, trimers and successive polymers. After oxidative modification, fragmentation of BSA led to molecular sizes ranging from 7 kD to 60 kD. In terms of proteolytic susceptibility, oxidatively modified BSA also exhibited several fold increased proteolysis when incubated with cell-free erythrocyte extracts or proteases (Davies 1987).

Apart from oxidative modification, CS also causes other modifications owing to the presence of several thousand compounds. The aldehydes present in CS have long been suggested to modify proteins. A study on HSA as the model protein modified by CS extract identified acrolein and crotonaldehyde Michael adducts at Cys34, Lys525, Lys351, and His39 residues of the protein using nanoscale capillary liquid chromatography and electrospray tandem mass spectrometry analysis (Colombo 2010). Another much investigated protein modification identified in smokers is homocysteine formation that has been suspected to play a significant
role in cardiovascular disease development (Reis 2000; Pagan 2001; McCarty 2000, O’Callaghan 2002). In a study on smokers and non smokers blood plasma, a yet another protein modification was detected namely 3-nitrotyrosine formation (Petruzelli 1997). CS has been found to be interfering with normal functioning of reverse cholesterol transport pathway by affecting lecithincholesterol acyltransferase (LCAT) activity and modifying high-density lipoprotein (HDL) with increased negative charge and cross linking with apolipoproteins AI and AII (McCall 1994). CS has also been identified to cause ethylene oxide adduct formation on the N-terminal valine in smoker’s haemoglobin leading to the significantly higher levels of hydroxyethylvaline adduct (Bailey 1988).

A study conducted among workers exposed to various concentrations of benzene in three factories in China revealed the formation of benzene oxide (BO) and 1, 4-benzoquinone (1,4-BQ) adduct with proteins. It was observed that compared to the control individuals, the exposed individuals had significantly higher levels of albumin BO and albumin 1,4-BQ adducts and hemoglobin BO adducts. An interesting observation from the study was that cigarette smoking increased 1,4-BQ adducts with albumin indicating cigarette smoking leads to the formation of 1,4-BQ adduct with proteins (Yeowell-O’Connell 2001).

Components of cigarette smoke (CS)

CS is a mixture of more than 4,000 compounds. Out of these, several components of CS received special attention being significantly associated with CS mediated effects. Such compounds include nicotine, which is the principal cause of cigarette dependence, carcinogens including polycyclic hydrocarbons and Nitrosamines, aldehydes and free radicals.

Nicotine

Being the principal addictive component of CS, nicotine has been a compound of several studies with reports indicating the role of nicotine in cigarette dependence to various physiological effects in the body. Nicotine is the primarily responsible component for cigarette smoke addiction and dependence. Smoking of cigarettes has been shown to increase the arterial and venous nicotine concentration indicating nicotine is distributed throughout the body following smoking (Henningfield 1993). Other physiological effects of nicotine include increase in heart rate, constriction of coronary blood vessels with increase in myocardial contractility.
Other physiological effects reported include lipid peroxidation, endothelial injury and proliferation of vascular endothelial cells (Gouaze 1998; Villablanca 1998). On an average, a single cigarette smoked delivers up to 3 mg of nicotine (Benowitz 1998).

**Nitrosamines**

N-Nitrosamines are generated by reaction of secondary amine like nornicotine, anabasine and anatabine and tertiary amine like nicotine with nitrosating agents (Hecht 1988). Amongst all the N-Nitrosamines present in CS, two were studied extensively: 4- (methylnitrosamino)-1- (3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). Several studies detected carcinogenic activity of these compounds *in vivo*. NNK given to mice through gavages induced development of multiple adenomas per mouse lung (Xu 1992). The induction of lung tumors by NNK and NNN was also demonstrated in A/J mouse (Castonguay 1983). In yet another study, subcutaneous injection of both NNK to F344 rats developed tumors of nasal cavity, liver and lung. The same study also showed that NNN injection induced tumors of nasal cavity but failed to induce liver and lung tumors (Hecht 1980). The amount of these N-Nitrosamines were calculated to be 0.017-0.43 µg/ cigarette and 0.39-1.44 µg/ cigarette for NNK and 0.066-1.01 µg/ cigarette and 0.19-0.86 µg/ cigarette for NNN in mainstream and sidestream cigarette smoke respectively (Adams 1987). Regarding the mechanism of carcinogenesis by N-Nitrosamines, it was reported that NNK undergoes hydroxylation (α-hydroxylation) on the carbon atom adjacent to N-nitroso group leading to the DNA adducts formation: 7-methylguanine or O-methylguanine methyl adducts and pyridyloxobutyl adducts (Hecht 1999).

**Polycyclic aromatic Hydrocarbons (PAHs)**

PAHs or polynuclear aromatic hydrocarbons are organic compounds with two or more aromatic rings fused with each other. The presence of four or more ring PAHs in CS was showed to be 60-200 ng per cigarette (IARC 1986). The most studied PAHs is benzo[a]pyrene that is metabolically activated through conversion to 7,8-diol-9,10-epoxides (BPDE) which forms adduct with N² of deoxyguanosine of DNA (Hecht 1999). Studies using BPDE-treated HeLa cells and bronchial epithelial cells indicated formation of BPDE adducts in the exons of p53 gene, particularly at guanine residues 157, 248 and 273 indicating the role of benzo[a]pyrene in DNA adduct formation and lung cancer induction (Denissenko 1996). Study utilizing
lymphocytes cultured from healthy subjects and lung cancer patients and treated with BPDE concluded that subjects with reduced activity of removing BPDE mediated DNA adduct may be at an elevated risk of developing lung cancer (Li 2001).

**Benzene and Aldehydes**

One of the much studied constituent of CS is benzene (6-70 μg/cigarette) and other aldehydes like acetaldehyde (18-1400 μg/cigarette), crotonaldehyde (10-20 μg/cigarette) and formaldehyde (70-100 μg/cigarette), acrolein (60-140 μg/cigarette) (IARC 1986; Geiss 2007). Benzene has been suggested to cause one third of all smoking-induced acute myeloid leukemia (Korte 2000). The benzene concentration in the blood of smokers is much higher than nonsmokers (Vineis 2004). Acrolein has been suggested to cause lung cancer by two mechanisms: direct DNA damage by binding to CpG site and formation of acrolein DNA adducts and by inhibiting DNA repair mechanism (Feng 2006). Acrolein and crotonaldehyde has been shown to mimic the effect of aqueous cigarette smoke extract in releasing IL-8, a neutrophil attractant and TNF-α from human macrophagic cell line U937 thus indicating a role in inflammation in COPD (Facchinetti 2007).

**Metals**

Several carcinogenic metals are present in CS although the amount is in ng quantities per cigarette namely Nickel, Cadmium, Cobalt, Chromium, lead, polonium-210 and arsenic (Hecht 1999; Geiss 2007). Exposure of CS to male C57B1 mice and Sprague-Dawley rats for almost a year has been shown to increase the levels of cadmium in lung and kidney in both the species (Gairola 1991). It was reported that concentration of lead-210 and polonium-210 in rib bones of smokers were twice more than nonsmokers and lead-210 concentration in alveolar lung tissue of smokers was twice that of nonsmokers indicating accumulation of metals within body by CS exposure (Holtzman 1966).

**Free radicals**

CS contains large amounts of free radicals and free radical generating components (Pryor 1983; Cosgrove 1985). Reports suggest presence of about $10^{17}$ stable long lived radicals per gram of tar phase and $10^{15}$ radicals per puff of gas phase (Pryor 1993). The free radicals are
broadly classified in two groups; first group generated during burning of tobacco and smoking process involving TPM and gas phase, and second group is formed by the oxidation of TPM constituents or gas phase including semiquinones, ROS and RNS. It was demonstrated later that hydroquinone present in tar phase generates semiquinone and superoxide radicals by autooxidation (Zang 1995). The EPR analysis of TPM radicals suggested the presence of semiquinone radicals (Pryor 1983 a b). The nitric oxide and superoxide present in the gas phase reacts with each other in aqueous phase to produce reactive nitroxide species and peroxynitrite (Papaharalambus 2007). Semiquinone radicals have been shown to undergo redox cycling and generating ROS causing oxidative damage to macromolecules (Valavanidis 2009).

**Previous Work Done in our Laboratory**

CS (CS) is a complex and dynamic mixture of about 4,000 compounds distributed in two phases; gas phase containing about 3,000 compounds and tar phase with about 1,000 compounds (Hoffmann 1997). Reports suggest presence of about $10^7$ stable long lived radicals per gram of tar phase and $10^{15}$ radicals per puff of gas phase (Pryor 1993). The most prominent deleterious
effect of CS is oxidative damage that leads to protein oxidation, lipid peroxidation and DNA damage (Frei 1991; Reznick 1992; Kiyosawa 1990). Using various in vitro and in vivo systems, investigations have been done on the effects of CS exposure both at the protein level as well as tissue level (Panda 1999; Panda 2000; Panda 2001; Banerjee 2008).

**CS induced proteolysis and protein oxidation in vitro: role of vitamin C**

Previously using in vitro systems, it had been observed that CS causes degradation of microsomal proteins (Panda 1999). When guinea pig lung, heart and liver microsomes were incubated with aqueous extract of CS, massive protein degradation was evidenced. Lung microsomal protein degradation increased with CS extract in a concentration dependent manner. Four antioxidants were tested for their potential role in inhibiting CS induced microsomal protein degradation; vitamin C, GSH, SOD and catalase. Of these four antioxidants, only vitamin C exhibited almost complete protection against CS induced proteolytic degradation under the experimental condition used. Inability of GSH, SOD and catalase to restrict microsomal protein degradation indicated that probably O$_2^·$, H$_2$O$_2$, OH·, singlet oxygen, peroxyl radicals are not involved in CS aqueous extract induced microsomal protein degradation (Panda 1999). Incubation of guinea pig lung microsomes also showed protein oxidation as evidenced by protein carbonyl formation with almost complete prevention by vitamin C. Among other antioxidants, GSH only partly inhibited the carbonyl formation and SOD, catalase, vitamin E, β-carotene, probucol, mannitol and uric acid almost failed to provide protection against CS induced protein oxidation. Oxidative damage of microsomal proteins was also indicated by tryptophan loss, bityrosine formation and thiol loss of microsomal proteins. CS also induced ascorbate inhibitable oxidation of human plasma protein, BSA and polypeptides. Compared to the earlier CS aqueous extract mediated oxidation, the oxidation by only gas phase is substantially low. The oxidants present in gas phases were found to be extremely unstable. Direct exposure of albumin solution to gas phase results in oxidation of protein however the instability of gas phase oxidants was detected when gas phase was passed through aqueous buffer and then used to treat protein solution. The oxidant system active in gas phase was detected using various reactive oxygen species scavengers like SOD, catalase, mannitol, ascorbate glutathione and α-tocopherol. Results indicated O$_2^·$/H$_2$O$_2$/OH· is involved in gas phase
induced protein oxidation. Further experiments revealed that interaction of tar phase component with $O_2^*/H_2O_2/\cdot OH'$ of gas phase generates stable oxidants in tar phase (Panda 1999).

The proposed mechanism of CS induced microsomal protein degradation involves two steps. Firstly, the CS results in oxidation of protein that subsequently leads to proteolytic degradation of oxidized proteins. Presence of PMSF and EDTA inhibits proteolytic degradation confirming involvement of microsomal proteases but failed to inhibit oxidation of proteins. Susceptibility of oxidized proteins to protease mediated degradation was evident from studies of oxidized microsomal proteins with trypsin and chymotrypsin. Native microsomal protein remains almost unaffected by the proteases but when CS treated microsomal proteins are incubated with these proteases, massive protein degradation was observed. Presence of inhibitors of these proteases failed to degrade the proteins. Presence of vitamin C in oxidized microsomal solution also failed to protect proteasomal degradation (Panda 1999).

Further study on the effect of CS on status of vitamin C revealed ascorbic acid is oxidized rapidly by CS. HPLC analysis of oxidized ascorbic acid when incubated with aqueous extract of CS revealed that 88% of total ascorbic acid oxidized during 60 minutes occurs instantly (Panda 1999).

**CS induced proteolysis and oxidation in vivo: role of vitamin C**

Using CS exposed marginal ascorbate-deficient guinea pigs, the *in vitro* effects observed were confirmed *in vivo*. As observed in the *in vitro* analysis, CS exposed marginal ascorbate-deficient guinea pig demonstrated severe widespread lung microsomal proteins loss. Similar observation was made for heart microsomal proteins also although to a much lesser extent. Supplementation with vitamin C at 5 mg per animal per day failed to substantially inhibit CS induced microsomal protein loss. However vitamin C at 15 mg per animal per day almost completely inhibited microsomal protein loss. As observed *in vitro* experiments, the cause of microsomal protein loss can be attributed to oxidative damage which was evident from carbonyl formation, bityrosine formation, tryptophan loss and thiol loss. Membranes of lung and heart microsomes also indicated the presence of lipid peroxidation products like dienes, malondialdehyde and fluorescent pigment. Similar to protein oxidation and degradation, the protective role of vitamin C was also equally effective with 15 mg vitamin C per day per animal almost completely inhibiting lipid oxidation phenomenon (Panda 2000).
Vitamin C was also found to be effective in reverting back the oxidative damage by CS exposure to its original values. After treating for 15 days, CS exposure was stopped and ascorbic acid supplementation was carried out for 10 days. Treatment with vitamin C restored the lung and heart microsomal protein to its original values. The carbonyl values of plasma and lung microsomes as well as lung maondialdehyde values also reverted back to normal levels (Panda 2000).

**Protein oxidation and proteolysis is caused exclusively by tar phase of CS: prevention by vitamin C**

Previously it had been observed that gas phase oxidants are unstable and labile with only limited effects on protein oxidation (Panda 1999). As CS is divided into two phases namely gas phase and tar phase by the size of the particles, further experiments to ascertain which phase is more effective in observed CS induced damage. Experiments with *in vitro* systems containing guinea pig lung microsomal proteins and human plasma revealed that effects of whole phase CS can be almost completely caused by the tar phase components. SDS PAGE profile of lung and heart microsome incubated separately with both tar phase and gas phase indicated tar phase oxidants are capable of proteolysis of microsomal protein similar to whole phase CS. Gas phase oxidants being transient in nature failed to cause protein degradation when incubated with microsome (Panda 2001).

Investigation on the capacity of inducing oxidative damage, compared to gas phase, tar phase produced carbonyl in a time dependent manner. Other markers of oxidative damage tested including tryptophan loss, bityrosine formation and thiol loss was found to significant in tar phase treated proteins. Gas phase failed to generate any protein carbonyl and oxidizing microsomal proteins. Inclusion of ascorbic acid in the reaction mixture completely inhibited such oxidation and GSH was partly effective in this context. Other antioxidants tested like SOD, catalase, vitamin E, β-carotene and mannitol failed to exhibit any protective role against tar phase induced oxidative damage. When human plasma protein and BSA were used, similar to microsomal proteins, tar phase oxidants resulted in oxidation and carbonyl formation that was almost completely inhibited by vitamin C. gas phase failed to produce substantial oxidation but when directly bubbled to protein solution, substantial protein carbonyl formation confirmed that gas phase radicals are actually transient in nature and unstable. Moreover, gas phase mediated
oxidation was found to be completely inhibited by SOD and catalase. Similar to earlier observation for aqueous extract of CS, tar phase induced degradation of microsomal protein was found to be a two step process. An initial oxidation of proteins is subsequently followed by degradation of oxidized proteins by proteases (Panda 2001).

Similar to earlier observations (Panda 1999), tar phase treated microsomal proteins were found to be susceptible to proteolytic degradation apparently by proteases present in the microsomes. Presence of ascorbic acid in the incubation mixture before addition of tar phase solution prevented completely protein oxidation and subsequent degradation (Panda 2001).

A significant observation from the study was that tar phase oxidants are capable of causing the effects of whole phase CS in protein oxidation and proteolysis of oxidized proteins. It was obvious that tar phase oxidants were not generated by interaction with atmospheric oxygen as similar oxidative potential was observed even when incubated in nitrogen atmosphere. Experiments with antioxidants clearly indicated that tar phase mediated oxidation did not involve $\text{O}_2^-$, $\text{H}_2\text{O}_2$ or $\text{OH}^-$. When compared with effects of whole phase CS, tar phase was found to be substantially causing formation of carbonyl and bityrosine as well as loss of tryptophan and thiols. A percentage comparison of oxidative effects indicated that 90% protein carbonyl formation, 77% bityrosine formation, 82% tryptophan loss and 82% thiol loss due to whole phase CS is caused by tar phase oxidants (Panda 2001).

**CS-induced emphysematous lung damage and role of p-benzosemiquinone of tar phase: inhibition by vitamin C**

The *in vitro* studies with microsomal proteins, human plasma and BSA showed that CS-induced oxidative damage and resulting proteolytic degradation is caused by the tar phase oxidants. Using *in vivo* analysis with ascorbate-deficient guinea pigs and *in vitro* analysis with lung carcinoma epithelial cells A549, we had also demonstrated that CS exposure causes progressive destruction of lung structure indicating development of emphysematous lung lesions accompanied by oxidative damage, induction of inflammation and activation and execution of apoptosis (Banerjee 2007; Banerjee 2008).

Using ascorbate-deficient guinea pig as a model animal, exposure to CS for 21 days revealed pulmonary parenchymal destruction characteristics of emphysematous lesions.
Histological slide examination and morphometric analysis of the lung sections revealed that the lung damage by CS was indeed progressive in nature. Determination of surface density by measuring area and perimeter of alveolar airspace indicated a significant and progressive decrease in the value in CS exposed guinea pig lung compared to air exposed sham control guinea pig lung. When vitamin C was added to the diet, no significant damage was noticed representing the protective role of vitamin C against CS induced lung damage. Calculations of mean linear intercept and destructive indices also confirmed significant lung structure destruction by CS exposure for 14 days compared to air exposed animals (Banerjee 2008).

Previous in vitro and in vivo observations about CS induced protein oxidation were confirmed again through immunoblot of DNPH derivative of carbonyl formed as a result of oxidative modification (Panda 1999; Panda 2000; Panda 2001; Banerjee 2007). Compared to air exposed guinea pig lung, CS exposure increased lung protein carbonyl indicating CS-mediated oxidative damage (Banerjee 2007). Even exposure to smoke from a single cigarette resulted in the formation of protein carbonyl that increased with increasing number of cigarettes (Figure 4). So the initial event involved with CS-induced lung damage is undoubtedly the oxidative damage. Investigation on whether apoptosis is induced by smoke from a single or a few cigarettes or not led to the observation that apoptosis was not the initial event as no translocation of cytochrome c from mitochondria to cytosol was detected, which is a hallmark of apoptosis induction (Banerjee 2008).

CS exposure also induced inflammation in the guinea pig lung. Histological slide examination of CS exposed guinea pig lung also indicated infiltration of inflammatory cells in the septal region along with accumulation of leukocytes (probably macrophages) in the alveolar spaces (Banerjee 2007). When total number of leucocytes and neutrophils were counted in the bronchoalveolar lavage fluid, a significant increase in number was detected compared to air-
exposed sham control guinea pig lung. As an obvious consequence of increase in inflammatory cells, the inflammatory proteases were also increased. Compared to sham control guinea pig lung, MMP-9 protein level in lung and MMP-12 level in lung and BALF were elevated as evidenced by immunoblot analysis (Banerjee 2008).

CS also induced apoptotic cell death in the lung tissue as detected by activation and execution of apoptosis and DNA fragmentation. Guinea pigs exposed to CS showed a significant increase in TUNEL positive cells indicating cells undergoing apoptosis as well as phosphorylation of p53, an increased expression of bax protein disturbing the bax/bcl2 ratio, activation and cleavage of caspase3 and degradation of PARP (Banerjee 2007; Banerjee 2008). Analysis of TUNEL positive cell number after exposure to CS revealed a more or less significant increase from 7 and 14 days and strictly significant increase on 21 day indicating activation and execution of apoptosis. Supplementation of guinea pigs with vitamin C reduced
the apoptotic cell death to a basal level and no significant change in number of TUNEL positive cells was noticed. Cleavage of caspase3, PARP degradation and increased expression of bax protein was also inhibited by vitamin C supplementation (Banerjee 2008).

Treatment of human adenocarcinomic alveolar epithelial cells A549 with aqueous extract of cigarette smoke also induced apoptotic cell death as evidenced by cleavage and activation of caspase3 and degradation of PARP. Number of TUNEL positive cells also increased in number by exposure to CS (Banerjee 2008).

**Isolation of p-benzosemiquinone (p-BSQ) as a major long-lived radical of cigarette smoke**

EPR analysis of aqueous extract of CS revealed the presence of the semiquinone species (Pryor 1998; Chouchane 2006). It had been observed in our laboratory that all commercial cigarettes examined contain fairly large amounts of p-benzosemiquinone (p-BSQ). p-BSQ is present exclusively in the tar phase of cigarette smoke. p-BSQ has been isolated from AECS by differential solvent extraction, thin layer chromatography (TLC) and HPLC and characterized by UV, mass, NMR and ESR spectroscopy (Figure 5) (Chatterjee US Patent 2005; Banerjee 2008).

![Figure 5](image)

**Conversion of p-benzosemiquinone to p-benzoquinone at physiological pH and comparison with effects of cigarette smoke**

Comparison of the effects of CS with p-benzosemiquinone established the fact that the major effects of CS are actually carried out by p-benzoquinone produced from p-benzosemiquinone of CS (Banerjee 2008).

Under physiological condition, p-BSQ is rapidly converted to p-BQ. Two mechanisms drive the conversion within the body, namely disproportionation and transition metal containing protein mediated direct oxidation (Figure 6). In the case of disproportionation reaction, two molecules of p-BSQ react to
form one molecule of p-BQ and one molecule of hydroquinone. A much faster process is direct oxidation by transition metal containing proteins where p-BSQ is oxidized to p-BQ. HPLC analysis of determination of p-BQ formed after incubation of p-BSQ alone or with cytochrome c and Cu, Zn- SOD indicated that in air, p-BQ is formed slowly, presumably by disproportionation and in the presence of transition metal containing proteins, a rapid conversion of p-BSQ to p-BQ occurs that is also reverted back with addition of vitamin C. Presence of vitamin C inhibited the formation of p-BQ by disproportionation reaction indicating reduction of p-BQ by vitamin C may contribute to preventing effects of p-BSQ and p-BQ mediated physiological effects (Banerjee 2008).

Similar to earlier observations, p-BSQ in equivalent amount of that present in AECS causes oxidative damage and formation of protein carbonyl in BSA and protein adduct formation thereby mimicking CS mediated protein modification. The carbonyl formation by aqueous extract of CS is closely mimicked by equivalent amounts of p-BSQ and also by p-BQ in amounts expected to form from disproportionation reaction only. The presence of vitamin C in the reaction mixture inhibits the formation of protein carbonyl by AECS, p-BSQ and p-BQ apparently by reduction of p-BQ and thereby restricting the oxidative damage (Figure 7). Formation of p-BQ from p-BSQ and following Michael adduct formation with nucleophilic groups of proteins was further confirmed by incubating BSA with p-BSQ and p-BQ and determining whole protein mass by MALDI analysis. When BSA was incubated with equal amount of p-BSQ and p-BQ, the adduct formation and increase in mass for p-BSQ was about half of that of p-BQ confirming disproportionation reaction mediated conversion of half amount of p-BSQ to p-BQ participated in the formation of protein adduct (Banerjee 2008).
Not only in the protein modification aspect, p-BSQ and p-BQ also mimicked the effects of CS in terms of pathological effects in vitro. Lung microsomal protein degradation by p-BSQ and p-BQ in equivalent amounts present in CS followed the similar pattern. The degradation observed earlier with AECS and tar phase oxidants were repeated for p-BSQ and p-BQ. The degradation even followed a time dependent manner and inclusion of vitamin C in the reaction mixture also inhibited the degradation as clearly depicted in the SDS PAGE profile of microsomal protein profile (Figure 8) (Banerjee 2008).

CS mediated pathological effects and the role of p-BSQ and p-BQ were further verified using in vivo analysis using ascorbate-deficient guinea pigs and in vitro analysis using A549 human lung epithelial cell line. Intratracheal instillation of p-BSQ in the guinea pig lung resulted in the activation of apoptosis in the lung and lung structural damage. TUNEL positive cells increased significantly within the lung of p-BSQ instillation compared to PBS instilled lung which was further confirmed by immunoblotting detection of cleavage of caspase3 and degradation of PARP. Guinea pig lung structural damage and emphysematous lesions were indicated by marked airspace enlargement. Supplementation of vitamin C inhibited the apoptosis induction as well as lung airspace enlargement (Banerjee 2008).
Summary of the previous studies carried out in our laboratory

Cigarette smoke causes oxidative damage and proteolysis both in vitro and in vivo (Panda 1999; Panda 2000). Compared to the gas phase oxidants, the tar phase oxidants were found to be mostly responsible for whole phase CS effects (Panda 2001). Further investigations on the tar phase oxidants revealed that the free radical p-BSQ is mostly responsible for effects of CS. CS mediated oxidative damage and apoptosis induction was mimicked by equivalent amount of p-BSQ both in vitro in A549 cell line and in vivo in ascorbate-deficient guinea pig. CS mediated lung emphysema development was also reproduced by intratracheal instillation of p-BSQ. Since p-BSQ is converted to p-BQ by disproportionation and oxidation by transition metal containing proteins (Banerjee 2008), it had been proposed that p-BSQ is probably converted to p-BQ in the lungs of smokers and that p-BQ derived from CS might be responsible for CS-related protein modification and emphysematous lung damage, as depicted in the scheme (Figure 9).

What is not known?

Although it has been unequivocally established that cigarette smoking causes a number of degenerative diseases, the molecular mechanisms of the diseases are not clear. This is particularly because cigarette smoke contains 4000 compounds, and until now identification of the disease-relevant chemical(s) in cigarette smoke has not been possible and its contribution to the pathophysiologies of the diseases is yet to be known.

In this thesis, the molecular mechanisms of cigarette smoke-induced pathogenesis of protein damage and emphysematous lung damage as well as their prevention have been elucidated.
Objectives of the thesis

I. To demonstrate that p-benzoquinone derived from cigarette smoke is a major factor responsible for cigarette smoke-induced protein modification with particular reference to human serum albumin, resulting in alteration of structure and conformation leading to impairment of its ligand binding properties.

II. To delineate the molecular, cellular and pathophysiological mechanisms involved in cigarette smoke-induced emphysematous lung damage in a guinea pig model developed in our laboratory and prevention by vitamin C.

III. To demonstrate that p-benzoquinone derived from cigarette smoke is responsible for cigarette smoke-induced emphysema and prevention by vitamin C.