CHAPTER 2

Literature Reviews

Air pollutions have been recognized as adverse effects for human around the world. PM is the one of air pollutants. Several studies have provided most of the evidence linking particulate air pollution to human health (Trenga *et al*., 2006; Anderson *et al*., 2012; Andreau *et al*., 2012; Sram *et al*., 2013).

This chapter reviews the currently available knowledge for the relationship between PM and health through the topics of:

2.1 Air pollution
2.2 Characterization of PM
2.3 Route of PM exposure
2.4 Adverse health effects associated with PM exposure
2.5 PM and the children' s health
2.6 Oxidative stress induced by PM
2.7 Pulmonary function test
2.8 PM and pulmonary function
2.9 Exhaled breath condensate
2.10 Biomarkers in exhaled breath condensate

2.1 Air pollution

Air pollutions occur when the air contains gases, dust, fumes or odor in harmful amounts. Air pollution can affect human health in many ways. In December, 1952, the most well-known cases occurred in London, when environmental conditions caused a 5-day accumulation of air pollution, especially sulfur dioxide (SO_2) and smoke, reaching 1,500 mg/m^3 and resulting in an increase in the number of deaths to around 4000cases.. And in 1966 at New York City, conditions similar to those occurring in London caused 400 deaths. The same event have been reported in Mexico City, Rio de Janeiro, Milan, Ankara, Melbourne, Tokyo and Moscow (Dockery and Brunekreef, 1996).

Air pollution consists of gas and particle contaminants that are present in the atmosphere. Gaseous pollutants include SO_2, NO_X, ozone (O_3), carbon monoxide (CO), volatile organic compounds (VOCs), certain toxic air pollutants, and some gaseous forms of metals. Particle pollution ($PM_{2.5}$ and PM_{10}) includes a mixture of chemical compounds and metals. Air pollutants can be classified as primary or secondary. Primary pollutants are substances that are directly emitted into the atmosphere from sources. The main primary pollutants known to cause harm in high enough concentrations are the following:

1) Carbon compounds, such as CO, CO_2, CH_4, and VOCs

2) Nitrogen compounds, such as NO, N_2O, and NH_3

3) Sulfur compounds, such as H_2S and SO_2

4) Halogen ions, such as chlorides, fluorides, and bromides

5) Particulate matter (PM).

Secondary pollutants are not directly emitted from sources, but instead form in the atmosphere from primary pollutants (also called "precursors"). The main secondary pollutants known to cause harm in high enough concentrations are the following:

1) NO_2 and HNO_3 formed from NO

2) Ozone (O_3) formed from photochemical reactions of nitrogen oxides and VOC_S

3) Sulfuric acid droplets formed from SO_2, and nitric acid droplets formed from NO_2

4) Sulfates and nitrates aerosols (e.g., ammonium (bi) sulfate and ammonium nitrate) formed from reactions of sulfuric acid droplets and nitric acid droplets with NH_3, respectively

5) Organic aerosols formed from VOCs in gas-to-particle reactions

2.2 Characterization of PM

PM is the term used for a mixture of solid particles and liquid droplets suspended in the air. PM in ambient air is a diverse mixture of substances with different chemical and physical characteristics. Particles in the air are classified by aerodynamic diameter size and also classified by their sources into two categories, primary and secondary particles as following.

2.2.1 Primary particles are composed of particles that are emitted directly into the atmosphere from sources such as sea spray, windblown soil, road traffic, coal burning, biomass burning, and industry.

2.2.2 Secondary particles are formed in the air, usually by chemical change of gaseous substances. Products from the transformation of nitrogen oxides are mainly emitted by transportation, some industrial processes, and sulfur dioxide release from combustion of high sulfur containing fuels. Secondary particles are mostly found in the fine PM fraction (Kelly, 2003).

In terms of aerodynamic diameter size, PM is usually categorized into these groups based on the aerodynamic diameter of the particles as following (Refs).

1) Particles less than 100 micrometers (μm), which are also called "inhalable" since they can easily enter the nose and mouth.

2) Particles less than 10 μm (PM_{10}). These particles are also called "thoracic" since they can penetrate deep in the respiratory system.

3) Particles less than 4 μm. These particles are often called "respirable" because they are small enough to pass completely through the respiratory system and enter the bloodstream.

4) Particles less than 2.5 μm ($PM_{2.5,}$ "fine particles").

5) Particles less than 0.1 μm ($PM_{0.1,}$ "ultrafine particles").

The larger particles of up to 100 μm in diameter are negligible health significance since they rapidly settle out of the air, and although it is possible to be inhaled, they do not generally penetrate beyond the nose and mouth as the natural human defense mechanisms such as mucous secretions capture and dispose of them. It is the particles of up to 10 μm in diameter that are of most health significance, since they manage to bypass the natural human defense mechanisms, are inhaled into the

lungs and pass deep into the respiratory tract of alveoli. The larger particles of up to 100 µm in diameter are comparable to the thickness of a human hair (Figure 2.1).

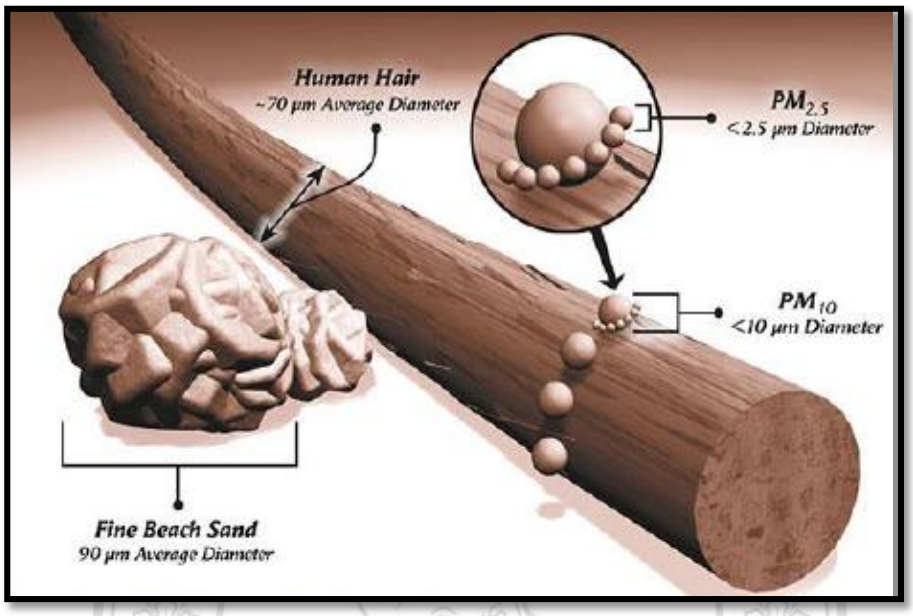

Figure 2.1 Comparison of size of particles to a strand of hair and beach sand (http://www.iaquk.org.uk/ResourcesParticulate.html, October 19, 2014)

Much of the literature also makes reference to PM in terms of coarse (PM_{10}), fine ($PM_{2.5}$), and ultrafine particles ($PM_{0.1}$). PM_{10} and coarse particles, synonymous terms, both refer to PM with an aerodynamic diameter of $\leq$ 10 µm. $PM_{2.5}$ and fine particles also both refer to PM with a diameter of $\leq$ 2.5 µm, while the ultrafine particles are those with a diameter of < 0.1 µm. The distinction between coarse and fine particles are made due to differences in sources, formation mechanisms, composition, atmospheric lifetimes, spatial distribution, indoor-outdoor ratios, temporal variability in addition to size, and health impacts (Kim *et al*., 2005; Schwarze *et al*., 2006; Chen and Xie, 2014). The comparison of fine and coarse mode particles is shown in Table 2.1.

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Table 2.1 Comparison of fine and coarse mode particles

	Fine mode particles	**Coarse mode particles**
Composition	- Sulfate, SO^{2-}_4; Nitrate, NO^{-3}; - Ammonium, NH^{+}_4; - Hydrogen ion, H^{+}; - Elemental carbon, C; - Organic Compounds; PAH; - Metals, Pb, Cd, V, Ni, Cu, Zn; - Particle-bound water; - Biogenic organics.	- Re-suspended dusts, soil dust, street dust; - Coal and oil fly ash; - Metal oxides of Si, Al, Mg, Ti, Fe; - $CaCO_3$, NaCl, sea salt - Pollen, mold spores, plant parts.
Sources	- Combustion of coal, oil, gasoline; - Transformation products of NO_x; - SO_2, and organics including biogenic organics; - High temperature processes; - Smelters, steel mills, etc.	- Re-suspension of soil tracked onto roads and streets; - Suspension from disturbed soils, e.g., farming, mining; - Re-suspension of industrial dusts; - Construction, coal and oil combustion, ocean spray.
Lifetime:	- Days to weeks	- Minutes to hours
Travel distance:	- 100s to 1000s of kilometers	- 1 to 10s of kilometers.

Source: Schwarze *et al*., 2006

PM comes primarily from two types of sources. The first is natural aerosolization of crustal matter such as during a windblown dust storm, during agricultural practices, and during excavations for construction which all result in relatively larger size particles closer to 10 μm in diameter. This category also includes re-suspended dust from roadways, coal and oil fly ash, sea salt, and biological material such as pollen, mold and fungi. The other source of PM is combustion which includes gasoline and diesel fuel vehicle combustion, industry combustion, coal combustion in the process of electricity generation, and burning of vegetative material such as wood

burning for residential heating and grass burning for clearing agricultural land (Andreau *et a*l., 2012).

Air pollution continues to be a growing concern globally, with increases in both population and energy consumption contributing to higher pollution levels. The World Health Organization assessment of the burden of disease due to air pollution states modestly that more than two million premature deaths occur each year as the result of the effects of urban outdoor and indoor air pollution. Deaths as the result of indoor air pollution are predominantly caused by the burning of solid fuels. Additionally, more than half of this disease burden is borne by the populations of developing countries. All of the major pollutants emitted to air, PM_{10} pollution is consistently and independently related to the most serious effects on human health worldwide in both developed and developing countries. The ranges of health effects is broad, but are predominantly linked to the respiratory and cardiovascular systems. All population is affected, but susceptibility to the population may vary with health or age (Mossman *et al*., 2007).

2.3 Route of PM exposure

Inhalation is the major route of air pollution exposure, while the minor routes are via contaminated food and water (Salvaggio, 1994).

The respiratory system can be divided into three regions (Figure 2.2) that are different in structure, airflow patterns, function, retention time and sensitivity to deposited particles. These regions are;

1) Nasopharynx region: the head region, including the nose, mouth, pharynx, and larynx

2) Tracheobronchial region: includes the trachea, bronchi, and bronchioles

3) Pulmonary (Alveolar) region: comprised of the alveoli; the exchange of oxygen and carbon dioxide through the process of respiration occurs in the alveolar region.

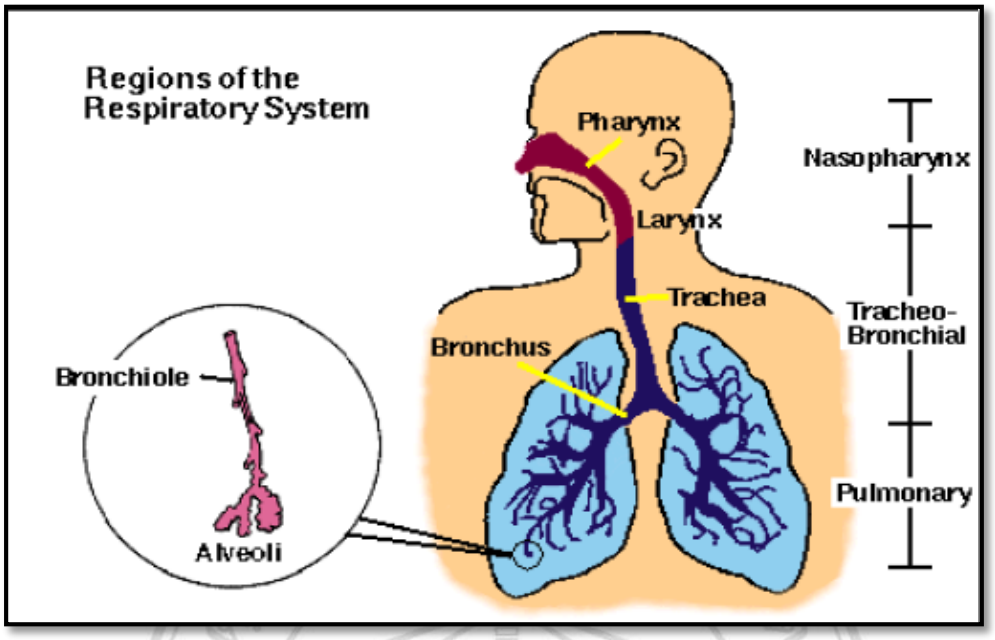

Figure 2.2 Diagrammatic representation of the human respiratory tract (http://www.mfg.mtu.edu/cyberman/environment/air/anatomy.html, October 19, 2014)

PM can penetrate into the respiratory airways depending on the aerodynamic diameter. The coarse particles or PM_{10} (>10 μm) are filtered in the nose, naso and oropharynx, and the larynx. Only those particles smaller than 10μm penetrate into the lung, and the smaller their aerodynamic diameter, the deeper they penetrate. PM of approximately 5–8 μm is deposited in the tracheobronchial tree and those of 1–5 μm deposit primarily in the respiratory bronchioles and alveoli. Interestingly, particles of approximately 0.5 μm penetrate to the alveoli during inhalation, but their diffusiveness and settling velocities are so low that they remain mostly airborne, and are exhaled rather than significantly deposited in the alveoli (Squadrito *et al*., 2001).

It is thought that smaller particles have a peak value for deposition in the alveoli because smaller particles are typically more numerous and have larger surface areas (Schwarze *et al.,* 2006). The three major deposition mechanisms affecting inhaled PM are inertial impaction, sedimentation and diffusion (Oberdörster, 2001). Deposition of inhaled PM in various regions within the respiratory system is an important aspect that determines the potential for the damage it can cause. The mechanism that clears particles in the respiratory system has several patterns. The

particles deposited in the trachea and bronchioles rise on the mucociliary ladder, and are expelled by coughing or swallowing. PM deposited beyond the terminal bronchioles are cleared largely by lung macrophages that, in turn, transport the ingested particles onto the mucociliary ladder or into the lymphatic system. A small fraction of these distally deposited particles migrate through alveolar tissue directly into the lymphatic circulation (Schmid *et al*., 2009).

2.4 Adverse health effects associated with PM exposure

2.4.1 Acute effects

The scientific literature on PM epidemiology suggests an association between ambient PM exposures and various acute health outcomes. In particular, pulmonary function studies are suggestive of short-term effects resulting from ambient PM exposures. Such outcomes include hospital admissions, inflammatory response in the respiratory tract, exacerbation of asthma, and decreased lung functions (Atkinson *et al.*, 2001; Brunekreef and Holgate, 2002; Anderson *et al*., 2012). A review of the findings indicates that:

1) Sensitive populations such as the patients with asthma and other respiratory diseases, cardiovascular disease, the elderly and children are susceptible to more severe symptoms, including cough, phlegm, wheezing, shortness of breath, bronchitis, increased asthma attacks, and aggravation of lung or heart disease;

2) Cardiovascular causes of death and hospitalization in older adults may be a component of PM-attributable mortality;

3) PM health effects have been associated with several different PM size fractions; and health effects may occur at different time scales for exposure to PM

Epidemiology findings indicate that risk of mortality due to lower respiratory disease (e.g., pneumonia) is increased by ambient PM exposure (Zhang *et al.*, 2011). This may be due to exacerbation by PM of existing respiratory disease. PM may increase susceptibility to infectious disease by decreasing clearance, impairing macrophage function, or through other effects on the immune system. The findings also indicate that individuals with preexisting infectious respiratory disease (e.g., pneumonia) are at increased risk for PM effects. Recent studies are generally consistent with regard to ambient PM associations of short-term exposures with

respiratory-related hospital admissions and medical visits (Atkinson *et al.*, 2001; Chen *et al.*, 2006; Wellenius *et al.*, 2006). This population includes both healthy individuals and those susceptible to respiratory problems. Epidemiologic findings also indicate that ambient PM exposures in the elderly are associated with increased risk for mortality and hospitalization due to cardiovascular causes (Jiménez *et al*., 2011; Zhang *et al*., 2011; Langrish *et al*., 2012). Researchers have hypothesized that cardiac arrhythmia contributes to increased mortality due to PM exposure. Thus, individuals with pre-existing cardiovascular disease have been identified as a susceptible group for increased risk from ambient PM effects. In addition to inhalation exposures to PM, dermal (skin) exposures can also produce short-term, reversible symptoms. Anecdotal information received from Gulf War veterans suggests that some personnel experienced rashes, skin irritation, and scaling. Particulates containing silica in particular are associated with specific types of dermatitis and skin inflammation.

2.4.2 Chronic effects

The recent literature contains the results of several studies that evaluate the effects of long-term PM exposure on respiratory illness, pulmonary function, cardiovascular morbidity, mortality, and cancer rates. In general, chronic respiratory illness and pulmonary function decrement studies are less numerous than acute studies, and the findings are inconclusive and inconsistent. Some studies show effects for some health endpoints (e.g., pneumonia, reduced lung function, and bronchitis) with high significant results, but other studies fail to find the same effects. For example, chronic pulmonary studies, looking for latent effects, by one group of researchers showed no effect for children from airborne particle pollution. In contrast, another group of researchers studying Canadian and United States' children found significant associations between pulmonary function and PM levels (Trenga *et al*., 2006; Liu *et al*., 2009). Results of studies conducted in urban environments provided data on the positive relationship between chronic respiratory disease and elevated long-term particulate matter levels. Study results suggest a potential long-term PM exposure effect on chronic respiratory disease (Peacock *et al*., 2011).Other studies report associations between PM exposures and bronchitis rates and/or lung function decrements or slowed lung function growth in children (McConnell *et al*., 1999).

Numerous PM epidemiology studies have implicated ambient PM levels as a likely contributor to mortality and morbidity effects, particularly among the chronically ill or elderly. For example, of particular interest with regard to PM-related effects on cause-specific mortality is a growing body of evidence linking long-term PM exposure with increased risk of lung cancer. Historical evidence includes studies of lung cancer trends, studies of occupational groups, comparisons of urban and rural populations, and case-control and cohort studies using diverse exposure metrics. These studies have generally indicated an elevated risk for lung cancer relative to living in urban areas where ambient PM levels exceed the standard (Cohen, 2000; Cohen, 2003; Hystad *et al*., 2013).

2.5 PM and the children's health

Adverse health effect from PM is especially high for some susceptible groups such as the children and the elderly persons, and who have diseases of the heart and lungs (Epton *et al*., 2008; Delfino *et al*., 2010; Bentayeb *et al*., 2012; Jia *et al*., 2012). The present study was focus on the health effects of PM_{10} exposure on school children. Children are more susceptible to PM pollutants than the adults. The special vulnerability of children to PM pollution exposure is related to several differences between children and adults (Gilliland *et al*., 1999; Guaderman et al., 2004; Schwartz, 2004)

1) Children generally spend more time and are also more active outdoors than adults. They are active outdoor during midday when air pollution levels tend to be higher. They have significantly higher metabolic rate and oxygen demands so their respiration rates are higher and consequently greater intake of air per unit body weight than adults.

2) Because of their smaller stature their breathing zone is lower, so they inhale air loaded with more particles. Typically, an adult's breathing zone is 1.5 to 2 meters above ground level. However, for a child this is much closer to the ground. This is particularly important since it is at these lower breathing zones that the large respirable particulates and heavier chemicals descend, including PM_{10}.

3) Diameters of their airways are smaller and therefore more likely to be affected by inflammation produced by air pollution.

4) Their lungs are still developing and hence are more vulnerable to airborne. The efficiency of detoxification system of the body develops in time-dependent pattern.

5) The metabolic rate of a child is higher than adult's due to a child's larger surface to volume ratio. Consequently a child's oxygen consumption is also greater than adult, which results in a greater exposure to any air pollutant. The metabolic rate per kilogram weight of children is much higher than that of an adult, in part because children are still developing and they are smaller. This means that their respiratory rate is proportionately greater and they breathe in much more pollution in relation to their body weight compared to an adult.

6) Their immune defense is immature and hence less active against inhaled pathogens. Because their organs are constantly undergoing growth, children are also different from adults with regard to susceptibility of their organs. The lungs and the brain are the main organs that have a prolonged period of development in early childhood. Alveolar is not complete until late childhood and this extended period of growth and development increases the susceptibility of these organs in a child.

2.6 Oxidative stress induced by PM

Oxidative stress is the condition that occurs when the steady-state balance of pro-oxidants is shifted in the direction of the former, in favor of the pro-oxidants and leading to potential damage (Auerbach and Hernandez, 2012). Oxidative stress has been implicated in the pathophysiology of a number of respiratory disorders such as asthma, chronic obstructive pulmonary disease and cystic fibrosis (Kharitonov and Barnes, 2002). Activation of eosinophils, neutrophils, and macrophages induces a respiratory burst, resulting in the production of reactive oxygen species (ROS) that cause tissue damage and cell death. Lung cells, in particular alveolar epithelial type II cells, are particularly susceptible to the injurious effects of oxidants. Therefore, oxidative stress and overall inflammatory response are a fundamental process involved in pathogenesis of many lung diseases (Antczak and Górski, 2002; Borrill *et al*., 2008; Celik *et al*., 2012). Oxidative stress can arise for many reasons, including consumption

of alcohol, medications, trauma, with a cold, air pollutants, toxins, and radiation (Kelly, 2003; Romieu *et al*., 2008b).

Ambient air contains a range of pollutants. Many of the individual pollutants that make up this ambient mix are free radicals (for example, nitrogen dioxide) or have the ability to drive free radical reactions (for example, ozone and PM). As a consequence, exposure to a wide range of air pollutants gives rise to oxidative stress within the lung, and this appears to initiate responses that are particularly dangerous to susceptible members of the population. One of these responses is the influx of inflammatory cells to the lung. These responses can lead to a second wave of oxidative stress in the lung, since activated inflammatory cells also generate and release large quantities of free radicals. In the absence of any invading organisms to kill, these free radicals attack local tissue components and cause cell injury.

The oxidative stress mediated by PM may arise of reactive oxygen species (ROS) from the surface of soluble compounds; altered function of mitochondria or reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase; and activation of inflammatory cells capable of generating ROS and reactive nitrogen species (RNS), as well as oxidative DNA damage (González-Flecha, 2004; Risom *et al*., 2005). The particle provides a template for electron transfer to molecular oxygen in these reduction and oxidation (redox) cycling events (Smith *et al*., 2005). In addition, target cells, such as airway epithelial cells and macrophages, generate ROS in response to particle uptake by biologically catalyzed oxidation reactions that occur in the cell membrane and mitochondria (Li *et al*., 2003). In vitro studies have shown that inhaled PM causes expression of nuclear factor (NF)-kB-related genes and oxidant-dependent NF-kB activation (Jiménez *et al*., 2000; Shukla *et al.*, 2000). The dose of bio-available transition metal, rather than particulate mass, may be the primary determinant of acute inflammatory response (Shukla *et al*., 2000; González-Flecha, 2004).

2.7 Pulmonary function test

The pulmonary function test (PFT) or spirometry is an important tool in the investigation and monitoring of patients with respiratory pathology. PFT used to determine the functional status of the lungs, as it relates to how much as can be moved in and out of then, how fast the gas can be moved, the stiffness of the lungs and chest wall, diffusion characteristics of the membrane through which the gas move, and how well the lungs responds to therapy. PFT is a noninvasive diagnosis instrument for screening and basic testing of pulmonary function. PFT can be performed fast at fairly low cost (MacIntyre, 2012).

A sitting position is typically used at the time of testing to prevent the risk of falling and injury in the event of the sudden and temporary loss of consciousness while pulmonary testing, although PFT can be performed in the standing position. Patients are advised not to smoke for at least one hour before testing, not to eat a large meal two hours before testing and not to wear tight fitting clothing as under these circumstances results may be adversely effected (Miller *et al*., 2005). Normal or predicted ranges of values are obtained from large population studies of healthy subjects. Values are taken for people matched for age, height, sex and where appropriate ethnicity. PFT should be performed three times to ensure that the results are reproducible and accurate. Spirometry is the most frequently used to measure lung function and is a measure of volume against time. It is a simple and quick procedure to perform: patients are asked to take a maximal inspiration and then to forcefully expel air for as long and as quickly as possible.

The following basic variables that have an impact on lung function must be considered such as height, weight, sex, and age. Other defined predictors of pulmonary function may include race, environmental factors, and altitude. The measurements that are usually made are as follows:

1) FVC (Force vital capacity) is the maximum volume of air that can be exhaled or inspired during a forced maneuver.

2) FEV_1 (Force expired volume in first second) is the volume expired in the first second of maximal expiration after a maximal inspiration and a useful measurement of the speed at which full lungs can be emptied.

3) FEV_1/FVC is the FEV_1 express a percentage of the FVC and gives a clinically useful index of airflow limitation.

4) $FEF_{25-75\%}$ is the average expired flow over the middle half of the FVC maneuver and is regarded as a more sensitive measurement of small airway narrowing than FEV_1. Unfortunately, in having a wide range of normality $FEF_{25-75\%}$, is less reproducible than FEV_1 and is difficult to interpret if the FVC is related or increased.

5) PEFR (Peak expiratory flow rate) is the maximal expiratory flow rate achieved, and this occurs very easy in the forced expiratory maneuver.

The present study was focus on FEV_1, FVC and the ratio of the two volumes (FEV_1/FVC). Spirometry and the calculation of FEV_1/FVC allow the identification of obstructive or restrictive ventilatory defects. A $FEV_1/FVC < 70\%$ where FEV_1 is reduced more than FVC signifies an obstructive defect. Common examples of obstructive defects include chronic obstructive pulmonary disease (COPD) and asthma. The FEV_1 can be expressed as a percentage of the predictive value which allows classification of the severity of the impairment (Fabbri *et al*., 2003). An $FEV_1/FVC> 70\%$ where FVC is reduced more so than FEV_1 is seen in restrictive defects such as interstitial lung diseases and chest wall deformities.

2.8 PM and pulmonary function

Several recent studies suggest that exposed PM levels may affect lung function and lung development. The Children's Health Study (Gauderman *et al*., 2004) followed 1,759 patients over 8 years, finding that children who lived in communities with the highest PM concentrations were five times more likely to have low FEV_1 than those in communities with the lowest PM concentrations. Moreover, children that moved from areas of higher to lower PM_{10} concentration had increased growth in lung function, and those that moved from areas of lower to higher PM_{10} concentration had decreased growth in lung function (Avol *et al.*, 2001). Even children with better lung function were susceptible to new onset asthma when exposed to elevated levels of $PM_{2.5}$ (Islam *et al*., 2007). Lower lung function has also been shown for children with cystic fibrosis exposed to elevated levels of PM_{10} and $PM_{2.5}$ (Goss *et al*., 2004). Similar inverse correlations between PM exposure and individual PEFR and FEV_1 measurements have been reproduced internationally (Barraza-Villarreal *et al*., 2008).

In the developing world, where indoor biomass burning can lead to PM levels exceeding 200 μg/m^3, researchers demonstrated that chronic exposure in children can lead to adult COPD, increased rates of lung infection, and impaired lung function. In adults, effects of PM on lung function have been found primarily in susceptible populations. Investigators showed that asthmatic Londoners taking walks in areas of high PM had significantly higher reduction in FEV_1, FVC, and increases in sputum biomarkers of inflammation (McCreanor *et al*., 2007). In elderly patients, increased PM_{10} and $PM_{2.5}$ concentrations were associated with the decrease in PEFR (Lee *et a*l., 2007). In COPD patients, decrements in lung function were associated with increases in $PM_{2.5}$ concentration (Lagorio *et al*., 2006). Some researcher (Downs *et al*., 2007) demonstrated that declines in PM_{10} concentration may actually lead to an attenuated decline in lung function in adult patients. However, research on healthy adults has not as consistently shown an association between PM and respiratory compromise (Gent *et al*., 2003).

2.9 Exhaled breath condensate

Traditional way to assess pathophysiological processes and the level of airway inflammation and oxidative stress are often using bronchoalveolar lavage and/or airway biopsies. The limitations of these procedures are invasive and not suitable for repeated use in children (Barnes *et al*., 2006). More recently, the less invasive procedure of sputum induction has been focused (Gibson *et al*., 2000) for the assessment of airway inflammation and oxidative stress in older children and adults. However, this method is limited by difficulties in obtaining adequate samples. In young children, the success rate of sputum production is lower, and sputum expectoration is impossible in infants (Spanevello *et al*., 1997).

The need for a noninvasive, rapid and easy-to-perform method that can be used repeatedly has been recognized. In recent years there has been increasing interest in different techniques to assess inflammation and oxidative stress in the airways and lungs indirectly by the analysis of various nonvolatile and volatile breath components. These methods are based on the hypothesis that aerosolized exhaled particles contain nonvolatile insoluble and soluble substances from the lower respiratory tract, which

reflect the composition of bronchoalveolar extracellular lining fluid (Barnes *et al*., 2006).

Exhaled air is saturated with water vapor. When this air is cooled below the dew point, for example on a chilled condenser surface, aerosolized particles and vapor will condense and collect on the condenser wall. To date, a wide range of markers of airway inflammation and oxidative stress have been reported to be present in exhaled breath condensate (EBC). EBC contains many biomarkers of inflammation and oxidative stress (Antczak and Górski, 2002; Montuschi and Barnes, 2002; Carraro and Baraldi, 2008). The principle of sampling the airways by EBC is that mediators from airways are released from the airway lining fluid, carried up by exhaled breath, and subsequently collected by condensation of the exhaled breath into a cooled tube. Collection of EBC is a non-invasive method of sampling the whole respiratory tract including the oral cavity, oropharynx, tracheobronchial system, and alveoli, although the proportion that each compartment contributes to the EBC has not yet been fully investigated. As it is non-invasive, EBC collection can be repeated in the same subject within a short period of time. The collection procedure does not influence airway function nor does it induce any inflammation. EBC collection is less expensive in terms of both equipment and personnel costs compared to collecting bronchoalveolar lavage and sputum. It can even be applied to both those who are breathing freely and those who are mechanically ventilated. Furthermore, it can be used across all age groups, from infant to the elderly. Abnormalities in condensate composition can reflect biochemical changes of airway lining fluid (Effros *et al*., 2004; Grob *et al*., 2008; Hoffmeyer *et al*., 2009a; Davis *et al*., 2012).

The collection of EBC is simple and easy to perform, and requires subjects to breathe tidally via a mouth piece that is connected to a condensing chamber for 10-15 minutes. Subjects are asked to inhale and exhale through the mouth only. The collecting system can be a condensing chamber with a double wall of glass, the inner wall of which is cooled either by ice or by liquid nitrogen (Mutlu *et al.*, 2001; Montuschi and Barnes, 2002; Montuschi, 2005; Montuschi, 2007) or with tubing of different materials (Teflon®, polypropylene) can be cooled to the required temperature. Exhaled air enters and leaves the condensing chamber through one-way valves at the

inlet and outlet while the chamber is kept closed so as to separate inspiratory and expiratory air, as well as to avoid unintentional inhalation of cold air from the condenser. Generally, 1-2 mL of breath condensate can be collected over a period of 10 to 15 minutes in an adult but this takes up to 20 minutes in children.

Several different EBC collecting devices have been designed by different research groups; however, the principle of rapid cooling of the exhaled air with consequent condensation of water vapor, as well as sedimentation and impaction of aerosol particles to cold surfaces, is similar for all systems used. Commercially manufactured condensers are also available such as EcoScreen® (Jaeger Tonnies, Hoechberg, Germany and RTube™ (Respiratory Research Inc., Charlottesville, VA, USA). The EcoScreen® is an electric refrigerated system which consists of a mouthpiece with a one-way valve and collecting system connected to a power supply by an extendable arm as shown in Figure 2.3B. This device can collect 1-2 ml of EBC at -20ºC within 10 minutes (Kharitonov, 2004; Kharitonov and Barnes, 2006). The RTube™ is another commercially available condenser which has the advantage of being portable. This device consists of a disposable polypropylene collecting system with an exhalation valve that also serves as syringe-style plunger to pull fluid off the condense wall (Figure2.3C). The RTube™ is portable, which makes it possible to collect EBC samples at home, which is particularly suitable for longitudinal studies or when collection of several samples a day is required; the EBC sample in a polypropylene tube can be stored in a freezer at home (Hunt, 2002; Montuschi, 2007).

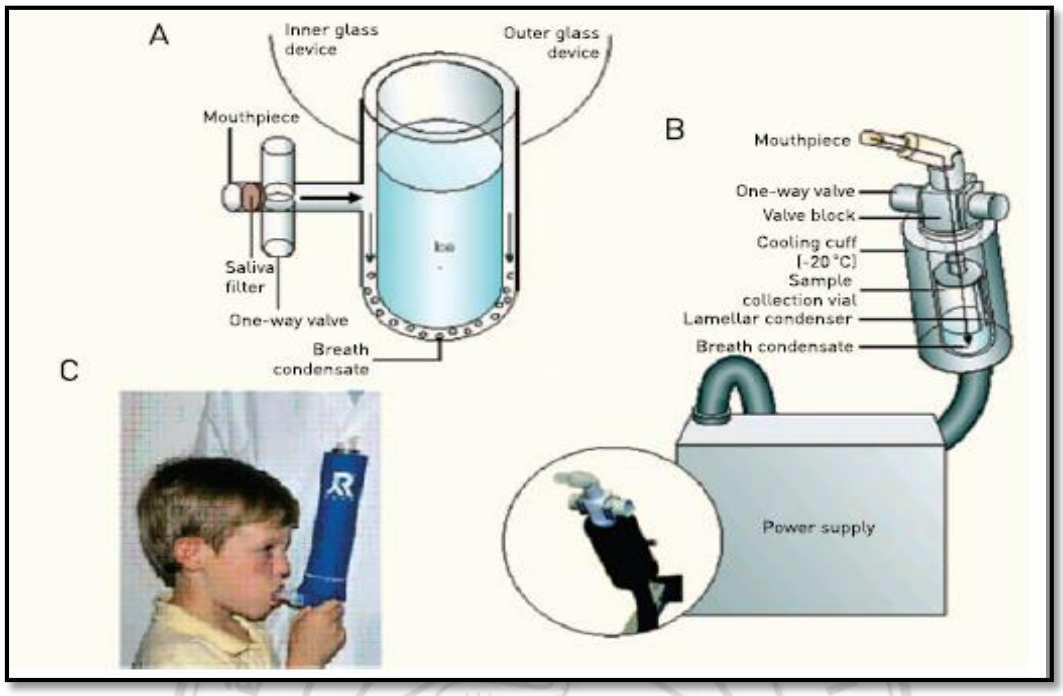

Figure 2.3 Representation of EBC collecting systems. (A) A homemade EBC collecting system. (B) A commercially available condenser EcoScreen®. (C) A portable EBC collecting system RTube™ (Montuschi, 2007)

In most studies, custom-made collection devices are constructed and used for EBC collection as shown in Figure 2.3A. Home-made equipment generally consists of a mouthpiece with a one-way valve connected to a collecting system which is placed in either ice or liquid nitrogen to cool the breath (Montuschi, 2007). The collecting system consists of a double wall of glass, the inner wall of which is cooled by ice. Alternatively, jacketed cooling pipes or tubes in buckets have been used (Mutlu *et al.*, 2001) as shown in Figure 2.4. Generally, subjects are asked to breathe tidally, with a nose-clip on, for 15 minutes through a mouthpiece connected to the condenser. Exhaled air enters and leaves the condensing chamber through one-way valves at the inlet and outlet while the chamber is kept closed. If the collecting system consists of two glass containers, EBC is collected between the two glass surfaces at the bottom of the outer glass container in a liquid form.

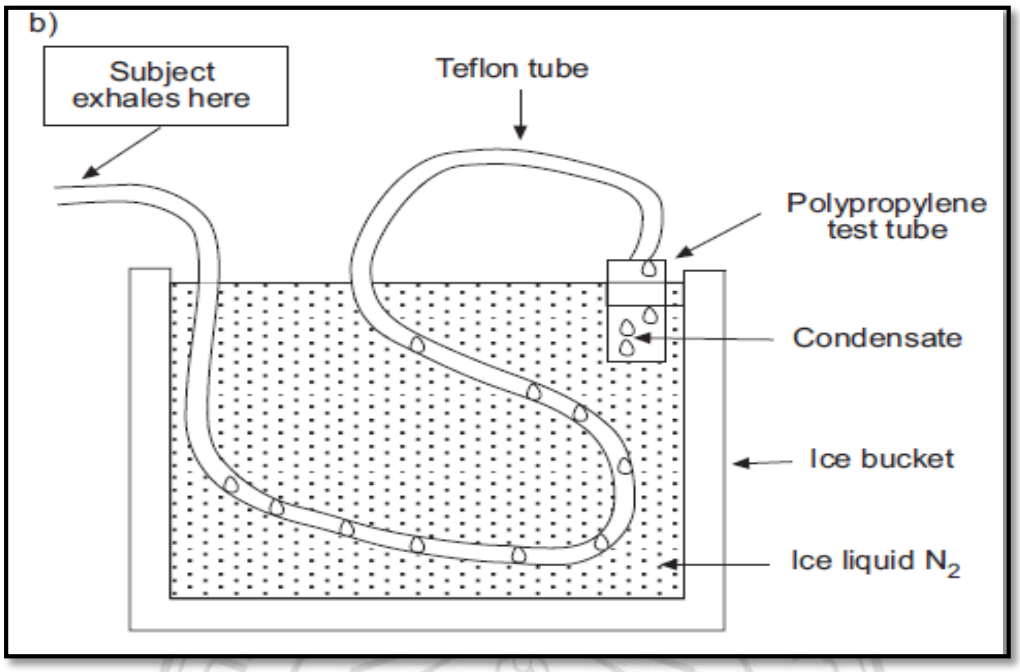

Figure 2.4 Schematic diagrams of a custom made EBC collecting devices (Mutlu *et al*., 2001)

2.10 Biomarkers in exhaled breath condensate

Biomarkers are substances used as indicators of a biologic state-normal or abnormal. In medicine, biomarkers are used to detect disease states. Detection indicates a change in the expression of a biomarker that has been found to correlate with a risk or progression of a disease or with a susceptibility of the disease to a given treatment. Several criteria for an ideal biomarker of oxidative stress/damage can be listed. The biomarker must first be measurable by a robust method or assay that is specific, sensitive and reproducible, and detectable even in normal, healthy individuals. Its levels shall not vary widely in the same subjects under the same conditions at different times. Ideally, it shall be predictive of the later development of the disease, though no biomarker has fulfilled this criterion as necessary experiments have not been done (Halliwell *et al*., 2004). In respiratory disease, biomarkers are used to reflect disease processes occurring in the lungs. Biomarkers can be detected in lung tissue, bronchoalveolar lavage, sputum, peripheral blood, urine, exhaled gases and exhaled breath**.** EBC contains many biomarkers of inflammation and oxidative stress that have been proposed to assess and monitor lung injury and disease (Mutlu *et al*.,

2001; Montuschi and Barnes, 2002) as shown in Table 2.2. The principle of sampling airways by EBC is that mediators from airways are released from the airway lining fluid, carried up by exhaled breath, and subsequently collected by condensation of the exhalation by breathing into a cooled tube.

Table 2.2 Selected biomarkers in exhaled breath condensate that have been proposed to assess and monitor lung injury and disease.

Biomarkers	**Meaning**	**Methods**
Hydrogen peroxide	- Cell release oxidant	- Colorimetric or fluorimetric methods
Nitrotyrosine	- Nitrosative stress biomarker	- Enzyme immune assay (EIA), mass spectrometry (MS)techniques
Eicosanoids	- Arachidonic acid derived inflammatory mediators	- EIA, radioimmunoassay, High performance liquid chromatography (HPLC)
8-isoprostane	- Lipid peroxidation biomarker	- EIA and gas chromatography/MS
Aldehydes	- Lipid peroxidation biomarker	- Liquid chromatography tandem/MS
Glutathione	- Antioxidant	- HPLC
pH	- Airway acidity	-pH electrodes and indicator dyes
Cytokine	- Inflammatory mediators	- EIA

Source s: Mutlu *et al*., 2001; Montuschi and Barnes, 2002

2.10.1 Hydrogen peroxide in EBC

Lung is the organ in the entire human body which has the highest exposure to atmospheric oxygen. Due to its large surface area and blood supply, the lung is susceptible to oxidative injury in the form of several reactive oxygen species (ROS) and free radicals. ROS may be produced endogenously by metabolic reactions or exogenously, such as air pollutants or cigarette smoke (Rahman *et al.*, 2006). At the biochemical level, ROS inactivate proteases, induce apoptosis, regulate cell proliferation and modulate the immune system in the lungs (Rahman and MacNee, 1999). At the molecular level, increased ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors, such as nuclear factor-kB and activator protein-1, signal transduction, chromatin remodeling and gene expression of proinflammatory mediators (Rahman and MacNee, 1998). Activated inflammatory cells respond with a "respiratory burst" which results in the production of reactive oxygen species (ROS). Naturally occurring free radicals have an oxygen- or nitrogen-based unpaired electron. Classical examples are superoxide anion (O_2^-), hydroxyl radical ($\bullet OH^-$), and nitric oxide (NO). O_2^- is formed from oxygen. The reaction of O_2^- and hydrogen peroxide (H_2O_2) in the presence of transition metal produces •OH. When catalyzed by neutrophil myeloperoxidase (MPO), H_2O_2 and a chloride form hypochlorous acid (HOCl) (Repine *et al*., 1997) (Figure 2.5). H_2O_2 acts as a central precursor. The main cellular sources for H_2O_2 are neutrophils, eosinophils, alveolar macrophages, epithelial cells and endothelial cells (Kinnula *et al*., 1992). H_2O_2 levels reflect the underlying state of oxidative stress in the lungs.

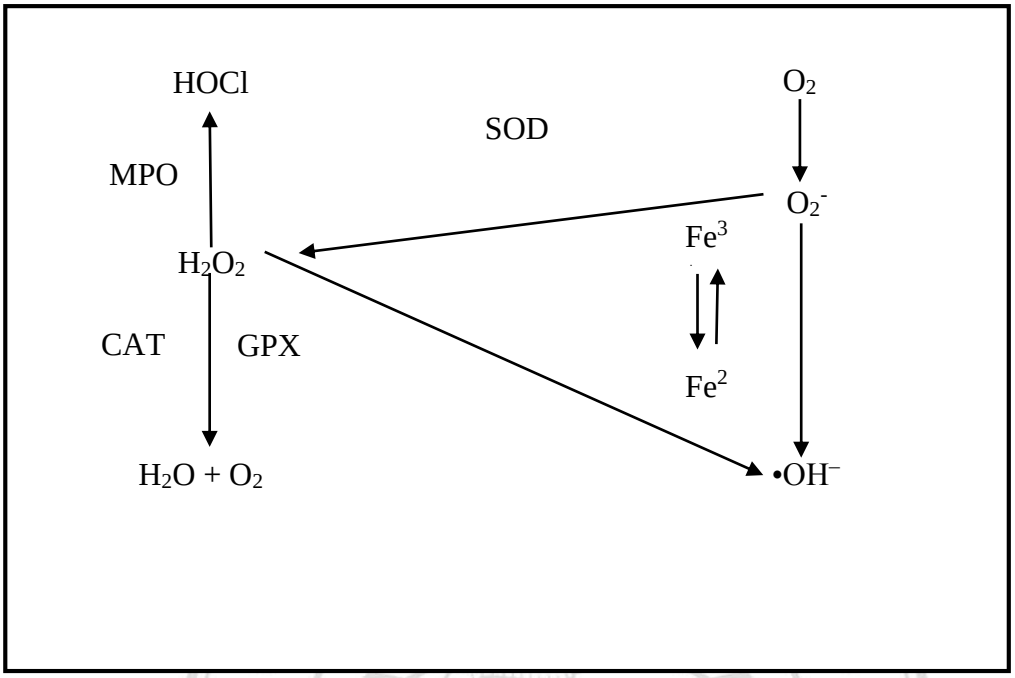

Figure 2.5 The formation of hydrogen peroxide (H_2O_2) and other ROS. CAT, catalase;Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion; GPX, glutathione peroxidase; O_2^-, superoxide anion; •OH^-,hydroxyl radical; SOD, superoxide dismutase; HOCl, hypochlorous acid.

The need for noninvasive assessment of airway inflammation is imperative, since inflammatory airway diseases, are usually characterized by variation in the clinical presentation. Moreover, there is an increasing trend for assessing the inflammatory pattern of inflammatory airway diseases through mediators measured by noninvasive techniques (Kharitonov and Barnes, 2006).

The purpose of collecting H_2O_2 in EBC was to provide a simple, cost-effective and non-invasive diagnostic/monitoring tool. H_2O_2 is one of the more abundant markers of inflammation in EBC. It is produced from a variety of sources including, but not limited to, inflammatory cells and various pathologic conditions. Due to its lack of specificity, the diagnostic usefulness of H_2O_2 has received considerable debate. Nevertheless, H_2O_2 in EBC remains a biomarker of keen investigation due to its likely association with numerous pulmonary pathologies having a common inflammatory component. These include bronchial asthma (Ueno *et al.*, 2008) and COPD (Montuschi, 2005; Inonu *et al*., 2012) and have been two of the most

widely studied disorders with respect to exhaled H_2O_2. Others disorders of respiratory system include adult respiratory distress syndrome (ARDS), cystic fibrosis, idiopathic pulmonary fibrosis, tuberculosis, community acquired pneumonia, systemic disorders with pulmonary involvement (scleroderma) and also those with extra-pulmonary location (uremia, breast cancer, asbestosis, and common cold) (Antczak and Górski, 2002; Kharitonov and Barnes, 2002; Vass *et al.*, 2003; Chow *et al.*, 2012; Nagaraja *et al*., 2012).

Hydrogen peroxide is produced by the enzymatic conversion of superoxide anions by superoxide dismutase in several cell types (Conner *et al*., 2002). In respiratory system, H_2O_2 can be released from both inflammatory and structural cells including neutrophils, eosinophils, macrophages and epithelial cells (Mossman and Churg, 1998). H_2O_2 is an important reactive oxygen species causing cellular injury via further reactions leading to more reactive species such as the hydroxyl radical (OH°) and lipid peroxidation products. H_2O_2 is less reactive than other reactive oxygen species but it has the propensity to cross cell membranes and enter other compartments. H_2O_2 is unstable in EBC; therefore EBC should be frozen quickly after collection and kept at -70 ºc until the determination of its peroxide level.

H_2O_2 in EBC is most commonly measured by spectrophotometrically or spectrofluorimetrically (De Benedetto *et al*., 2000; Gerritsen *et al.*, 2005; Horváth *et al*., 2005; Brooks *et al*., 2006) but various techniques have been used, including the development of automated amperometric biosensor (Ecocheck^{TM}; Filt GmbH, Berlin, Germany) (Gerritsen *et al*., 2005). The spectrofluorimetric method is based on the reaction of H_2O_2 with the enzyme HRP.

2.10.2 Methods of H_2O_2 determination

Various colorimetric, chemiluminescent and fluorometric methods have been developed to measure H_2O_2 in EBC. Methods of H_2O_2 measurement in EBC are based on the ability of H_2O_2 to react with suitable substrates, leading to the release of color, light, or fluorescence. Two methods of analysis have been used most: the spectrophotometric method according to Gallati and Pratch (1985) and the fluorimetric method Hyslop and Sklar (1986) or Ruch (2012).

EBC may contain H_2O_2 concentration <0.1 μmol/l which is close to the detection limits of some analytical methods. Spectrophotometric techniques have detection limit ≥0.1 μmol/l (Horváth *et al*., 1998) and thus show lack of analytic sensitivity. Measurement of H_2O_2 requires derivatization because it lacks a chromophore and itself emits very weak light (Svensson *et al*., 2004). This method typically requires a horseradish peroxidase (HRP) catalyzed reaction and a substrate with subsequent formation of highly fluorescent compound or product in an excited state that exhibits intense luminescence. The performance characteristic of current methods for determination is rather low H_2O_2 concentration. Some assays have been validated for H_2O_2 measurement in EBC (Nowak *et al*., 2001; Zappacosta *et al*., 2001; Svensson *et al*., 2004; Gerritsen *et al*., 2005; Brooks *et al*., 2006; Hu *et al*., 2008) and others could be adapted (Nozaki and Kawamoto, 2000; Onoda *et al*., 2006; Deng *et al*., 2009; Xia *et al*., 2009). Fluorometric methods are based on HRP catalyzed oxidation of 4-hydroxyphenylacetic acid by H_2O_2 into 2,2′-dihydroxybiphenyl-5,5′-diacetate, a highly fluorescent product. This assay is recommended for studies with exhaled H_2O_2 due to its sensitivity (limit of detection 3.4 nM) precision and rapid 10 min derivatization procedure (van Beurden *et al*., 2002; Brooks *et al*., 2006).

2.10.3 Malondialdehyde in EBC

Oxidative stress plays an important role in the pathogenesis of many inflammatory lung disorders. Among the many biological targets of oxidative stress, membrane lipids are the most commonly involved class of biomolecules. Lipid peroxidation yields a number of secondary products able to boost oxidative damage (Louhelainen *et al*., 2008). In addition to their cytotoxic properties, lipid peroxides are increasingly recognized as being important in signal transduction for a number of events in the inflammatory response (Rahman and MacNee, 1998). Malondialdehyde (MDA) has been widely studied as a product of polyunsaturated fatty acid peroxidation. High MDA levels have been observed in several biological fluids from patients with different airway diseases including asthma (Corradi *et al*., 2003; Ercan *et al*., 2006; Dut *et al*., 2008; Romieu *et al*., 2008a), COPD (Cobanoğlu *et al*., 2002; Barnes *et al*., 2006), and bronchiectasis (Cobanoğlu *et al*., 2002). MDA in EBC has been measured in relatively few studies (Corradi *et al*., 2003; Romieu *et al*., 2008a; Bartoli *et al*., 2011), and only few of them include large number of subjects (Romieu *et*

al., 2008a; Bartoli *et al*., 2011). Several hypotheses describing the formation of MDA in vivo have been proposed.

1) Oxidized lipids are able to produce MDA as a decomposition product. The mechanism is thought to involve formation of prostaglandin-like endoperoxides from polyunsaturated fatty acids with two or more double bonds.

2) This hypothesis is based on successive hydroperoxide formation and β-cleavage of polyunsaturated fatty acids chain to give a hydroperoxy aldehyde; MDA is then generated by β- scission or by reaction of the final acrolein radical with a hydroxyl radical (Esterbauer *et al*., 1991).

3) MDA can be also generated in vivo by enzymatic processes from various prostaglandins such as byproducts of free radical generation by ionizing radiation and of the biosynthesis of prostaglandins (Valenzuela, 1991).

2.10.4 Methods of MDA determination

Most assays to determine MDA have been developed on the basis of its derivatization with thiobarbituric acid (TBA) which can be easily assessed with a spectrophotometer, but the specificity of the test based on this reaction is low, as TBA may react with several compounds other than MDA also derived from oxidation. Moreover, the treatment of biological samples to obtain the condensation product is usually carried out at high temperature (around 100°C) and may generate further oxidation of the matrix with obvious overestimation of the results. To minimize matrix oxidation, most of these methods involve the precipitation of protein prior to the TBA reaction as a pre-treatment of plasma samples. One of the first and still most widely used methods to detect MDA is that developed by Yagi (1976), carrying over the TBA reaction on a blood lipid and protein precipitate at 95°C in acidic conditions. Based on this method, results are often reported as ‘‘TBA reacting substances’’ (TBARS) instead of MDA. TBARS measurements continue to be assessed in clinical trials and often give positive results, apparently demonstrating levels of oxidative stress higher in pathological than in healthy conditions. However, in the chemical conditions usually applied for the MDA-TBA reaction, the measure obtained by the TBARS assays gives an idea of sample oxidizability, rather than sample oxidation**.** In the last few years several innovations have been introduced to improve the specificity and avoid the common biases of these old procedures.

As indicated above, the TBA assay has frequently been criticized for its lack of specificity and artifact formation. To improve specificity, a range of HPLC methods has been developed either based on direct measurement of MDA or the measurement of a genuine MDA adduct. Direct measurement of MDA by HPLC takes advantage of its UV absorbing properties (Agarwal and Chase, 2002; Del Rio *et al*., 2003). Consequently, UV-based methods usually interfere from a high background which compromises the sensitivity. On the other hand, interference from other substances is limited when the derivatization step. The MDA-TBA adduct is also highly fluorescent and several HPLC methods are based on fluorescence detection (typically excitation, 515 nm; emission, 553 nm) (Templar *et al*., 1999). The HPLC methods significantly improve the specificity of MDA measurement as indicated by much more homogenous results obtained in various laboratories. MDA has also been quantified by other means than spectrophotometry and HPLC. A gas chromatography-mass spectrometry method based on derivatization with pentafluorophenylhydrazine to form the stable adduct N-pentafluorophenylpyrazole allows the quantification of femtomole amounts of MDA using isotope MDA as internal standard (Sheu *et al*., 2003). Direct measurement of MDA by high-performance capillary electrophoresis apparently offers both high specificity and sensitivity (Karatas *et al.*, 2002). However, although these methods may appear superior to those mentioned above from an analytical point of view, they have so far not been widely used presumably due to their requirement of expensive equipment and well- trained operator.