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DAILY FINE AND ULTRAFINE PARTICULATE MATTER EXPOSURE AFFECTS INFLAMMATORY AND COAGULATORY MARKERS AMONG SUSCEPTIBLE SUBJECTS

Settore disciplinare MED44

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INDEX

ABSTRACT

Current concerns about the health effects of airborne particles are largely based on the results of epidemiological studies, suggesting effects on mortality and morbidity for cardiovascular and respiratory causes at very low levels of particulate matter exposure. The mechanisms behind these effects are still unknown, although some hypothesis have been postulated. The studies support the idea that inhalation of PM can instigate extra-pulmonary effects by the release of pro-inflammatory mediators (eg. cytokines, activated immune cells, platelets), vasculoactive molecules (eg. ET, histamine, microparticles) from lung-based cells; and/or by translocation of PM (UFPs) or particle constituents (organic compounds, metals) into the systemic circulation. Subsequently, these events may contribute to a systemic inflammatory state, which may in turn be capable of activating haemostatic pathways, impairing vascular function, and accelerating atherosclerosis.
Which fraction of PM is the most harmful is still controversial, and few studies investigated the role of personal exposure to different fractions, in particular fine and ultrafine particles.

Aims. My doctoral thesis aims at characterize the effects of PM exposure on inflammatory and coagulatory indices in susceptible subjects (with chronic heart diseases or chronic respiratory diseases), focused on the role of the fine and ultrafine particles in determining changes in these markers.

Materials and methods. 27 healthy individuals (*Healthy group*), 34 individuals with chronic ischemic heart disease (*Heart group*), 18 with chronic asthma or COPD (*Lung group*) underwent a 24-hour exposure/clinical evaluation protocol during their habitual activities, both in the warm season (no heating period) and in the cold season (heating period). Individual exposure to UFPs (0.02–1 μm, Aerodynamic Diameter, D.a), fine and coarse particles number concentration (0.3-0.5; 0.5-1.0; 1.0-2.5; 2.5-5.0; 5.0-10 μm D.a), gravimetric $PM_{0.5}$, PM_1, $PM_{2.5}$ and PM_{10} was assessed for each subject, along with measurement of total blood cells count and blood markers of inflammation [TNF-alfa, sRI- and sRII-TNF-alfa, IL-8, IL-10, hs-CRP] and coagulation [fibrinogen, aPTT, INR, D-dimer, vWF, tPA, F1+2, closure time measured with PF 100 Analyzer® (PFA100 C-EPI CT e PFA100 C-ADP CT)].

Since the three groups had different clinical status, each group were analyzed independently using mixed effects models for repeated measurements to evaluate the associations between particles exposure and clinical parameters. Models include time varying factor (PM, temperature and relative humidity) and time-invariant subjects specific characteristics (age, gender, BMI, drug assumption). Pollution effects were expressed as percent changes by interquartile range (IQR) changes of PM (Chuang K. et al. 2007).

Results. The mean age for the overall studied population was 64±10 years at the beginning of the study and the gender was male for the 63% of individuals.

The median (25°-75° percentiles) 24h concentration of PM_{10} during the no-heating period was 35.5 (29.3-51.1) $\mu g/m^3$ and during the heating period 58.0 (41.7-79.0) $\mu g/m^3$. For $PM_{2.5}$, the median concentration (25°-75° percentiles) during the no-heating period was 26.8 (21.4-37.7) $\mu g/m^3$ and during the heating period 49.8 (33.7-66.3) $\mu g/m^3$. Comparing the data from the two monitoring periods, the results showed a significant increase for particulate matter concentrations in the heating ignition power-on period. The PM_{10} percentage variation was 63.4% and for $PM_{2.5}$ was 85.8%. The three groups of subjects were exposed to similar PM concentration, except for fine particles ($PM_{0.5}$, $FP_{0.3\text{-}1}$ D.a), that were higher in the *Healthy group*.

The subjects in the three groups provided different values of total leukocytes count, inflammatory parameters and coagulation parameters. *Healthy group* showed lower values of inflammatory markers than those in *Heart* and *Lung groups*, which in particular is characterized by slight higher levels of inflammatory markers and lower levels of an antinflammatory marker (IL-10). Concerning coagulation parameters, *Heart group* presented longer closure time (PFA-100 CT) than *Lung* and *Healthy groups.*

The results of the mixed models in the *Heart group* showed significant increase in monocytes number associated with fine and coarse particles. The monocytes increased of 7.9% (p=0.06) in association with PM_{10}. Erythrocytes number increased of 2.14% (p=0,02) in association with $FP_{0.3\text{-}0.5}$ and of 1,9% (p=0.01) in association with $CP_{5\text{-}10.}$ Platelets number increased of 6.77% (p=0.08) in association with $PM_{0.5}$, and of 5.19% (p=0.08) with $CP_{5\text{-}10}$, although the relation is slight significant.

In *Healthy group*, variations of lymphocytes number were associated with fine and coarse particles. The lymphocytes increased of 8,71% (p=0,01) in association with PM_{10}, and of 5.68% (p=0.004) in association with $FP_{0.3-0.5}$.

In the *Lung group* there is a negative association with monocytes and lymphocytes with fine and coarse particles and only with fine particles respectively. The number of monocytes decreased of 14,73% (p=0,01) in association with PM_{10}, and decreased of 14.45 % (p=0.02) in association with $FP_{0.3-0.5}$. The lymphocytes decreased of 11.48% (p=0.04) in association with $FP_{0.3-0.5}$.

There were negative associations between closure time (PFA-100 C-EPI CT) and fine particles in *Healthy group*, and between fine and coarse particles in *Heart group*. In *Healthy group*, the closure time was shortened of 10% (p=0.06) in association with PM_1 and of 6.81 % (p=0.05) with $FP_{0,5-1}$. In *Heart group* closure time was shortened of 17.84% (p=0.02) in association with PM_1 and of 14.98 % (p=0.004) with $FP_{0,5-1}$.

In *Healthy group* the t-PA is positive associated with ultrafine, fine and coarse particles. The t-PA varied of 22.47% (p=0.07) in association with PM_1 and of 22.38% (p=0.05) with $FP_{0,3-0,5}$

No changes were present in the *Lung Group*.

No statistical associations were found for cytokines, interleukins, hsPCR and the others parameters.

Discussion. Investigated subjects experienced high levels of individual exposure to $PM_{2.5}$ and PM_{10}. In particular, exposure to $PM_{2.5}$ exceeded the 24-hour mean of 25 $\mu g/m^3$ suggested by WHO in both the investigated periods, while the limit suggested for PM_{10} (i.e 50 $\mu g/m^3$) was exceeded only in cold season (WHO, 2005). Observed PM_{10} concentrations were similar to twenty four hours average reported for urban background in Europe (Larssen S. et al. 2005), and $PM_{2.5}$ concentrations were similar to the previously reported for personal monitoring indoor and outdoor in Milan (Rotko T. et al. 2002). As expected, higher levels of exposure to almost all particles were observed in the cold season. The great contribution of these higher winter levels is mainly due to particles in the accumulation mode ($FP_{0.3-1}$ μm). In cold season the PM levels are strongly affected by the stability of the atmosphere, by the low degree of air convection (characteristics of the warm season), and also by the heating which is one of the major sources of the particles in the accumulation mode.

The different levels of exposure among the three groups were mainly caused by fine particles in the accumulation mode ($FP_{0.3\text{-}1}$ μm) and the *Healthy group* seemed to be the higher exposed. An analysis of the activity of these subjects showed that they have spent more time outdoor than the subjects of other groups, so they were much exposed to particles from outdoor origin, the particles in the accumulation mode, confirming the outdoor origin of these particles.

A comparative analysis of the biological data among the three groups shows that the *Healthy group* presented strongly different values from the other groups, as expected, confirming a great status of health of these subjects compared with the others. The *Heart group* showed an impairment of the coagulation parameters in comparison to the others, probably due to the characteristics of the diseases and the assumed anti-clotting therapy. The *Lung group* seemed to have an impairment of the activation of the antinflammatory markers with a persistent low grade condition of inflammation.

The increase of monocytes, erythrocytes and platelets number in *Heart group* and lymphocytes number in *Healthy group* in association with fine and coarse particles could suggest an increased bone marrow activity, involving a variety of cell types, as a result of the effects of cytokines and chemokines from the lung that spill over into the circulation and trigger a cascade of inflammatory reaction signals generated in the lung. A leukocytosis associated with the activation of bone marrow activity was demonstrated in human exposed to PM, that reacted mobilizing leucocytes into circulation as a part of systemic exposure (Tan WC. et al. 2000; Sakai M. et al. 2004). On the other hand we observed a negative association between PM and monocytes and lymphocytes number in the *Lung group* that could be explain by the presence of allergic subjects in this group; the summer PM in Milan is more rich in pollens, endotoxins and biological materials (Camatini M. et al. 2010), so we could hypothesize a pro-allergic effects of no-heating PM levels, an increase of the inflammatory pattern during the no-heating period.

The activation of the platelets aggregation capacity, measured as closure time with PF 100 Analyzer, in *Heart* and *Healthy groups* in association with fine particles could suggest that particles with little aerodynamic diameter could pass directly from the alveoli to the blood and interact with the platelets, impairing their aggregability. Another possible mechanism of

platelets activation might reside in the pulmonary oxidative stress and the activation of subsets of white blood cells, that lead to a systemic lowering of endothelial- and platelet-derived nitrogen oxide and concomitant platelets activation (Brook RD et al 2008). Moreover, the increment of the tissue type plasminogen activator in the *Healthy group* in association with fine particles could suggest an increased thrombin generation and a reduced fibrinolytic activity.

The lack of consistencies in the association with PM and cytokines and interleukins could be explain by the fact that we have measured these factor after about 12 hours of the higher PM exposure levels, missing probably the concentration peaks in the blood (they have very short half-life). While the lack of consistencies in the association with PM and fibrinogen and C reactive protein could be due to the time necessary for the *ex-novo* synthesis of these proteins in the liver, that requires an induction time of 1-2 days (Seaton A. et al. 1999, Ruckerl R. et al. 2006).

The strength of this study is that it is one of the few using individual monitoring of gravimetric and number concentration of particles. The use of fixed site monitoring stations could not be representative of personal exposure resulting in imprecise associations (Delfino RJ. et al. 2008), therefore the individual monitoring is the only way to measure the real exposure of the subjects. A limitation of our study is that we enrolled a small number of subjects with different pathologies and drug therapies, that had a large impact on the biological parameters. Moreover we monitored these subjects only twice, resulting in few data for each subjects. Despite of these limitation, this work supports the hypothesis that exposure to PM results in a systemic inflammatory response, characterized by stimulation of bone marrow activity, that could increase the blood coagulability. It could also support the hypothesis that small particles may translocate form the lung into circulation and directly activate platelets and blood vessels. Together these mechanisms may account for the increase of cardiovascular events associated with episodes of air pollution.

Conclusion. The results suggest that PM exposure could contribute to the risk of cardiovascular events, in particular in elderly and subjects with cardiovascular diseases. Since there are evidences linking PM air pollution exposure and cardiovascular mortality and morbidity, may we consider PM as a risk factor for cardiovascular diseases or not? Particulate

matter exposure is ubiquitous, it may continuously enhance acute cardiovascular risk among susceptible people worldwide; moreover it may further elicits numerous adverse biological responses that could augment cardiovascular risk over the long term. Therefore, PM could be surely considered as a factor that modify and contribute to cardiovascular mortality and morbidity.

Despite the huge amount of studies about health effects of PM exposure, some issues remain open: to define the role of particles with different aerodynamic diameters and their chemical composition; to characterize the contribution of other co-pollutants (ozone, nitrogen dioxide, sulphur dioxide); to assess the importance of regional and intra-city differences in composition and combination of pollutants; to better define the susceptible subjects and define recommendations to help to reduce PM exposure; and finally to define whether there is a safe PM threshold concentration that eliminates both acute and chronic effects in susceptible subjects but also in general population.

1 INTRODUCTION

Air pollution is one of the primary health emergencies all over the word, and the Particulate Matter (PM) is one of the more relevant pollutant according to its toxicological pattern. The WHO estimated that PM contributes to approximately 800 000 premature deaths per year, ranking it as the 13th leading cause of worldwide mortality (WHO, 2002). Hence, the airborne PM appears to be an important modifiable factor that affects the public health on a global scale.

Cohort and time series studies have shown that environmental exposure to PM is associated with both acute and chronic cardiovascular and respiratory effects, that are more relevant among susceptible subjects such as the elderly, individuals with COPD and asthma, recent myocardial infarction, ischemic heart disease, hypertension, diabetes and obesity.

The association between PM and health effects appears to be more evident as particle size gets smaller because of the characteristics of the smaller particles (higher number concentration, better lung deposition, larger surface area).

Although controlled human exposure and human panel studies have been conducted in order to identify the mechanisms underlying the epidemiologic studies, the exact mechanisms linking the inhalation of ambient particulate matter and increased risk of cardiovascular or respiratory events remain unclear.

I had looked at susceptible groups (subjects with chronic ischemic heart disease, with chronic asthma or COPD) and subjects without diagnosis of the afore mentioned diseases, living in the urban area of Milan, to provide insight into the ways in which PM individual exposure, during warm and cold season, may cause alteration in inflammatory and coagulatory haematological parameters.

1.1 PARTICULATE MATTER

1.1.1 DISTRIBUTION

Particulate matter (PM) is the general term used for a mixture of solid particles and liquid droplets found in the air. These suspended particles vary in size, composition and origin, so that it is convenient to classify particles by their aerodynamic properties because they (WHO, 2003):

- govern the transport and removal of particles from the air;
- govern their deposition within the respiratory system;
- are associated with the chemical composition and source of particles.

These properties are summarized by the aerodynamic diameter, that is the size of a unit-density sphere with the same aerodynamic characteristics. The aerodynamic diameter is usually called simply particles size.

Aerosol scientists use three different approaches or conventions in the classification of particles by size (EPA, June 2003):

1. Modes, based on the observed size distributions and formation mechanisms;
2. Cut point, usually based on the 50% cut point of the specific sampling device, including legally specified, regulatory sizes for air quality standards;
3. Dosimetry or occupational health size, based on the entrance into various compartments of the respiratory system.

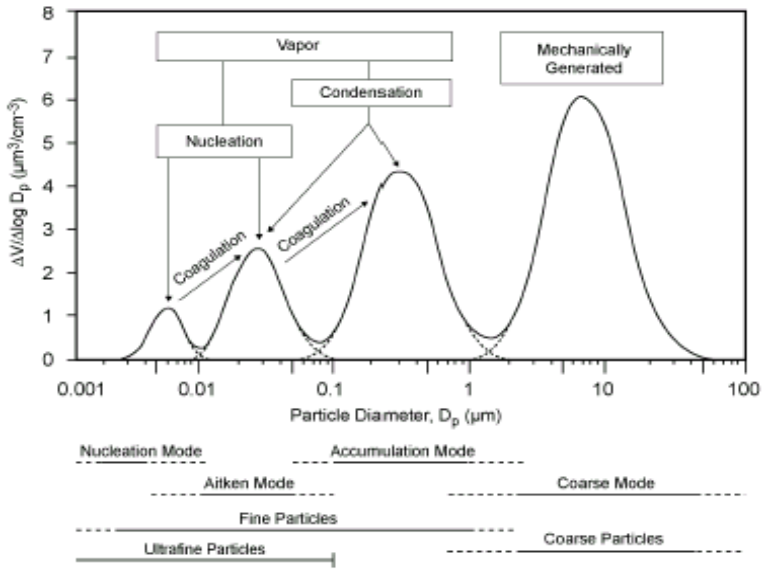
Whereas, among the common people the most known classification method is the cut point, which divided the particles into “classes” according to their dimension and quantify their presence in the air with mass expressed as microgram per cubic meter (μg/m3).

MODAL

The modal classification was proposed first by Whitby in 1978. Whitby observed that the size distribution typically had three peaks which he called “modes”. The entire size distribution could be characterized by a trimodal model consisting of three additive log-normal distributions. The peak between 5 and 30 μm was named the coarse particles mode; the mode

with a peak between 0.15 and 0.5 μm was called the accumulation mode; the mode with a peak between 0.015 and 0.04 μm was called the transient nuclei or Aiken nuclei range, subsequently shortened to the nuclei mode. An idealized size distribution showing modes and formation mechanisms is shown in the Figure 1.

Figure 1 An idealized size distribution, as might be observed in traffic, showing fine and coarse particles an the nucleation, Aitken, and accumulation modes that comprise fine particles. Also shown are the major formation and growth mechanisms of the four modes of atmospheric particles (EPA, August 2004)



The *Nucleation Mode* is freshly formed particles with diameters below 10nm, observed during active nucleation events. The lower limit, where particles and large molecule overlap, is uncertain. The *Aitken Mode* contains larger particles with diameter between 10 and 100 nm. The Aitken mode may results from growth of smaller particles or nucleation from higher concentrations of precursor. The *Accumulation Mode* includes particles with diameter from about 0.1 μm to just above the minimum in the mass or volume distributions which usually occurs between 1 and 3 μm. Accumulation-mode particles normally do not grow into coarse mode. Nucleation-mode and Aitken-mode particles grow by coagulation (two particles combining to from one) or by condensation (low-equilibrium vapour pressure gas molecule condensing on a particle) and "accumulate" in this size range.

The *Coarse Mode* or *Coarse Particles* are particles with diameters mostly greater than 1 or 3 μm. These particles are usually formed by mechanical break-up of larger particles or bulk material.

The *Fine particles* are particles including nucleation, Aitken and accumulation mode. These particles are generated during combustion or formed from gases.

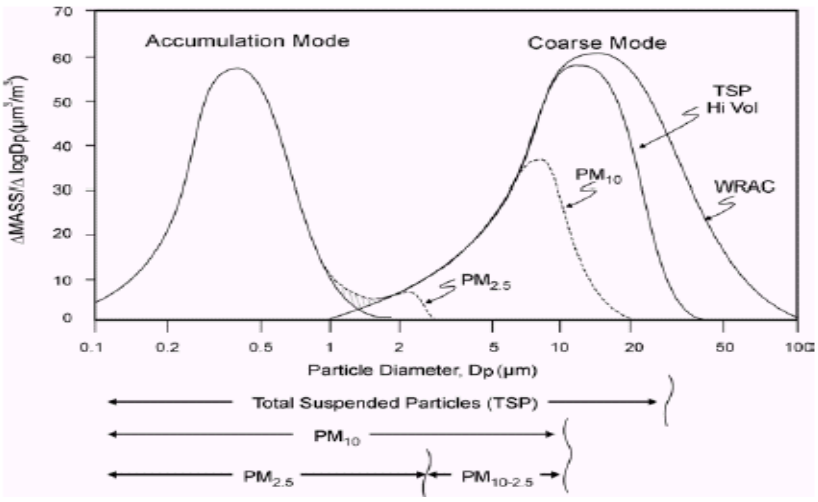
The *Ultrafine particles* are that portion of fine particles with diameter below about 0.1 μm (100 nm), for example Aitken and nucleation modes.

SAMPLER CUT POINT

Another way to define particle size is to refer at size-selective sampling; size-selective sampling refers to the collection of particles below or within a specific aerodynamic size range. Size fraction is usually specified by the 50% cut point size; for example, $PM_{2.5}$ refers to particles collected by sampling device that collects 50% of the 2.5 μm particles and rejects 50% of 2.5 μm particles. However, size fractions are defined, not merely by the 50% cut point, but by the entire penetration curve.

An idealized distribution with the normally observed division of ambient aerosol into fine-mode particles, coarse particles and the size fractions collected by the Wide Range Aerosol Classifier (WRAC), Total Suspended Particles (TSP), PM_{10}, $PM_{2.5}$ and $PM_{10-2.5}$ samplers is shown in Figure 2.

Figure 2. An idealized distribution of ambient particulate matter showing fine-mode particles and coarse-mode particles and the fractions collected by size-selective samplers. (EPA, June 2003)



1.1.2 FORMATION AND SOURCES OF PARTICLES

Particles are made up of different chemical components. The major components, or species, are carbon, sulphate and nitrate compounds, crustal materials such as soil and ash. The different components that make up particle pollution come from specific sources and are often formed in the atmosphere.

Particulate matter includes both *primary* PM, which is directly emitted into the air, and *secondary* PM, which is formed indirectly from fuel combustion and other sources. Generally, coarse particles are made up of primary particles, while fine particles is dominated by secondary particles.

The coarse fractions are mechanically produced by the break-up of larger solid particles. These particles can include wind-blown dust from agricultural processes, uncovered soil, unpaved roads or mining operations. Traffic produces road dust and air turbulence that can stir up road dust. Near coasts, evaporation of sea spray can produce large particles. Pollen grains, mould spores, and plant and insect parts are all in this larger size range.

The fine fractions are largely formed from gases. The smallest particles, less than 0.1 μm, are formed by nucleation, that is, condensation of low-vapour-pressure substances formed by high-temperature vaporization or by chemical reactions in the atmosphere to form new particles (nuclei). Four major classes of sources with equilibrium pressures low enough to form nuclei mode particles can yield particulate matter: heavy metals (vaporized during combustion), elemental carbon (from short C molecules generated by combustion), organic carbon and sulphates and nitrates.

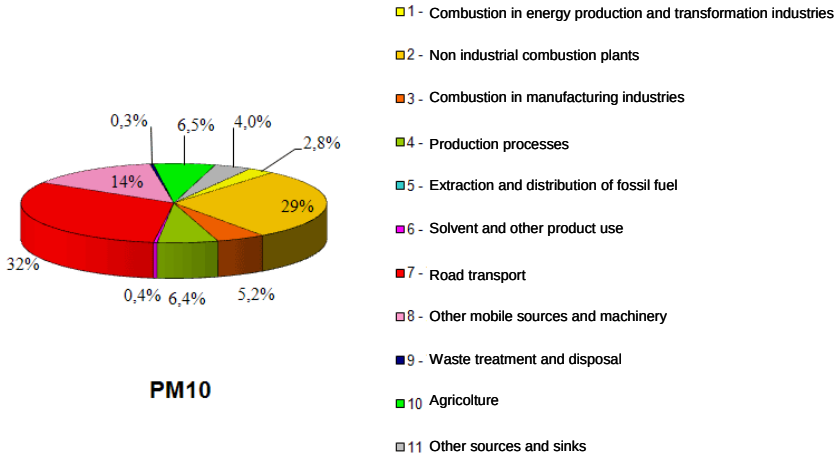
Sub micrometre-sized particles can be produced by the condensation of metals or organic compounds that are vaporized in high-temperature combustion processes. They can also be produced by condensation of gases that have been converted in atmospheric reactions to low-vapour-pressure substances. For example, these particles could include sulphates formed from sulphur dioxide emissions from power plants and industrial facilities, or nitrates formed form nitrogen oxide emissions from cars, truck, and power plants, or carbon formed from reactive organic gas emissions from cars, trucks, industrial facilities, forest fires, and biogenic sources

such as trees. Secondary sulphate and nitrate particles are usually the dominant component of fine particles.

In the atmosphere, coarse and fine particles behave in different ways. Larger coarse particles may settle out from the air more rapidly than fine particles and usually will be found relatively close to their emission sources. Fine particles, however, can be transported long distance by wind and can be found in the air thousands of miles from where they were formed.

The contribution of different sources of PM_{10} in Lombardy region is presented in Figure 3

Figure 3. Emissions in Lombardy region for 2007 (adapted from ARPA Lombardia, Inemar, INventario delle EMissioni ARia nel 2007,public review http://ita.arpalombardia.it/ITA/inemar/regione.htm)



Ultrafine particles are the result of nucleation of gas phase species to form condensed phase species with very low equilibrium vapour pressure. In the atmosphere there are four major classes of substances that yield particulate matter with equilibrium vapour pressures low enough to form nuclei mode particles: heavy metals, elemental carbon (EC) or soot, organic carbon (OC), sulphates.

Nuclei mode particles of metal oxides or other metal compounds are generated when metallic impurities in coal or oil are vaporized during combustion and the vapour undergoes nucleation. Metallic ultrafine particles also may be formed from metals in lubricating oil or fuel additives that are vaporized during combustion of gasoline or diesel fuels.

EC particles are formed primarily by condensation of C_2 molecules generated during the combustion process. Recent smog chamber studies and indoor experiments show that

atmospheric oxidation of certain organic compounds found in the atmosphere can produce highly oxidized organic compounds with an equilibrium vapour pressure sufficiently low to result in nucleation. Sulphuric acid molecules are generated in the atmosphere by conversion of sulphur dioxide (SO2) to H_2SO4. As H_2SO4 is formed, it can either nucleate to form new ultrafine particles, or it can condense on existing ultrafine or accumulation mode particles. Vehicle engine exhaust may include all these substances. Ultrafine particles are observed in the emissions from spark, diesel, and jet engines. In these cases it seems likely that elemental carbon, organic compounds, ammonia and sulphuric acid from sulphur in the fuel, as well as metal additives in the fuel or fuel oil, may contribute to the formation of ultrafine particles.

The presence of metals (in particular Fe and/or transitional metals) and acid compounds on the surface of the UFPs could have an important role in the determination of the biological effects. Some scientists argue that ultrafine particles may lead to some health effects may be associated with particle number or particle surface area. The more the dimension decreases the more the surface area increases, hence the ultrafine particles exhibit a greater surface area than the larger particles with the same mass. Increasing of the surface area leads to an increasing also of the proportion of atoms/molecules that are on the surface in comparison of those in the internal part. The increasing of the ratio surface/volume could lead to an increasing of the superficial energy that could make the particles biologically more reactive (Oberdoster G. et al. 2005b).

1.1.3 DEPOSITION OF PARTICULATE MATTER

Deposition of inhaled PM depends primarily on exposure concentrations, particle characteristics (size, shape, electrical charge, density and hygroscopicity), anatomy of the respiratory tract (lung size, airway branching pattern, airway diameters, lengths and frequency, depth and flow rate of breathing), tidal volume and breathing pattern.

Larger particles (>10μm) are mainly deposited in the extrathoracic part of the respiratory tract (above the larynx) and the main proportion of particles of 5–10 μm in aerodynamic diameter are deposited in the larger conductive airways. Particles of between 2.5 μm and 5 μm are deposited in the smaller conductive airways in proximity to the fine airways (respiratory bronchioles), with normal nasal breathing. Changing from nasal breathing to mouth breathing, the regional deposition pattern changes markedly, extrathoracic deposition being reduced and

tracheobronchial and pulmonary deposition enhanced. During mouth breathing, fine particles (< 2.5 μm) are deposited primarily in the pulmonary region; between about 3 and 5 μm significant deposition in both the pulmonary and the tracheobronchial regions occurs (Figure 4) (WHO, 2004). The proportion of mouth breathing to nose breathing increases with exercise and conversation.

An important observation is that large particles (>2 μm aerodynamic diameter) are not deposited uniformly in the airways, but that there are prominent deposition "hot spots" at airway bifurcation and other airway surfaces directly downstream of high-velocity air flows. Studies have shown that also smaller particles that penetrate deeply into the lung tend to be deposited preferentially near airway bifurcation (Lippmann M, 1994).

There are other factors that affect the total and regional respiratory tract particle deposition: ventilation rate, gender, age and respiratory disease status. In general, because of faster breathing rates and likely smaller airway size, women have a greater deposition of inhaled particles than men in upper tracheobronchial airways, but somewhat lower alveolar region deposition than for men. Overall, given that children have smaller lungs and higher minute volumes relative to lung size, they likely receive greater doses of particles per lung surface area than adults for comparable ambient PM exposures. This and the propensity for young children to generally exhibit higher activity levels and associated higher breathing rates than adults, likely contribute to enhanced susceptibility to ambient particle effects (EPA, 2003).

Altered PM deposition patterns due to respiratory disease status may put certain groups of adults (including some elderly) and children at greater risk for PM effects. One of the disease that could affect the deposition pattern is chronic obstructive pulmonary disease (COPD); the pathophysiologic characteristics of it contribute to more heterogeneous deposition patterns and differences in regional deposition. People with COPD tend to breath faster and deeper than those with normal lungs (i.e about 50% higher resting ventilation) and had about 50% greater deposition than age-matched healthy adults under typical breathing conditions, with average deposition rates 2.5 times higher under elevated ventilation rates (EPA, 2003)

Particle Clearance and Translocation

Particles depositing on airway surfaces may be cleared from the respiratory tract completely or translocated to other sites within this system by regionally specific clearance mechanisms (EPA, 2003). Insoluble particles deposited on ciliated airways are generally cleared from the

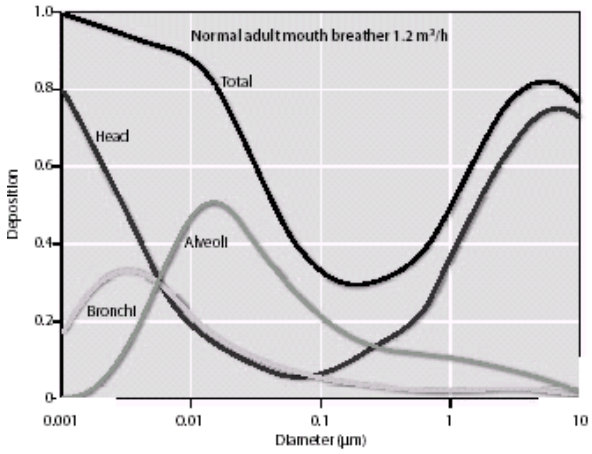
respiratory tract by mucociliary activity in 24-48 hours. All of the conditions that could affect this activity, such as chronic sinusitis, bronchitis, rhinitis, bronchial carcinoma, asthma, COPD and various acute respiratory infections, could bring to an increase of the retention of deposited particle material and thereby, to arise the probability of toxic effects from inhaled ambient PM components.

Soluble particles in the tracheobronchial region are absorbed mostly into the blood and some by mucociliary transport. Although tracheobronchial clearance is generally fast, the slow component of this clearance (likely associated with bronchioles <1mm diameter) retains deposited particles for more than 24 hours and has clearance half-times of about 50 days (EPA, 2003).

Clearance of particles from the alveolar region by alveolar macrophages and their mucociliary transport is usually rapid (< 24 h). A small proportion of the particles are transported to cilial airways and cleared by mucociliary clearance. A larger proportion is cleared by absorptive pathways, which include direct penetration into epithelial cells with transport to the mucociliary escalator or to the interstitial space, transport through the epithelial cell wall by transcellular and paracellular pathways, phagocytosis and destruction by the phagocytic system, or transport to lymphatics (Squadrito GL et al, 2001). Penetration of uningested particles into the interstitium increases with increasing particle load and results in increased translocation to lymph nodes. Soluble particles, that aren't absorbed quickly into the blood stream and traslocated to extrapulmonary organs (e.g. the heart) within minutes, may also enter the lymphatic system. Translocation into the lymphatic system is quite slow, and elimination from lymph nodes even slower (half-times estimated in decades).

Alveolar region clearance rates are decreased in human COPD sufferers and slowed by acute respiratory infections, and the viability and functioning of alveolar macrophages are reduced in human asthmatics. These observations suggest that persons with asthma or acute lung infections are likely at increased risk for ambient PM exposure effects.

Figure 4 Deposition probability of inhaled particles in the respiratory tract according to particle size (WHO, 2004)



1.2 EPIDEMIOLOGICAL STUDIES

Current concerns about the health effects of airborne particles are largely based on the results of epidemiological studies, suggesting effects on mortality and morbidity at very low levels of exposure of particulate matter. A wide amount of epidemiological studies all over the world have evidenced that the day variations of the concentration of fine and ultrafine particles in the air of the city areas are associated to variation of the population morbidity and mortality. Epidemiological studies can be separated into those that have investigated the health effects of acute exposure and those of chronic exposure. In the studies related to the adverse effects of short-term exposure, population-wide changes in acute outcomes (mortality, symptomatology, hospitalization, and health-care visits) are linked to short-term variations (in terms of days) in ambient pollutant concentrations. Studies on health effects of long-term exposure have involved analysis of data (e.g. total mortality) from large cohorts from multiple geographic locations that differ in the average chronic ambient concentrations and mixtures of air pollutants.

The acute effects of air pollution are generally investigated by time-series and case-crossover studies that explore the association between daily changes in health outcomes (e.g. mortality) in relation to day-to-day variations in ambient air pollution concentrations. The sum of the current evidence supports the findings of a review that demonstrated that short-term elevation in daily PM levels leads to a greater absolute risk for cardiovascular disease (CVD) related mortality than for other causes (69% increase in absolute mortality rate for CVDs compared

with 28% for pulmonary diseases) (Pope CA. 2000). To address concerns about city selection bias, publication bias, influence of co-pollutants, several large multicity daily time-series studies have been conducted worldwide. Two of the largest were the National Morbidity, Mortality, and Air Pollution Study (NMMAPS) in the United States (Samet JM. et al. 2000; Dominici F. et al. 2003) and the Air Pollution and Health: a European Approach (APHEA-2) project (Katsouyanni K. et al. 2001).These studies produced remarkably consistent results. The NMMAPS observed outcomes in 50 million people in the 20 largest cities in the United States. Average mortality rates were independently associated with particle concentrations the day before death. Each 10 $\mu g/m^3$ elevation in PM_{10} was associated with an increase of 0.21% (± 0.06 SE) and 0.31% (± 0.09 SE) for daily all-cause and cardiopulmonary mortality, respectively. The NMMAPS investigators reported no differences among various lag time periods from to 2 days and therefore based their estimates solely on the prior 24-hour period (1-day lag).

The APHEA-2 study demonstrated slightly more robust associations between adverse health outcomes and air pollution. For 43 million people in 29 European cities, the estimated increase in daily mortality was 0.6% (95% CI 0.4% to 0.8%) for each 10 $\mu g /m^3$ increase in PM_{10}. Cardiovascular deaths were increased by 0.69% (95% CI 0.31% to 1.08%) (Zanobetti A. et al. 2003). APHEA-2 based estimations on average particle concentrations the day of and 1 day before observed health outcomes (a day exposure time window).

Additional analyses of the APHEA-2 mortality data, based on lag periods up to 40 days, found that the risk of adverse health effects was associated with air pollution more than doubled (eg, 1.97% increase in cardiovascular mortality [95% CI 1.38% to 2.55%] per 10 $\mu g/m^3$ elevation PM_{10}) (Zanobetti A. et al. 2003). This finding indicated that the increase in cardiopulmonary mortality was not simply the result of "harvesting" (also called mortality displacement), which refers to the advancement of death by no more than a few days for severely ill individuals (Brook RD. et al. 2004).

In attempt to evaluate the coherence of multicity studies across continents, the Air Pollution and Health: A Combined European and North America Approach (APHENA) study analyzed data from APHEA, NMMAPS and Canadian studies (Samoli E. et al. 2008). The combined effect on all-causes mortality ranged from 0.2% to 0.6% for 10 $\mu g/m^3$ in daily elevation of PM_{10}, with the largest effects observed in Canada. Among individuals older than 75 years, the

effects were greater for cardiovascular mortality than for overall and pulmonary mortality (0.47% to 1.30%). Higher NO_2 levels were associated with larger PM_{10} effects on mortality, particularly in Europe. Finally, there appeared to be no lower-limit threshold below which PM_{10} was not associated with excess mortality across all regions.

Some time series studies were conducted also in Asia and they have confirmed increases in cardiovascular mortality related to short term PM exposure in China (Wong CM. et al. 2008a) and in several Asian cities (Wong CM. et al. 2008b).

The overall evidence from time-series analysis confirmed the association between increased mortality in occurrence of increased PM_{10} and $PM_{2.5}$. A meta-analysis has estimated that increased daily mortality due to a 10μg/m^3 elevation in $PM_{2.5}$ in the previous 1 to 5 days is approximately equal to a 0.4% to 1.0% (Brook RD. et al. 2010) .

The EpiAir Project has investigated the relationship between air pollution (PM_{10}, NO_2, O_3) and all natural mortality, as well as cardiac, cerebrovascular and respiratory in 10 Italian cities. Specific issues were addressed on the latency of the effects and the susceptibility (Stafoggia M. et al. 2009). Short-term effects of PM_{10} on mortality have been detected for all the groups of causes, with a latency ranging from lag 0 for cerebrovascular mortality to lag 0-3 for respiratory mortality. The elderly subjects were especially vulnerable to the effects of particles.

Effects of long-term exposure to PM on mortality are also of concern. Although short-term changes in PM concentrations have harmful health effects, long term exposure may have a more relevant clinical health effect on lung cancer and cardiovascular morbidity and mortality. It has been estimated that long-term exposure to moderate levels of fine PM can be associated with a reduction in life expectancy of up to several months.

Data **on chronic effects** of airborne particulate matter on mortality comes from cross-sectional studies, comparing air pollution exposure and mortality rates between locations, and from cohort studies documenting the mortality experience of differentially exposed subjects over time.

Dockery and collaborators (Dockery DW. et al. 1993) followed a cohort of more than 8000 adults living in six US cities (Harvard Six Cities study) with varying levels of air pollution exposure for periods of 14–16 years, between 1974 and 1991. After adjustment for age, sex, smoking, education, occupational exposure and body mass index, a significant relationship was

found between exposure to fine particles and lung cancer and cardiopulmonary mortality. The closest association was found for $PM_{2.5}$ and sulphate; the estimated effect was a mortality-rate ratio of 1.26, comparing the most polluted city (Steubenville, OH) with the least polluted city (Portage, WI).

An analysis of mortality occurring on low- and high-pollution days conducted on the data from Philadelphia showed a disproportionate increase in mortality among the elderly (Schwartz J. 1994). Mortality due to chronic lung disease and cardiovascular disease was also disproportionally increased. Interestingly, respiratory conditions were also more often mentioned on death certificates as contributing causes to cardiovascular deaths on high-pollution days. An analysis of location of death revealed that deaths outside the hospital were disproportionally increased as compared to death of hospitalized patients. This pattern is very similar to the pattern of mortality seen during and following the 1952 London smog (WHO, 2001).

An analysis from the Harvard Six Cities study was published addressing the question of whether fine particulate mass ($PM_{2.5}$) is a better predictor of mortality than coarse particulate mass (Schwartz J. et al. 1996). The results indicate that mortality is strongly associated with $PM_{2.5}$ but not with coarse mass. Because of the high correlation between $PM_{2.5}$ and PM_{10}, mortality was also strongly associated with PM_{10}, and the results of this particular analysis suggest that the associations between PM_{10} and mortality observed in other studies may very well be due to the effects of fine rather than coarse particulate mass. The pooled estimate was a relative risk of 1.015 (95% confidence limits 1.011 to 1.019) for each 10 $\mu g/m^3$ increase in $PM_{2.5}$.

An extended analysis of the US Six Cities Study was performed by Laden (Laden F. et al. 2006), that has extended the mortality follow-up for an additional 8 years, and has found the similar association between $PM_{2.5}$ and all-cause and CVD mortality. Moreover, the reduction in $PM_{2.5}$ concentrations for the extended follow-up period was associated with a reduced mortality risk.

Pope and collaborators (Pope CA. et al. 1995) analysed data from a large cohort study conducted by the American Cancer Society since 1980 (ACS study). Pollution data from 151 US metropolitan areas were linked to 8 years of follow-up data from about 500 000 subjects. After adjustment for age, sex, race, active and passive smoking, occupational exposure, education, body mass index and alcohol intake, a significant association between fine particulate air pollution exposure and survival emerged. The primary results showed that each 10 $\mu g/m^3$

increase in annual $PM_{2.5}$ mean concentration was associated with increases in all-causes, cardiopulmonary, and lung cancer mortality of 4%, 6% and 8% respectively. The relationship between $PM_{2.5}$ and adverse effects was linear and without a discernible lower "safe" threshold. Comparing the highest polluted area with the lowest polluted area, an adjusted mortality-rate ratio of 1.17 was found for $PM_{2.5}$.

An extended analysis of the ACS study was conducted to emphasize the control for the effects of other covariates and risk factors (Pope AC. et al. 2002). This analysis has confirmed the elevated mortality risk more strongly associated with $PM_{2.5}$ than PM_{10} and gaseous pollutants (except SO_2).

In a subsequent analysis of the ACS study (Pope CA. et al. 2004), the investigators reported PM-mortality associations with the specific cause of death; a statistically robust association between $PM_{2.5}$ and overall cardiovascular mortality was confirmed for 10 $\mu g/m^3$ increase in long-term exposure (RR 1.12, 95% CI 1.08 to 1.15). The single largest increase in risk was for ischemic hearth disease (RR 1.18, 95% CI 1.14 to 1.23), which also accounted for the largest proportion of deaths. In addition, the risk for arrhythmia, hearth failure, or cardiac arrest mortality was also increased (RR 1.13, 95% CI 1.05 to 1.21).

In a recent analysis of the Adventist Health Study of Smog (AHSMOG) fatal coronary heart disease was significantly associate with $PM_{2.5}$ among female but not male (Chen LH. et al. 2005). The observation that women may be at special risk from PM exposure is confirmed by other studies (Women's Health Initiative Study, the German women cohort, the intracity Oslo study) (Miller KA. et al. 2007, Gehring U. et al. 2006, Naess Ø. et al. 2007). So, the women seem to be a part of the susceptible population. Susceptible population include elderly, obese, individuals with low education or socioeconomic status, individuals with diabetes, with chronic cardiac disease and chronic respiratory disease (Zanobetti A. et al. 2000; O'Neil MS. et al. 2005; Sullivan KH. et al. 2005; Zeka A. et al. 2006; Halonen J. et al. 2008; Ostro BD. et al. 2008). In short-term studies, elderly subjects, and subjects with pre-existing heart and lung disease were found to be more susceptible to effects of ambient PM on mortality and morbidity; in panel studies, asthmatics have been shown to respond to ambient PM with more symptoms, larger lung functions changes and with increased medication use than non-asthmatics. In long term studies, it has been suggested that socially disadvantaged and poorly educated populations respond more strongly in terms of mortality (WHO, 2003). The APHENA study of European

and North American cities recently confirmed that elderly and unemployed individuals are at greater risk of short-term PM exposure (Samoli E. et al. 2008). Two cohort studies have shown that a greater body max index enhances the susceptibility for PM, at least in women (Miller KA. et al. 2007, Puett RC. et al. 2008).

1.3 PANEL AND EXPERIMENTAL STUDIES

A large amount of epidemiological studies have shown the association between variations of PM concentrations in urban air and variations of daily mortality and morbidity. The increase of mortality has been associated with both cardiovascular and respiratory harmful effects. These cardiovascular effects are acute (some hours after the exposure), latest and chronic (at least some days after the exposure); the acute effects seem to be related to a direct action of the PM on blood, circulation and lungs; while the latest ones are the consequence of the inflammation, at first pulmonary and subsequently systemic, that could active the haemostasis, influence the vascular functions and accelerate the atherosclerosis (Brook R.D. et al., 2004).
Over the last several years, many studies have been conducted to understand the pathophysiological mechanisms of health-related PM effects; some of these have investigated changes in respiratory parameters, others in cardiovascular system, others both.

Studies related to respiratory changes

One of the most important studies is the panel study conducted in Erfurt, Germany. This study has investigated the association between particulate air pollution and asthma medication use and symptoms among 53 adult asthmatics in winter 1996/1997. The exposure was assessed measuring number concentrations of ultrafine particles and mass concentrations of fine particles. The associations between ambient particle concentrations and the prevalence of inhaled β2-agonist, corticosteroid use and asthma symptoms, were analysed separately with logistic regression models, adjusting for trend, temperature, weekend, holidays, and first order autocorrelation of the error. Cumulative exposures over 14 days of ultrafine and fine particles were associated with corticosteroid use; β2-agonist use was associated with 5-day mean number concentrations of ultrafine particles and mass concentrations of fine particles. The prevalence of asthma symptoms was associated with ambient particle concentrations. The results suggested that reported asthma medication use and symptoms increase in association

with particulate air pollution and gaseous pollutants such as nitrogen dioxide (von Klot S. et al. 2002).

Pope CA. and collaborators have explored a possible pathophysiological pathway: transient declines in blood oxygenation and/or changes in cardiac rhythm following particulate exposure. In this study, blood oxygen saturation using pulse oximetry (SpO2) and pulse rate were measured daily on a panel of 90 elderly subjects during the winter of 1995-1996 in Utah Valley. Associations of SpO2 and pulse rate with respirable particulate pollution (particles with an aerodynamic diameter ≤ a nominal 10 μm) were evaluated. The results showed a little evidence of pollution-related hypoxia and alterations in pulse rate, that could reflect cardiac rhythm changes and may be part of the pathophysiology linking particles to cardiopulmonary mortality (Pope CA. et al. 1999).

Other authors have studied the health effects on children as a susceptible population. Timonen KL. and collaborators investigated how daily variations in ambient air pollution, especially in particles, during the cold of winter affect repeated measurements of baseline lung function and exercise induced bronchial responsiveness among primary school children with chronic respiratory symptoms. 33 children took part in exercise challenge tests conducted outdoors in a school yard in the centre of Kuopio, Finland. Spirometric lung functions were measured indoors before the exercise, and 3 and 10 minutes after. Daily mean concentrations of PM_{10}, black smoke, NO_2, CO, SO_2, and particle size and numbers were monitored at a nearby fixed monitoring site.

Increased concentrations of black smoke, PM_{10}, particle numbers, NO_2, and CO were consistently associated with an impairment of baseline lung functions [forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1)] (Timonen KL. et al. 2002).

Studies related to cardiovascular changes

Some studies have highlighted the effects on hearth function. Pekkanen J. and collaborators have assessed the associations between levels of the three main modes of urban aerosol distribution and the occurrence of ST-segment depressions during repeated exercise tests. Repeated biweekly sub maximal exercise tests were performed during 6 months among 45 adult subjects with stable coronary heart disease in Helsinki, Finland. Simultaneously, particle mass of $PM_{2.5}$ and the number concentrations of ultrafine particles and accumulation mode

particles were monitored at a central site. Levels of particulate air pollution 2 days before the clinic visit were significantly associated with increased risk of ST-segment depression during exercise test. The association was most consistent for measures of particles reflecting accumulation mode particles (odds ratio 3.29; 95% CI, 1.57 to 6.92 for accumulation mode and 2.84; 95% CI, 1.42 to 5.66 for $PM_{2.5}$), but ultrafine particles also had an effect (odds ratio 3.14; 95% CI, 1.56 to 6.32), which was independent of $PM_{2.5}$ (Pekkanen J. et al. 2002).

Some studies have assessed whether PM-related deaths may be a consequence of alterations in the blood, secondary to pulmonary inflammation caused by the action of fine particles on alveolar cells, by repeatedly measuring haematological factors and relating them to measurements of exposure to airborne particles. In an early study, Seaton and collaborators have investigated 112 individuals aged up to 60 years in two UK cities providing repeated blood samples over 18 months and measuring, each three day period prior to blood sampling, the exposure at PM_{10} with a mathematical model based on activity diaries and comparative measurements of PM_{10} at multiple sites and during a variety of activities. They showed that the changes in haemoglobin adjusted for albumin suggest that inhalation of some component of PM_{10} may cause sequestration of red cells in the circulation. They also proposed that an action of such particles either on lung endothelial cells or on erythrocytes themselves may be responsible for changing red cell adhesiveness (Seaton A. et al, 1999).

Blood parameters were measured also in the MONICA study (MONItoring of trends and determinants in CArdiovascular disease). The first MONICA survey was carried out on 4022 subjects in Augsburg (Germany) during the winter 1984/1985 while an air pollution episode occurred throughout Central Europe; blood collected during this period has been compared to blood collected other sample days in the 1984/1985 survey and to follow-up periods for the same individuals. The results were an increased risk of extreme values of plasma viscosity observed in both men and women. The authors assumed that altered blood rheology due to inflammatory processes in the lung that induce an acute-phase reaction might therefore be part of the pathological mechanisms linking air pollution to mortality (Peters A. et al, 1997). In 1987/1988, participants in the first MONICA survey were re-examined: the levels of C-reactive protein were measured by a high sensitivity assay in serum samples of 631 men aged 45 to 64 years. At ambient concentrations of pollution, as noted during the 1985 air pollution episode, the odds of observing C-reactive protein concentrations above 5.7 mg/l (>90th

percentile) tripled, and increases of 26 μg/m^3 total suspended particles (mean of 5 days) raised the odds of C-reactive protein levels 50% above the 90th percentile. The conclusion is that the exposure to current levels of particulate matter in the atmosphere elicits an acute phase response in randomly selected healthy middle-aged men, which may contribute to the increased cardiovascular risk caused by air pollution (Peters A. et al, 2001).

It has been hypothesized that altered autonomic function and pulmonary/systemic inflammation may play a role in the pathophysiologic mechanisms of the PM-health effects. Some studies explored the effects of air pollution on autonomic function measured by both electrocardiographic monitoring and blood tests. In a US study, 88 elderly subjects were involved in multiple sessions of 24-hr ambulatory electrocardiographic monitoring and blood tests. Regression analysis was used to evaluate associations between fine particulate matter ($PM_{2.5}$) and HRV, C-reactive protein (CRP), blood cell counts, and whole blood viscosity. There was found that the $PM_{2.5}$–HRV associations were reasonably consistent and statistically robust, while associations between CPR and $PM_{2.5}$ were small but statistically significant (Pope A et al, 2004b).

In North Carolina, US, nine highway patrol troopers (male age 23 to 30) were monitored on four successive days while working a three pm. to midnight shift. Each patrol car was equipped with air-quality monitors for $PM_{2.5}$. Blood was drawn 14 hours after each shift, and ambulatory monitors recorded the electrocardiogram throughout the shift and until the next morning. In-vehicle $PM_{2.5}$ (average of 24 μg/m^3) was associated with decreased lymphocytes (11% per 10 μg/m^3) and increased red blood cell indices (1% mean corpuscular volume), neutrophils (6%), C-reactive protein (32%), von Willebrand factor (12%), next-morning heart beat cycle length (6%), next-morning heart rate variability parameters, and ectopic beats throughout the recording (20%). Although, controlling for potential confounders had little impact on the effect estimates, the observations in these healthy young men suggest that in-vehicle exposure to $PM_{2.5}$ may cause pathophysiologic changes that involve inflammation, coagulation, and cardiac rhythm (Riediker M. et al, 2004).

1.4 MECHANISMS OF PM-RELATED ADVERSE HEALTH EFFECTS

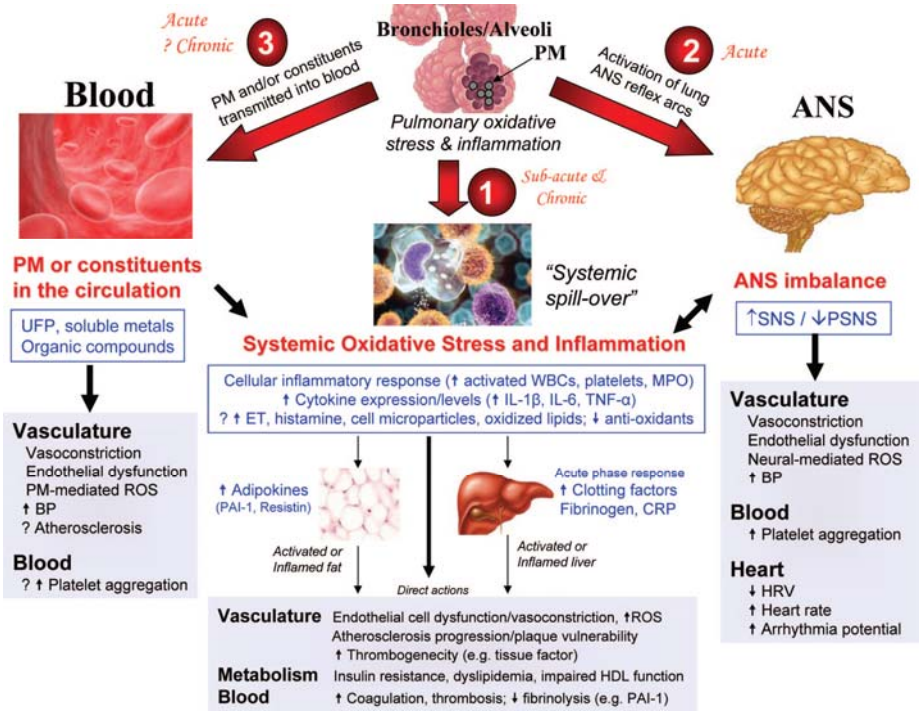
A number of experiments have demonstrated very rapid effects of PM which argues for the existence of pathways that convey signals systemically within hours of PM inhalation. On the other hand, there is also support for chronic effects, such as the promotion of atherosclerosis.

The presumed biological mechanisms linking PM air pollution to heart and respiratory diseases involve direct effects of PM on the cardiovascular system, blood, and lung receptors, and/or indirect effects mediated through pulmonary oxidative stress agents, such as gases, ultrafine particles along with soluble constituents of $PM_{2.5}$ (eg, transition metals), that readily cross the pulmonary epithelium into the circulation. In addition, activation of pulmonary neural reflexes secondary to PM interactions with lung receptors may play a role. The alterations in autonomic tone, under appropriate circumstances, might contribute to the instability of a vascular plaque or initiate cardiac arrhythmias. These direct effects of air pollution represent a plausible explanation for the occurrence of rapid (within a few hours) cardiovascular responses, such as increased myocardial infarctions. Less acute (several hours to days) and chronic indirect effects may occur via pulmonary oxidative stress/inflammation induced by inhaled pollutants. Subsequently, this may contribute to a systemic inflammatory state, which may in turn be capable of activating haemostatic pathways, impairing vascular function, and accelerating atherosclerosis.

The studies support the idea that inhalation of PM can instigate extra-pulmonary effects by three general pathways (Figure 5) (Brook RD et al 2010):

1. the release of pro-inflammatory mediators (eg. cytokines, activated immune cells, platelets) or vasculoactive molecules (eg. ET, histamine, microparticles) from lung-based cells;
2. perturbation of the systemic autonomic balance or heart rhythm by particle interactions with lung receptors or nerves;
3. potentially traslocation of PM (UFPs) or particle constituents (organic compounds, metals) into the systemic circulation.

Figure 5 Biological pathways linking PM exposure with CVDs. (A question mark (?) indicates the pathway/mechanism with a weak or mixed evidence or a mechanism of likely yet primarily theoretical existence based on the literature)



The molecular events triggering pulmonary oxidative stress and inflammation, along with the interactions between lung, immune cells and the inhaled PM are highly complex. Size, charge, solubility, aggregation, ROS-producing potential and chemistry of the particles play roles in the determining the responses. These include the particle fate (eg. lung clearance versus retention rates), the nature of the PM-cell interactions (eg. immune versus lung cell uptake, host cell responses, and intracellular sequestration/location), and the dose (likely typically a small percentage of inhaled PM) and pathways of potential systemic transmission of PM or its constituents, such as in the circulation (free, intracellular within circulating cells, (lipo)protein-bound) or via lymphatic spread. Because of their nano-scale size, UFPs may directly enter multiple lung cell types via nonphagocytic pathways and adversely affect organelles, such as mitochondria (Møller P et al. 2010). Larger unopsonized fine particles are more typically taken up by phagocytes through interactions with innate immunity receptors (Møller P et al. 2010). Certain particle compounds may directly generate ROS in vivo because of their surface chemistry (eg, metals, organic compounds, and semiquinones) or after

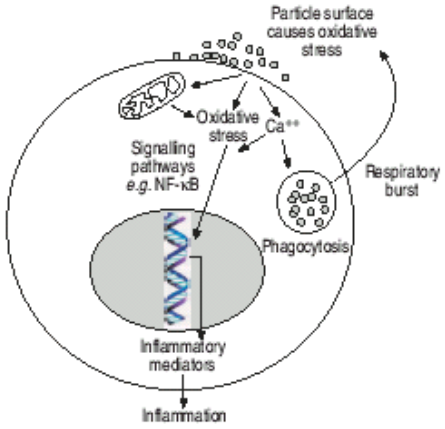
bioactivation by cytochrome P450 systems (eg, polycyclic aromatic hydrocarbon conversion to quinones) (Møller P. et al. 2010).

ROS in the lungs has been shown to augment the signal transduction of membrane ligand (eg, epidermal growth factor by disrupting phosphatases) or pattern recognition receptors (eg, toll-like receptors [TLR]) (Hollingsworth JW et al. 2004; Becker S. et al. 2005) and/or stimulate intracellular pathways (eg, mitogen-activated protein kinases) that lead to the activation of proinflammatory transcription factors (eg, nuclear factor-κβ), that upregulates expression of a variety of cytokines and chemokines. Alteration in lung cell redox status may itself stimulate nuclear factor-κβ (Figure 6). Biological components within coarse PM could also directly trigger inflammation (eg, nuclear factor-κβ pathways) by binding to TLR2 or TLR4 receptors or other innate immune pattern-recognition receptors (Becker S. et al. 2005).

The transcription factor NF-kB induces the expression of genes for:

- cytokines: TNF-α, IL-1β, IL-6, IL-11, GM-CSF (granulocytes macrophage colony-stimulating factor);
- chemotactic factors: IL-8, RANTES (Regulates upon Activation T-cell Expressed and Secreted);
- adhesion proteins: ICAM-1 (Intracellular Adhesion Molecule-1), VCAM-1 (Vascular Cell Adhesion Molecule-1) E-selectin;
- enzymes: iNOS (inducible nitric oxidase synthase), COX-2 (cycloxigenase-2), cPLA2 (citosolic phospholipase-2) 5-LO (5-lipoxigenase);
- receptor for: α-chain of the receptor for IL-2, β-chain of the receptor for T-cells.

Figure 6 Hypothetical sequence of events leading from the contact of particles with the cells (composite macrophage/epithelia cell) surface to inflammation (MacNee W., Donaldon K. , 2003)



Thus, the release of TNF-α and IL-1 can activate NF-kB, creating a mechanism of positive self-regulation for the expansion of the inflammatory response. So the activation of NF-kB system can be seen to be highly pro-inflammatory (Jimenez LA. et al. 2000).

Both macrophages and epithelial cells respond to PM exposure with increase release of cytokines. Deposition of PM into alveolar spaces in the lungs may trigger the release of cytokines from alveolar macrophages, which may stimulate the epithelial cells to produce specific mediators, further enhancing the inflammatory response. In vitro studies show that macrophages release TNF when exposed to PM; upon binding to its receptors subtype (TNF-R55 and TNF-R77), TNF evokes a complicated array of intracellular signals ranging from adaptation and protection of oxidative insult to cytotoxicity in the form of apoptosis or necrosis. In lung epithelial cells, exposure to TNF cause an increase the secretion of IL-8 (Flecha B. 2004).

Increased concentrations of IL-6 are associated with an increased risk of cardiovascular events (Ridker PM. et al. 2000; Lindmark E. et al. 2001) and mortality (Volpato S. et al. 2001). Serum IL-6, IL-1β, and granulocyte macrophage colony stimulating factor are increased in healthy male subjects after exposures to increased air pollution due to forest fires and are increased in vitro with exposure of human lung macrophages to urban PM_{10} (van Eden S. et al. 2001). IL-6 is directly involved in regulation of the synthesis of C-reactive protein in the liver. CRP concentration has been shown to be positively associated with exposure to total suspended particles and PM_{10} (Seaton A. et al. 1999; Peters A. et al. 2001).

Moreover, although the dominant source of cytokines likely represents the alveolar macrophages and lung epithelial cells, the role of other innate and adaptive immune cells cannot be ruled out (Fujii T et al. 2002; Hollingsworth JW et al. 2004). Available studies support important contributions to pulmonary inflammation from innate immune cells such as neutrophils and macrophages (TNF-α, IL-6), as well as from the adaptive immune system, such as T cells (IL-1, IL-4, IL-6, and IL-10). This large number of inflammatory mediators released from the lung cells after contact with PM could spill over to the general circulation or could induce a increase production of acute phase proteins (eg. C reactive protein, fibrinogen) from the liver. Stimulation of the liver that responds with the production and the release of acute phase proteins is an early and integral part of the systemic inflammatory response. Recent studies have suggested that C-reactive protein (CRP) is an important and independent

predictor of cardiovascular disease (Albert et al. 2002). Peters et al. (2001) showed that exposure to current ambient levels of particulate matter in the atmosphere elicited an acute phase response (e.g. CRP increase) in randomly selected healthy middle-aged men. This was further supported by data showing that plasma CRP levels were positively associated with higher levels of PM10 (Seaton et al., 1999). PM10 and ultrafine carbon black exposure enhance the expression of CRP in human lung epithelial cells, and the increased CRP is able to increase the infiltration of monocytes into arterial wall hence leading to atherogenesis by amplifying inflammatory and pro-coagulant response (Yeh ET. et al. 2001; Willerson JT. et al. 2002).

An integral component of the systemic inflammatory response is the stimulation of the hematopoietic system, specifically the bone marrow, which results in an increase in circulation leukocytes. The leukocytosis is the result of the accelerated release of PMN, band cells and monocytes. Military recruits exposed to high levels of PM_{10} during the forest fire in Southeast Asia in 1997, developed a leukocytosis that was associated with bone marrow stimulation (Tan WC. et al. 2000). Two separated studies on healthy subjects that resided in region with low PM10 (i.e. the South Pole) for prolonged periods, showed that circulating white blood cell count fell below the normal range shortly after the subjects entered this environment, remained low for the entire period, and than returned to normal levels once went back in the US (Mazzera DM. et al. 2001) and Japan (Sakai M. et al. 2004).

Emigration of inflammatory cells from blood to tissue site involves up regulation of adhesion molecules on vascular endothelium (E-selectin, P-selectin, intercellular adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1(VCAM-1) and on circulating leukocytes (L-selectin, lymphocyte function associated antigen-1, ICAM-1) (Frampton MW. 2001). Increased levels of CRP following exposure to particles may play a role in the impairment of endothelial function by attenuating NO reactivity and up-regulating expression of ICAM-1, VCAM-1 and E-selectin (Peters A. et al. 2001). Mills and colleagues have demonstrated that inhalation of diesel exhaust impairs vasomotor response to endothelium dependent and -independent vasodilators at 6 hours and endogenous fibrinolysis (Mills N. et al. 2005). Although the biological pathways responsible for PM-induced endothelial dysfunction have not yet been elucidated, an abnormal balance between the release of endothelium-derived constricting (ET-1) and relaxing (NO) substances has been proposed. Endothelial dysfunction

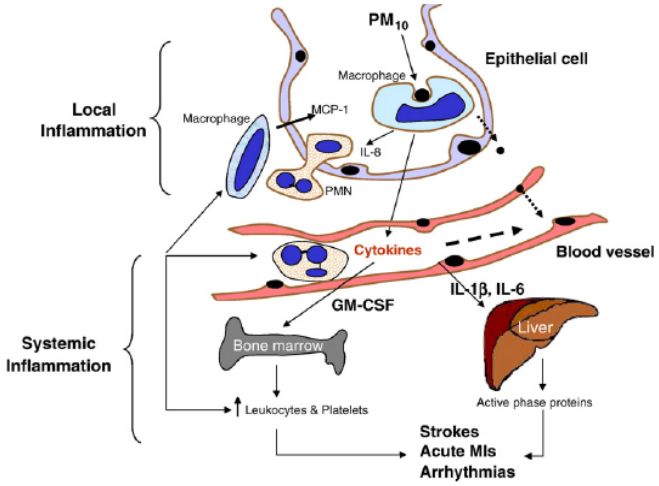
could significantly contribute to the development of atherosclerosis, acute coronary syndromes and MI associated with PM.

Human AM exposed to PM10 produce IL-1β and TNF-α that up-regulate the secretion of monocyte chemoattractant protein-1 (MCP-1) and promote the accumulation of monocytes and T lymphocytes in atherosclerotic lesions, hence accelerating the progression of atherosclerosis into an advanced stage (van Eeden S. et al. 2001).

A schematic summary of the events that could lead to cardiovascular events is presented in Figure 7. Seaton A. and collaborators proposed in 1995 (Seaton A. et al. 1995) a general hypothesis that exposure to inhaled particles induced alveolar inflammation, leading to exacerbation of pre-existing lung disease, increased blood coagulability, and associated risk of cardiovascular events. Some years later, Peters and collaborators (Peters A. et al. 1997) have investigated plasma viscosity, which is determined largely by plasma fibrinogen concentration, in a population in relation to a severe air pollution episode (MONICA study, Augsburg). They have found an increase on plasma viscosity during the pollution episode.

An experiment in rats have shown an increase in factor VII following a exposure to ultrafine particles. Increased in any proteins of clotting cascade present an increased possibility of coagulation. In addition, raised concentrations of fibrinogen and factor VII are recognized long-term risk factors for myocardial infarction.

Figure 7. Assumed pathways of inflammatory response elicide by PM (Bai et al. 2007)



Several others studies have evaluated the effects of PM exposure on haemostatic markers, such as fibrinogen, platelets count, D-dimer, von Willebrand factor. Some of those have not observed changes after PM exposure others reported increases coagulatory parameters. Ruckerl and colleagues have observed an increase of C- reactive protein (CRP), vWF, prothrombin 1+2 but not fibrinogen, d-dimer, FVII after in association with air ambient particles (PM10 and PM2.5) (Ruckerl R. et al. 2006). Mills and colleagues have demonstrated a significant effect of diesel exhaust on fibrinolytic function in response to intermittent exercise both in healthy men and in men with coronary artery disease (Mills N. et al. 2005). Rudez and colleagues have demonstrated that PM10 is associated with a increased platelets aggregation as well as coagulation activity but had not clear effects on CRP (Rudez G. et al. 2009). Carlsten and colleagues, in a ranodmized controlled crossover study, have recorded no effects on d-dimer, vWF, CRP, platelets count after PM exposure (Carlsten. C. et al. 2007).

The results related to to thrombosis/coagulation are quite variable given the differences in study designs, patients, biomarkers evaluated, and pollutants; however, these adverse effects appear somewhat more consistent among higher-risk individuals.

Mortality associated with air pollution might be further explained, at least in part, by alterations in the autonomic input to the heart. Decreased heart rate variability (HRV) predicts an increased risk of cardiovascular morbidity and mortality in the elderly and those with significant heart disease (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Because overall HRV decreases in response to ambient PM exposure (Pope CA et al. 1999; Liao D. et al. 1999) decreased parasympathetic input to the heart may provide an important mechanistic link between air pollution and cardiovascular mortality by promoting fatal tachyarrhythmias.

2 AIM OF THE PHD PROJECT

My PhD project forming a part of the PM-CARE study, a more comprehensive investigation designed to examine the mechanisms through which urban air particle exposures worsen cardiovascular and respiratory diseases.

Specific objectives of the PM-CARE study were:

- identify the pathogenetic mechanisms of the cardiac and respiratory injury induced by urban PM;
- define the role in this process of exposure levels and the single components of urban PM;
- define the deterministic and probabilistic PM risk models both at individual and population level;
- identify the criteria that physicians can use to identify PM as an environmental risk factor in cardio-respiratory patients and establish a relation with worsening of the disease in single patient.

My doctoral thesis aims at characterize the effects of PM exposure on inflammatory and coagulatory indices in susceptible subjects (with chronic heart disease or chronic respiratory disease), focused on the role of the fine and ultrafine particles in determining changes in these markers.

3 MATERIALS AND METHODS

Individual exposure and health measurements were made under a protocol approved by the Ethic Committee of the "Luigi Sacco" University Hospital (Milan, Italy) and the "San Gerardo" University Hospital (Monza, Italy), for individuals living in the urban and suburban area of Milan, Italy, between July 2005 and July 2006.

Three groups of subjects were studied: i) subjects suffering from chronic ischemic heart disease (*Heart group*), ii) subjects suffering from asthma or chronic obstructive pulmonary disease (COPD) (*Lung group*) and iii) subjects without diagnosis of the afore mentioned diseases (*Healthy group*).

Each participant underwent a twenty-four hours protocol, during normal unrestricted out-of-hospital activity, that included evaluation of exposure and health parameters twice, in the warm season (no-heating period, May – October) and in the cold season (heating period, November – April). A written informed consent was obtained.

The monitoring protocol was started by trained physicians and technicians in the morning at each participants' home, and ended the following day at the hospital.

3.1 STUDIED SUBJECTS

Studied subjects were recruited from the patients community of two Italian hospitals ("Luigi Sacco" University Hospital and "San Gerardo" University Hospital), and grouped according to their health status into subjects suffering from chronic ischemic heart disease, subjects suffering from asthma or chronic obstructive pulmonary disease (COPD) and subjects without diagnosis of the afore mentioned diseases. During an initial ambulatory medical visit a questionnaire was administered regarding the subject's demographics, physiological and medical history, current medication use, educational qualification and job position, usual sport activities intensity, alcohol consumption, smoking history and second-hand smoke (SHS) exposure at home. A 12-lead ECG and lung function tests were performed. A blood sample was collected for blood test analysis.

General exclusion criteria included current smokers (or ex-smokers < 6 months), poor glycemic control (sHbA1c > 7%, as defined by the "American Diabetes Association" Guidelines (Standard of medical care in diabetes, 2007), severe renal failure (serum creatinine

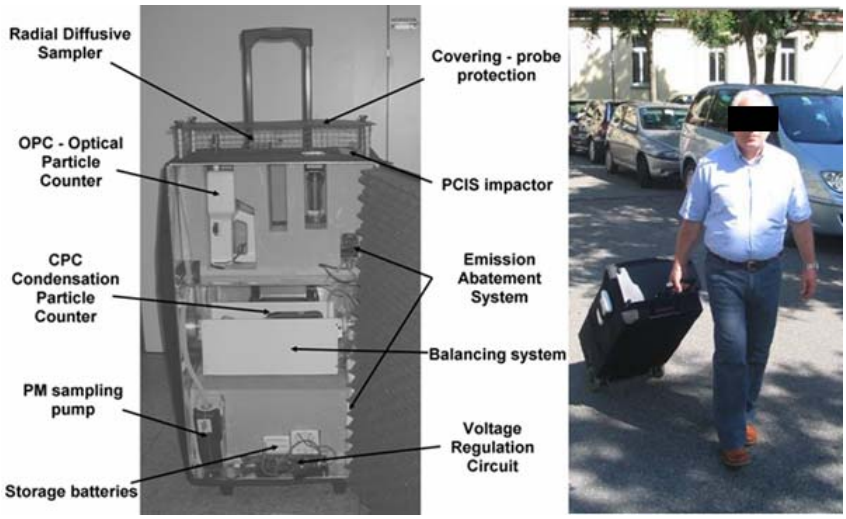
> 2,5 mg/dL), moderate-to-severe anemia (Hb < 10 mg/dL), coagulation disorders (PLT < 150.000 mg/dL; PT-INR < 0,8 or > 1,2; PTT < 21 or 36"; FG < 200 or > 400 mg/dL), electrolytic imbalance (Na+ < 135 or > 145 mEq/L; K+ < 3,4 or > 4,8 mEq/L).

Subjects in the *Heart Group* were eligible if they had chronic ischemic heart disease defined as 1) diagnosis of coronary artery disease (CAD) by at least one of the following: a) a positive coronarography, b) a positive provocative test (male), c) a positive myocardial scintigraphy (female); 2) a history of chronic stable angina; 3) a prior percutaneous coronary intervention (PTCA) or a prior coronary artery bypass surgery at least 6 months before recruitment; or 4) a previous myocardial infarction at least 6 months before recruitment. Specific exclusion criteria for this group included unstable angina, angina in CANADIAN class 3a or 4a, acute cardiovascular events in the previous 6 months, heart failure in NYHA class III or IV, anticoagulation therapy, paced rhythm (both pacemaker and implantable cardioverter defibrillator > 5% of R), chronic atrial fibrillation, moderate-to-severe COPD or asthma. Subjects in the *Lung group* were eligible if they had 1) mild-to-severe COPD (as defined by GOLD guidelines [Pauwels R.A. et al.,2001a; Pauwels R.A. et al., 2001b]); 2) mild-to-severe asthma (as defined by GINA guidelines, Bousquet J., 2000). Specific exclusion criteria for this group included a vital capacity less than two liters and chronic ischemic heart disease. Subjects in the *Healthy Group* were eligible if they had 1) normal ECG and no heart disease; 2) normal lung function tests and no lung disease. Specific exclusion criteria for this group included upper airways disease (included nasal and paranasal sinus disease), renal disease, allergy, and diabetes mellitus.

3.2 EXPOSURE ASSESSMENT

Exposure measurements were performed using individual monitoring instruments. All devices were assembled on a mobile monitoring unit (MMU), that was equipped to provide self-contained and unattended 24-hour monitoring, and that was easily transportable by the participants during their displacements (Figure 8). All the probes were placed on the top of the MMU, at 90 cm from the ground, allowing an individual exposure monitoring.

Figure 8 The Mobile Monitoring Unit (MMU) [size 90x45x31 cm, weight about 25 Kg]



The protocol included measurements of ultrafine particles number concentration (UFPs), fine particles and coarse particles number concentration, $PM_{0.5}$, PM_1, $PM_{2.5}$, PM_{10} mass fractions and microclimatic parameters (temperature and relative humidity).

UFPs number concentration was continuously measured with a Condensation Particle Counter (CPC, P-TRAK® Ultrafine Particle Counter, TSI Model 8525) for particles in the size range 0.02–1 μm in D_A. Data were recorded by an internal data-logger and downloaded on computer at the end of each 24-hour monitoring period with TrakPro software (Version 3.6.2.0, TSI Inc.).

An Optical Particle Counter (OPC, Lighthouse Handheld 3016 Particle Counter) was selected to measure fine (from 0.3 to 2.5 μm) and coarse (from 2.5 to 10 μm) particles number concentration. It measured the number of particles having D_A> 0,3 μm, sorted into six dimensional classes (0.3-0.5; 0.5-1.0; 1.0-2.5; 2.5-5.0; 5.0-10 μm).

Twenty four hours time weighted gravimetric measurements of PM were performed with a personal cascade impactor sampler (PCIS), with three impaction stages and an after-filter that allows the separation and collection of airborne particles in the following aerodynamic particle diameter ranges: <0.5, 0.5–1.0, 1.0–2.5 and 2.5–10 μm. The PCIS operates in combination with the Leland Legacy Sample Pump (SKC Inc., Cat. No. 100–3000) at a flow rate of 9 L/min. PTFE membranes with PTFE support were used for collection of PM fractions. Membrane filters were weighted according to UNI EN 12341. The filters were weighted

according to the D.M. April 2nd 2002, n. 60 and the European Legislation (EN12341). The filters were stored at 20°c with 50% of humidity for at least 48 hours both prior and after the monitoring.

Temperature and Relative Humidity (RH%) were continuously measured with a miniaturized microclimatic probe (Lighthouse Worldwide Sol.).

The MMU was delivered at each subject' home during the morning of the monitoring day (from 8 to 10 a.m.). Our personnel registered some information about house characteristics (presence of chimneys, gas heater or other kinds of heaters, carpets, storey, traffic intensity, density and height of the surrounded buildings, quality of the windows casing etc..). Each subject filled in a questionnaire recording the activity (walking, flame cooking, relax etc..), drugs assumption and symptoms occurring in the day of the monitoring.

3.3 HEALTH MONITORING

3.3.1 INFLAMMATORY MARKERS

Fasting blood samples were obtained from the antecubital vein without tourniquet at the end of each 24-hour monitoring protocol at the hospital, using standardized procedures for phlebotomy, and were collected into vacuum tubes.

Blood samples for inflammatory markers were stored at ambient temperature until 4 hours from the collection. 2,5 ml of blood were centrifuged at 2400 rpm for 10' at room temperature. Plasma was distributed into aliquots and stored at -20°C until the analysis.

TNF-alfa, IL-8, soluble receptor I and II of TNF-alfa and IL-10 levels were estimated both in plasma and in vitro following stimulation with phytohemagglutinin (PHA) or lipopolysaccharide (LPS), using a commercially available ELISA kits (Immunotools, Germany e R&D, USA). The blood samples (2 ml) for the in vitro stimulation of cytokine were diluted 1:10 with RPMI 1640 (Sigma, USA), added with 2 mM of L-glutamine, 0.1 mg/ml streptomycin and 100 IU/ml penicillin (Sigma), and incubated with 5% CO_2 at room temperature in polypropylene tubes in presence or not of LPS (1 mcg/ml; Sigma Cat. L3129) or PHA (1.2 mcg/ml; Gibco, Cat. 10576-015) during 24 hours for the release of IL-8 e TNF-alfa and for 72 h for the release of IL-10. The samples were centrifuged at 1500 rpm for 10

minutes at room temperature and the surnatant was distributed into aliquots and stored at -20°C until the analysis.

High-sensitivity C reactive protein (hsCRP) serum concentrations were measured using a near infrared particle immunoassay method using IMMAGE® Immunochemistry Systems Reagent (Beckman Coulter Inc., Fullerton, CA).

3.3.2 COAGULATION PARAMETERS

Blood samples were collected as previously described (see above). The blood was centrifuged at 3000rpm for 20 minutes at 4°C, then plasma was distributed into four aliquots and stored at -80°C until the analysis. The measure of D-dimer, von Willebrand factor (vWF), tissue plasminogen activator (tPA) and prothrombin fragment F1+2 (F1+2) were performed using available commercially ELISA kits.

The platelet activation was determined using the Dade Behring (Miami, FL) platelet function analyzer (PF-100®). Using this instrument, the blood–citrate mixture is aspirated under a constant negative pressure and contacts an ADP/epinephrine and collagen-coated membrane. The blood then passes through an aperture that induces high shear and simulates primary hemostastis after small blood vessel injury under flow conditions. The time to aperture occlusion (closure time, PFA 100 C-EPI CT, PFA 100 C-ADP CT) is recorded in seconds and is inversely related to the degree of platelet activation (Kundu Sk et al. 1995, Mammen EF et al. 1995).

Fibrinogen, aPTT and INR were measured with standardized methods used in the H. Sacco Hospital.

3.3.3 COMPLETE BLOOD CELLS COUNT

Complete blood count with differential leukocytes analysis were performed using standardized method used in the H. Sacco Hospital.

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3.4 STATISTICAL ANALYSIS

All data were tested for normality with Kolmogorov-Smirnov Test. Data were ln-transformed to achieve normality for statistical analysis purpose, when necessary. Parametric test (paired Student's T test) or non parametric test (Wilcoxon test) were used, depending on the distribution of data, to assess differences in clinical parameters between the two monitoring periods (cold and warm season).

Since the three groups had different clinical status, each group were analyzed independently using mixed effects models for repeated measurements to evaluate the associations between particles exposure and clinical parameters. The structure with repeated measures takes into account the variable time between the two monitoring period and visits. Model selections were based on the criteria of minimizing Akaike's Information Criterion (Akaike H. 1974).

The daily average PM exposure was matched with the biological parameters measured at the end of the 24 hours monitoring. Models include time varying factor (PM, temperature and relative humidity) and time-invariant subjects specific characteristics (age, gender, BMI, drug assumption). Pollution effects were expressed as percent changes by interquartile range (IQR) changes as $[e^{(\beta*IQR)}-1]*100$ for ln-transformed data and $[\beta*IQR/Media]*100$ for parametric data, where β and M are the estimated regression coefficient and the mean of each blood marker, respectively (Chuang K. et al. 2007).

Statistical computations were performed with SPSS software version 18.

4 RESULTS

4.1 STUDIED SUBJECTS

Out of the 112 volunteers contacted at the beginning of the study, 98 resulted eligible according to the established inclusion criteria of the study but, successively, 5 subjects dropped out for clinic reasons and 12 for scarce compliance. Then, the total group of volunteers enrolled for the 24h-monitoring was composed by 81 individuals: 34 suffering from chronic ischemic heart disease (*Heart Group*), 20 from chronic obstructive pulmonary disease or asthma (*Lung Group*) and 27 were characterized by absence of before mentioned heart or lung pathologies *(Healthy Group*).

The mean age for the overall studied population was 64±10 years at the beginning of the study and the gender was male for the 63% of individuals. Considering groups, the mean age and the male gender were respectively 61±7 years and 48% within the *Healthy group*, 66±10 years and 82% within the *Heart group* and 65±11 years and 50% within the *Lung Group*.

Subjects were generally overweight in all groups. Current smokers were not included in the study, although about half of them were former smokers (56%). Diabetes mellitus is totally absent in *Healthy Group*, while 24% and 15% out of individuals in the *Heart* and in the *Lung Group*, respectively, suffered from diabetes mellitus. In the total group 51% out of individuals suffered from hypertension, corresponding to the 26%, 71%, 50% in the *Healthy*, in the *Heart* and in the *Lung Group* respectively. In the *Heart Group* the majority of subjects underwent a PTCA or coronary artery bypass surgery (79%) or had a history of myocardial infarction (68%). Out of the *Lung Group,* 56% suffered from chronic obstructive pulmonary disease (COPD), 56% from asthma. Medications use differed in the three groups, depending on health status. General characteristics, as anthropometric description, physiological and medical history, drug assumption of studied population, are resumed in Table 1.

Table 1 Demographic characteristics, physiological and medical history, drug assumption of studied individuals (total sample and three groups). [Data are shown as mean±SD or as n (%)].

	Total Group	Healthy Group	Heart Group	Lung Group
	n (%)	n (%)	n (%)	n (%)
Subjects	81	27	34	20
Age (mean±SD)	64±10	61±7	66±10	65±11
BMI (mean±SD)	27±5	26±4	26±4	29±6
Male gender	51 (63%)	13 (48%)	28 (82%)	10 (50%)
Alcohol assumption[a]	39 (48%)	16 (59%)	16 (47%)	7 (35%)
Physical activity[b]	35 (43%)	23 (85%)	7 (21%)	5 (25%)
Former smoker	45 (56%)	13 (48%)	22 (65%)	10 (50%)
Hypertension	41 (51%)	7 (26%)	24 (71%)	10 (50%)
Dyslipidemia	39 (48%)	14 (52%)	18 (53%)	7 (35%)
Diabetes mellitus	11 (14%)	0 (0%)	8 (24%)	3 (15%)
ACE-inhibitor	27 (33%)	3 (11%)	20 (59%)	4 (20%)
β-blockers	29 (36%)	1 (4%)	27 (79%)	2 (10%)
Calcium channel antagonist	11 (14%)	1 (4%)	6 (18%)	4 (20%)
Diuretic drugs	22 (27%)	3 (11%)	15 (44%)	5 (25%)
Other anthypertensive treatment	40 (49%)	5 (19%)	27 (79%)	9 (45%)
Statins	29 (36%)	1 (4%)	26 (76%)	2 (10%)
Aspirin (anticlotting)	34 (42%)	2 (7%)	31 (92%)	1 (5%)
Anticoagulants	6 (7%)	-	6 (18%)	-
Long acting β2-agonist	11 (14%)	0 (0%)	0 (0%)	11 (55%)
Inhaled corticosteroids	9 (11%)	1(4%)	-	8(40%)
Hypoglycemic Agents	10 (12%)	-	7 (21%)	3 (15%)
NSAIDs	4 (5%)	-	1 (3%)	1 (5%)
Vitamins	6 (7%)	3 (11%)	0 (0%)	3 (15%)

Notes: [a] Alcohol assumption more than two glasses per day; [b] Physical activity from moderate to intense.

4.2 EXPOSURE DATA

The median (25°-75° percentiles) 24h concentration of PM_{10} during the no-heating period was 35.5 (29.3-51.1) µg/m^3 and during the heating period 58.0 (41.7-79.0) µg/m^3. For $PM_{2.5}$, the median (25°-75° percentiles) concentration during the no-heating period was 26.8 (21.4-37.7) µg/m^3 and during the heating period 49.8 (33.7-66.3) µg/m^3. The mean temperature was 25.3 (23.0-28.5)°C and the relative humidity was 37.4 (33.5-43.3) (Table 2).

Comparing the data from the two monitoring periods, the results showed a significant increase for particulate matter concentrations in the heating ignition power-on period (Wilcoxon test, $p<0.05$). The PM_{10} percentage variation was 63.4% and for $PM_{2.5}$ was 85.8 % (Table 2).

Table 2 Air particulate pollutants levels (median [25°-75°]) of the two 24h-monitoring set during both the period with heating ignition power-on and power-off for the whole sample of volunteers involved in the study. Percentage variation of medians level between the two set of 24-h-monitorings and relative difference significance are also reported (Wilcoxon test, * p<0.05).

Environmental parameters [24 h TWA (µg/m3)]	No heating Median		Heating Median		No H vs H	
	25°	75°	25°	75°	Δ%	Wilcoxon test
PM0,5	**19.74**		**38.70**		**96.5**	*
	15.37	28.12	24.53	52.41		
PM1	**22.72**		**43.72**		**92.5**	*
	17.81	31.78	28.67	58.72		
PM2,5	**26.79**		**49.81**		**85.8**	*
	21.38	37.67	33.65	66.25		
PM10	**35.54**		**58.04**		**63.4**	*
	29.27	50.98	41.73	79.02		
T °C	**28.5**		**23.1**		**- 20**	*
	26.9	30.3	27.5	23.1		
Relative Humidity	**42.5**		**34.3**		**- 20**	*
	37.9	45.0	31.6	37.3		

Environmental parameters [24h mean (#/cm3)]	No heating Median		Heating Median		No H vs H	
	25°	75°	25°	75°	Δ%	Wilcoxon test
UFP 0.02-1 µm	**16969**		**24958**		**32**	*
	13526	22082	17290	34025		
FP 0.3-0.5 µm	**73.100**		**172.900**		**58**	*
	41.100	101.100	102.100	249.300		
FP 0.5-1 µm	**4.700**		**15.400**		**69**	*
	3.100	9.000	9.300	25.100		
FP 1-2.5 µm	**0,388**		**0,764**		**49**	*
	0.238	0.550	0.563	1.099		
CP 2.5-5 µm	**0.177**		**0.233**		**24**	*
	0.131	0.275	0.163	0.369		
CP 5-10 µm	**0.015**		**0.018**		**17**	*
	0.010	0.093	0.092	0.138		

The three groups of subjects were exposed to similar PM concentration, except for fine particles ($PM_{0.5}$, $FP_{0.3-1}$ D.a), that were higher in the *Healthy group* (Table 3).

Table 3 Air particulate pollutants levels (median [25°-75°]) in total population, in each studied group and differences among groups (RA: ANOVA for repeated mesurements, * p<0.05)

Environmental parameters	Total Population		Healthy	Heart	Lung
Heating+ No Heating	Median 25°-75°	RA	Median 25°-75°	Median 25°-75°	Median 25°-75°
PM 0.5	27.1 17.8-42.4	*	31.3 19.8-50.3	28.1 18.1-37.1	20.6 15.3-41.7
PM 1	31.1 20.5-46.7		35.5 23.0-53.3	31.4 20.1-40.3	23.5 17.8-46.8
PM 2.5	35.2 24.6-50.7		40.0 27.2-57.9	35.7 25.2-46.1	29.0 20.7-50.4
PM 10	45.7 32.5-63.0		50.3 35.4-69.7	48.3 34.8-59.8	38.5 27.5-60.1
UFP 0.02-1	19443 14520-30328		19441 15269-28912	19545 13898-30739	19084 14139-30515
FP 0.3-0.5	101.2 58.0-184.8	*	149.2 74.9-223.5	100.8 67.8–156.9	63.8 42.3-175.3
FP 0.5-1.0	9.1 4.2-17.3	*	13.2 5.6 – 20.3	8.9 4.7 -12.8	5.1 3.0- 19.3
FP 1.0-2.5	0.552 0.335-0.871		0.526 0.357-0.915	0.595 0.382- 0.875	0.444 0.263-0.808
CP 2.5-5.0	0.203 0.147-0.326		0.220 0.155- 0.305	0.205 0.142- 0.351	0.192 0.138- 0.294
CP 5.0-10	0.017 0.009-0.118		0.019 0.012- 0.130	0.014 0.009- 0.103	0.015 0.008- 0.121
Temperature	25.3 23.0-28.5		25.3 23.1-28.5	25.2 22.7-28.9	25.4 23.1-28.3
Relative Humidity	37.4 33.5-43.3	*	39.7 34.1-44.2	38.2 34.3-43.7	34.6 31.6-40.4

4.3 ASSOCIATION BETWEEN CLINICAL AND EXPOSURE DATA

Descriptive data for the haematological parameters are shown in Table 4, Table 5 and Table 6 (the last in the Appendix). All the parameters of complete blood cells count, coagulatory parameters and C reactive protein were in the normal range value given by the laboratory where the analysis were done. Reliable reference value are generally lacking for cytokines.

The subjects in the three groups provided different values of total leukocytes count, inflammatory parameters and coagulation parameters (Table 4, Table5). *Healthy group* showed lower values of leucocytes, neutrophils, monocytes and eosinophils than those in *Heart* and *Lung groups*. As regard to inflammatory parameters, *Healthy group* displayed lower values of sRII-TNF-alfa than *Heart group*, and *Lung group* showed lower values of IL-10 (after stimulation with PHA) than those in *Heart* and *Healthy groups*. Concerning coagulation parameters, *Heart group* presented longer closure time (PFA-100 C-EPI CT) than *Lung* and *Healthy groups*, and *Healthy group* showed lower values of d-dimer than those in other two groups.

A preliminary analysis of heating and no-heating period results showed a spread distribution inflammatory parameters during the heating period (Table 6, Appendix). A comparison between heating and no-heating data showed significantly higher heating values in the three separated groups. The *Heart group* showed increased values of erythrocytes, haematocrit, leukocytes, monocytes, sRII-TNF-alfa, TNF-alfa and IL-8 (both after stimulation with LPS), d-dimer, while the *Lung group* showed IL-8 increased. The *Healthy group* showed increased values of erythrocytes, platelets, sRII-TNF-alfa, IL-8 (after stimulation with LPS), d-dimer and a shorter closure time (PFA-100 C-EPI CT).

Table 4 Descriptive statistic and differences between groups of hematological parameters in total population, Healthy, Heart and Lung group. (results expressed as median, 25°-75° percentiles; RA: ANOVA for repeated measurements, * p<0.05)

	Total sample	RA	"Healthy" group	Heart group	Lung group
Subjects [n]	**81**		**27**	**34**	**20**
	Median 25°-75°		**Median 25°-75°**	**Median 25°-75°**	**Median 25°-75°**
Eritrocytes [10^6/μL]	4.7 4.41-5.06		4.66 4.33-5.12	4.6 4.38-4.90	4.83 4.54-5.07
Platelets [10^3/μL]	225 187-268	*	246 212-278	209 174-229	253 188-295
Haemoglobin [g/dL]	14.3 13.27-15.30		14.25 13.20-15.42	14.35 13.3-15.2	14.3 13.2-15.13
Haematocrit [%]	42.55 39.68-45.30		42.15 39.43-45.73	42.3 39.58-44.92	43.5 40.28-45.25
Leukocytes [10^3/μL]	6.41 5.51-7.28	*	5.77 5.03-6.91	6.56 5.85-7.37	6.54 5.93-8.19
Neutrophils [10^3/μL]	3.55 2.91-4.40	*	3.01 2.61-3.86	3.80 3.17-4.52	3.83 3.02-4.76
Lymphocytes [10^3/μL]	1.99 1.62-2.36		1.99 1.65-2.27	1.93 1.48-2.45	2.04 1.70-2.41
Monocytes [10^3/μL]	0.46 0.38-0.58	*	0.40 0.35-0.50	0.52 0.40-0.60	0.49 0.41-0.63
Eosinophils [10^3/μL]	0.16 0.10-0.28	*	0.12 0.09-0.18	0.15 0.07-0.28	0.23 0.16-0.31
Basophils [10^3/μL]	0.03 0.02-0.03		0.02 0.02-0.03	0.03 0.02-0.04	0.03 0.02-0.04
sRI-TNFα [pg/mL]	402 308-557		422 317-554	440 311-593	356 296-483
sRIITNFα [pg/mL]	916 400-1993	*	428 360-1648	1457 405-2058	854 403-2013
TNFα [pg/mL][a]	689 351.25-1132.25		580 320-895	778 368-1270	724 395-894
IL8 [pg/mL][a]	7113 4350-14355		6800 4363-10963	6680 4057-14400	9200 4500-18870
IL10 [pg/mL][b]	25.0 7.0-59.0	*	24.0 12.0-60.0	36.0 7.0-91.5	8.0 0.5-33.5
Hs PCR [mg/L]	0.69 0.39-1.50		0.59 0.55-1.37	0.44 0.51-1.28	1.34 0.53-1.77

[a] in vitro following stimulation with LPS; [b] in vitro following stimulation with PHA

Table 5 Descriptive statistic and differences between groups of hematological parameters in total population, Healthy, Heart and Lung group. (results expressed as median, 25°-75° percentiles; RA: randomized one way ANOVA, * p<0.05)

	Total sample	RA	"Healthy" group	Heart group	Lung group
Subjects [n]	**81**		**27**	**34**	**20**
	Median		**Median**	**Median**	**Median**
	25°-75°		**25°-75°**	**25°-75°**	**25°-75°**
D-dimer [ng/mL]	169	*	131	224	166
	111-299		80-203	136-393	123-239
PFA-100 C-EPI CT [s]	135	*	121	278	120
	111-263		111-149	130-301	105-152
PFA-100 C-ADP CT [s]	83		87	82	84
	73-97		77-96	69-100	73-98
vWF [%]	119		112	132	114
	85-159		82-142	94-197	79-138
F1+ 2 [pmol/L]	137		138	134	140
	111-171		113-161	109-172	111-194
tPA [ng/mL]	6		6	6	6
	4-8		4-8	5-9	4-8
Fibrinogen [mg/dL]	404	*	394	398	469
	357-481		342-448	359-492	393-515
INR	1.10	*	1.00	1.10	1.10
	1.00-1.10		0.98-1.10	1.00-1.11	1.00-1.10
aPTT [s]	30.05		29.40	30.90	30.70
	28.00-32.58		27.37-31.98	28.00-33.10	28.90-32.70

Association between hematological parameters and PM

Total blood cells count

The results of the mixed models showed significant changes in monocytes number in the *Heart group* associated with $PM_{0,5}$, PM_1, $PM_{2,5}$, PM_{10} (Table 7 Appendix). The monocytes increased of 7.90 % (p=0.06) in association with PM_{10} (**Figure 9**).

Variations in lymphocytes number in *Healthy group* were associated with a large number of PM fraction ($PM_{0,5}$, PM_1, $PM_{2,5}$, PM_{10}, UFP, $FP_{0,3-1}$) (Table 8, Appendix). The lymphocytes increased of 8,71 % (p=0,01) in association with PM_{10} (Figure 11).

Figure 9. Percentage changes in monocytes number for an interquartile range change of PM10

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, statins, FANS, temperature and relative humidity.

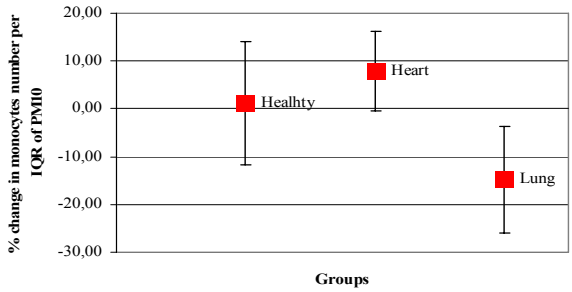
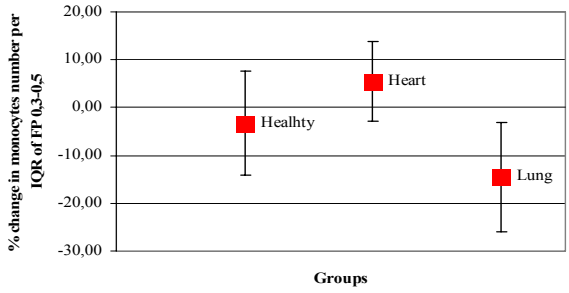


Figure 10 Percentage changes in monocytes number for an interquartile range change of $FP_{0.3-0.5}$

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, statins, FANS, temperature and relative humidity.



In the Lung group there is a negative association with monocytes and lymphocytes with $PM_{0,5}$, PM_1, $PM_{2,5}$, PM_{10}, $FP_{0,3-2,5}$ and $FP_{0,3-2,5}$ respectively (Table 7, Table 8 Appendix). The number of monocytes decreased of 14.73% (p=0,01) in association with PM_{10} (Figure 9); and decreased of 14.45% (p=0.02) in association with $FP_{0.3-0.5}$ (Figure 10). The lymphocytes decreased of 11.48 % (p=0.04) in association with $FP_{0.3-0.5}$ (Figure 12).

Figure 11. Percentage changes in lymphocytes number for an interquartile range change of PM_{10}

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, statins, FANS, temperature and relative humidity.

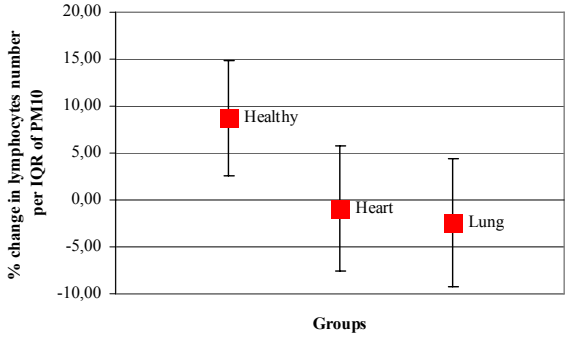
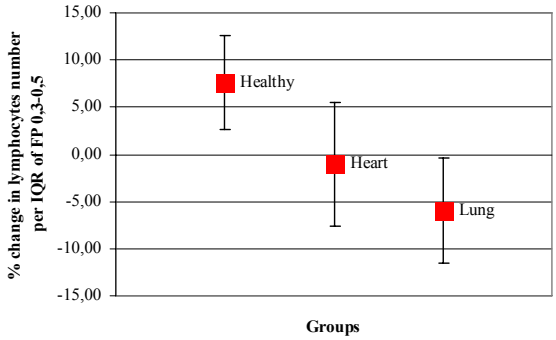


Figure 12 Percentage changes in lymphocytes number for an interquartile range change of $FP_{0.3-0.5}$

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, statins, FANS, temperature and relative humidity.



Erythrocytes number increased in *Heart group* of 2.14 % (p=0,02) in association with $FP_{0.3-0.5}$ (Figure 13) and of 1,9 % (p=0.01) in association with CP_{5-10} (Figure 14) (Table 9, Appendix).

Figure 13 Percentage changes in erythrocytes number for an interquartile range change of $FP_{0.3-0.5}$

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, FANS, temperature and relative humidity.

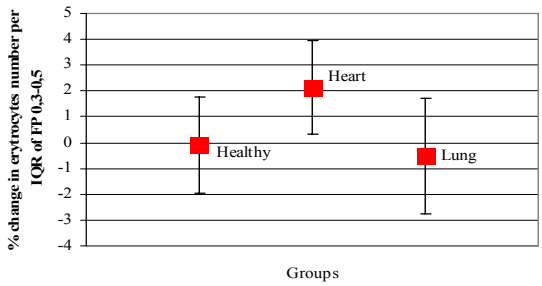
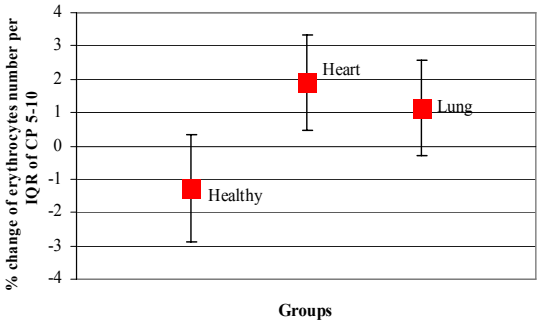


Figure 14 Percentage changes in erythrocytes number for an interquartile range change of CP_{5-10}

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, FANS, temperature and relative humidity.



Platelets number increased in *Heart group* of 6.77 % (p=0.08) in association with $PM_{0.5}$ (Figure 15) and of 5.19 % (p=0.08) with $CP_{5\text{-}10}$ (Figure 16), although the relation is slight significant (Table 10, Appendix).

Figure 15 Percentage changes in platelets number for an interquartile range change $PM_{0.5}$.

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anticlotting therapy, temperature and relative humidity.

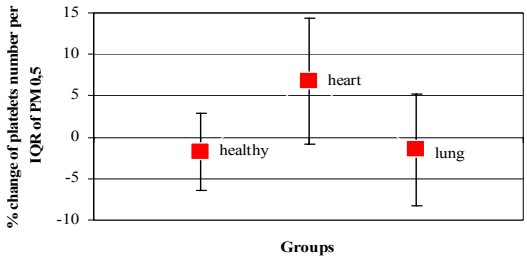
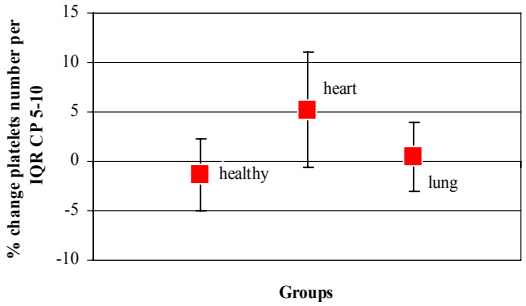


Figure 16 Percentage changes in platelets number for an interquartile range change $CP_{5\text{-}10}$.

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anticlotting therapy, temperature and relative humidity.



No statistical associations were found for other parameters.

Inflammatory parameters

No statistical associations were found for cytokines, interleukins and C reactive protein.

Coagulatory parameters

Among the coagulatory parameters there were negative associations between closure time (estimated with PFA-100 C-EPI) and $PM_{0,5}$, PM_1, $PM_{2,5}$, $FP_{0,3\text{-}1}$, $CP_{2.5\text{-}10}$ in Healthy group, and with $PM_{0,5}$, PM_1, $PM_{2,5}$, PM_{10}, $FP_{0,3\text{-}2.5}$, $CP_{2.5\text{-}5}$ in Heart group (Table 11, Appendix). The

closure time was shortened of 10 % (p=0.06) in association with PM_1 and of 6.81 % (p=0.05) with $FP_{0,5-1}$ in *Healthy group* (Figure 17, Figure 18). In *Heart group* closure time was shortened of 17.84 % (p=0.02) in association with PM_1 and of 14.98 % (p=0.004) with $FP_{0,5-1}$ (Figure 17, Figure 18).

No changes were present in the *Lung Group*.

Figure 17 Percentage changes in closure time for an interquartile range change of PM_1

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity.

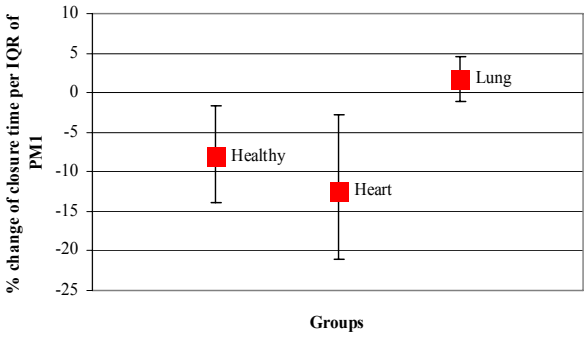
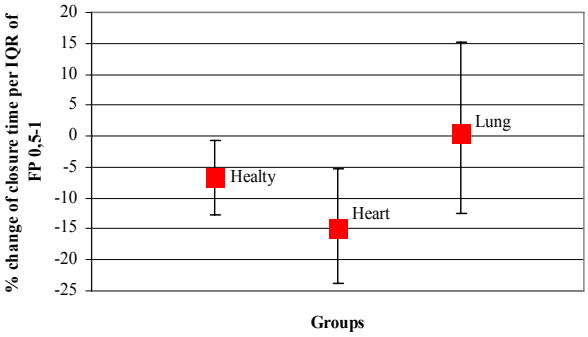


Figure 18 Percentage changes in closure time for an interquartile range change of $FP_{0.5-1}$

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity.



In Healthy group the tissue type plasminogen activator is positive associated with $PM_{0,5}$, PM_1, $PM_{2,5}$, PM_{10}, $UFP_{0.02-1}$, $FP_{0,3-0,5}$ $FP_{0,5-1}$ (Table 12, Appendix). The tPA varied of 22.47% (p=0.07) in association with PM_1 and of 22.38% (p=0.05) with $FP_{0,3-0,5}$ (Figure 19, Figure 20).

Figure 19 Percentage changes in tPA an interquartile range change of PM_1.

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity.

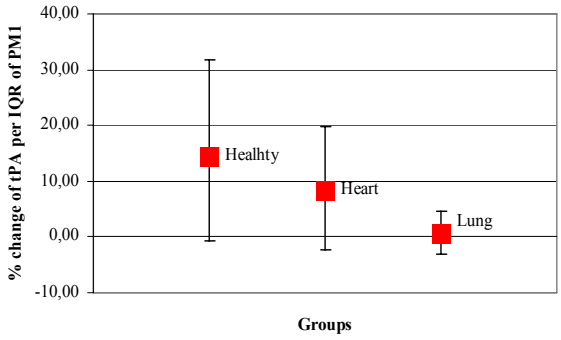
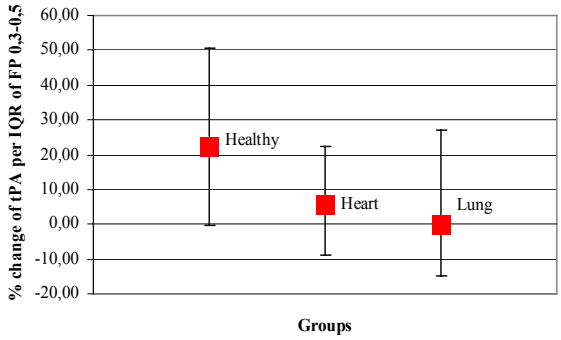


Figure 20 Percentage changes in tPA an interquartile range change of $FP_{0,3-0,5}$

The values are presented as percentage changes and 95% confidence intervals for interquartile range changes after adjusting for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity.



No statistical associations were found for other parameters.

5 DISCUSSION

Investigated subjects experienced high levels of individual exposure to $PM_{2.5}$ and PM_{10}. In particular, exposure to $PM_{2.5}$ exceeded the 24-hour mean of 25 $\mu g/m^3$ suggested by WHO in both the investigated periods, i.e. warm and cold season, while the limit suggested for PM_{10} (i.e 50 $\mu g/m^3$) was exceeded only in cold season (WHO, 2005). Observed PM_{10} concentrations were similar to twenty four hours average reported for urban background in Europe (Larssen S. et al. 2005), and $PM_{2.5}$ concentrations were similar to the previously reported for personal monitoring indoor and outdoor in Milan (Rotko T. et al. 2002). Moreover, UFPs levels could be considered in the high range, given that number concentration of particles smaller than 100 nm in urban background environments can range from a few thousand to some 20.000 particles per cm^3 (WHO 2005; Morawska, L et al. 2003). As expected, higher levels of exposure to almost all particles were observed in the cold season. The great contribution of these higher winter levels is mainly due to particles in the accumulation mode ($FP_{0.3\text{-}1}$ µm). In cold season the PM levels are strongly affected not only by the stability of the atmosphere and a low degree of air convection (characteristics of the warm season), but also by the heating which is one of the major sources of the particles in the accumulation mode. The contribution of the higher PM cold season levels of the coarse particles was relatively low because their sources are linked to mechanical or natural processes, quite independent from the seasonal phenomena.

The different levels of exposure among the three groups were mainly caused by fine particles in the accumulation mode ($FP_{0.3\text{-}1}$ µm) and the *Healthy group* seemed to be the higher exposed. An analysis of the activity of these subjects showed that they have spent more time outdoor than the subjects of other groups, so they were much exposed to the particles from outdoor origin, the particles in the accumulation mode, confirming the outdoor origin of these particles. No differences were recorded for ultrafine particles that in fact have indoor sources predominantly.

A comparative analysis of the biological data among the three groups shows that the *Healthy group* presented strongly different values from the other groups, as expected. The *Healthy* subjects' data showed a narrow distribution respect to those of the other subjects and the median values of the majority of the parameters were generally lower, confirming a great status of health of these subjects compared with the others. The *Heart group* showed an

impairment of the coagulation parameters in comparison to the others , probably due to the characteristics of the diseases and the assumed anti-clotting therapy. The *Lung group* seemed to have an impairment of the activation of the antinflammatory markers with a persistent low grade condition of inflammation, characterised by higher levels of total leukocytes count, an increased level of fibrinogen and a reduced release of an anti-inflammatory interleukin, the IL-10.

The increase of monocytes, erythrocytes and platelets number in *Heart group* and lymphocytes number in *Healthy group* in association with fine and coarse particles could suggest an increased bone marrow activity, involving a variety of cell types, as a result of the effects of cytokines and chemokines from the lung that spill over into the circulation and trigger a cascade of inflammatory reactions signals generated in the lung. The leukocytosis associated with the activation of bone marrow activity was demonstrated in human exposed to PM, that reacted mobilizing leucocytes into circulation as a part of systemic exposure (Tan WC. et al. 2000; Sakai M. et al. 2004).

On the other hand, subjects in *Lung group* show very little differences between heating and no-heating inflammatory results probably due to a low grade systemic inflammation persistent both in no-heating and heating period, which is likely because of the characteristics of the pathologies of the subjects belonging this group. Moreover they were exposed to small excursion of PM levels that may result in a not appreciable changes in blood measures of inflammation and coagulation. We observed a negative association between PM and monocytes and lymphocytes number that could be explain by the presence of allergic subjects in this group; the summer PM in Milan is more rich in pollens, endotoxins and biological materials (Camatini M. et al. 2010) so we could hypothesize a pro-allergic effects of no-heating PM exposure, an increase of the inflammatory pattern during the no-heating period and a slow decrease during the heating period.

The activation of the platelets aggregation capacity, measured as closure time with PF 100 Analyzer®, in *Heart* and *Healthy groups* in association with fine particles could suggest that particles with little aerodynamic diameter could pass directly from the alveoli to the blood and interact with the platelets, impairing their aggregability. Another possible mechanism of platelets activation might reside in the pulmonary oxidative stress and the activation of subsets of white blood cells that lead to a systemic lowering of endothelial- and platelet-derived

nitrogen oxide and concomitant platelets activation (Brook RD et al 2008). Moreover, although fibrin formation were not confirmed by elevated d-dimer and protrombin fragments levels, the increment of the tissue type plasminogen activator in the *Healthy group* in association with fine particles could suggest an increased thrombin generation and a reduced fibrinolytic activity; this because the immunoassay of t-PA measures, to a large extend, the circulating complex between t-PA and the main fibrinolysis inhibitor plasminogen activator inhibitor-1 (Nordenhem A. et al. 1998).

The lack of consistencies in the association with PM and cytokines and interleukins could be explain by their very short half life (2-6 hours). We have measured these factor after about 12 hours of the higher PM exposure levels, missing probably the concentration peaks in the blood. While the lack of consistencies in the association with PM and fibrinogen and C reactive protein could be due to the time necessary for the *ex-novo* synthesis of these proteins in the liver, that requires an induction time of 1-2 days (Seaton A. et al. 1999, Ruckerl R. et al. 2006).

The strength of this study is that it is one of the few using individual monitoring of both gravimetric and number concentration of particles with different aerodynamic diameters (from ultrafine to coarse particles). The use of fixed site monitoring stations could not be representative of personal exposure resulting in imprecise associations (Delfino RJ. et al. 2008), therefore the individual monitoring is the only way to measure the real exposure in particular of our subjects, that were already retired and have spent the greater part of the day within their home.

A limitation of our study is that we enrolled a small number of subjects with different pathologies and drug therapies, that had a large impact on the biological parameter. Moreover we monitored these subjects only twice, resulting in few data for each subjects.

Despite of these limitation, this work supports the hypothesis that exposure to PM results in a systemic inflammatory response, characterized by stimulation of bone marrow activity, that could increase the blood coagulability. It could also support the hypothesis that small particles may translocate form the lung into circulation and directly activate platelets and blood vessels. Together these mechanisms may account for the increase of cardiovascular events associated with episodes of air pollution.

6 CONCLUSION

The results suggest that PM exposure could contribute to the risk of cardiovascular events, in particular in elderly and subjects with cardiovascular diseases. The cardiovascular diseases representing 29% of the total deaths in 2004, and of these deaths, an estimated 7.2 million were due to coronary heart disease and 5.7 million were due to stroke (WHO, Cardiovascular disease fact sheet, 2009). Since there are evidences linking PM air pollution exposure and cardiovascular mortality and morbidity, may we consider PM as a risk factor for cardiovascular diseases or not? Particulate matter exposure is ubiquitous, it may continuously enhance acute cardiovascular risk among susceptible people worldwide; moreover it may further elicits numerous adverse biological responses that could augment cardiovascular risk over the long term. Therefore, PM could surely considered as a factor that modify and contribute to cardiovascular mortality and morbidity (Brook RD et al. 2010).

Despite the huge amount of studies about health effects of PM exposure, some issues remain open:

- to define the role of particles with different aerodynamic diameters and their chemical composition;
- to characterize the contribution of other co-pollutants (ozone, nitrogen dioxide, sulphur dioxide);
- to assess the importance of regional and intracity differences in composition and combination of pollutants;
- To better define the susceptible subjects and define recommendations to help to reduce PM exposure;
- To define whether there is a safe PM threshold concentration that eliminates both acute and chronic effects in susceptible subjects but also in general population.

7 BIBLIOGRAFY

Akaike H. (1974) A new look at the statistical model identification. IEEE Transactions on Automatic Control;19:716–723.

Albert C.M et al. (2002) Prospective study of C-reactive protein, homocysteine, and plasma lipid levels as predictors of sudden cardiac death. Circulation; 105: 2595−2599.

Alfaro-Moreno E. et al. (2007) Particulate matter in the environment: pulmonary and cardiovascular effects. Curr Opin Pulm Med;13:98-106.

ARPA Lombaria, Inemar, Inventario delle emissioni in Lombardia nel 2007- dati per revisione pubblica (http://ita.arpalombardia.it/ITA/inemar/regione.htm).

Becker S. et al. (2005) TLR-2 is involved in the air way and epithelial cell response to air pollution particles. Toxicol Appl Pharmacol;203:45-52.

Bousquet, J.(2000) Global initiative for asthma (GINA) and its objectives. Clin Exp Allergy; 30 Suppl 1: p. 2-5.

Brook RD. et al. (2004) Air pollution and cardiovascular disease; a statement for healthcare professionals from the expert panel on population and prevention science of the American Heart Association. Circulation; 109:2655-2671.

Brook RD. et al. (2010) Particulate matter air pollution and cardiovascular disease; an update to the scientific statement from the American Heart Association. Circulation;121:2331-2378.

Camatini M et al. 2010 . PM10-biogenic fraction drives the seasonal variation of proinflammatory response in A549 cells. Environ Toxicol. 2010 May 26. [Epub ahead of print].

Carlsten C. et al. (2007) Coagulation markers in healthy human subjects exposed to diesel exhaust. Thromb Res;120:849-855.

Chen LH et al. (2005) The association between fatal coronary heart disease and ambient particulate air pollution: are female at greater risk? Environ health Perspect;113:1723-1729.

Chuang K. et al. (2007) The effects of urban air pollution on inflammation, oxidative stress, coagulation and autonomic dysfunction in young adults. Am J Respir Crit Care Med; 176:370-376.

Churg, A.; Brauer, M. (1997): Human lung parenchyma retains PM2.5. Am. J. Respir. Crit. Care Med. 155:2109–2111.

Delfino RJ. et al. 2008. Circulating biomarkers of inflammation, antioxidant activity, and platelets activation are associated with primary combustion aerosol in subjects with coronary artery disease. Environ Health Perspect; 116:898-906.

Dockery DW et al. (1993) An association between air pollution and mortality in six U.S. cities. N Engl J Med;329:1753-1759.

Dolmetsch RE. et al. (1998): Calcium oscillation increase the efficiency and specificity of gene expression. Nature;392:933-6.

Dominici F. et al. (2003): Mortality among residents of 90 cities. In: Special Report: Revised Analyses of Time-Series Studies of Air Pollution and Health. Boston, Mass: Health Effects Institute;9–24.

EPA (August, 2004): Fourth external review draft of air quality criteria for particulate matter. Revised Chapter 9. Taken from http://cfpub2.epa.gov/ncea/cfm/recordisplay.cfm?deid=85763.

EPA (June, 2003): Fourth external review draft of air quality criteria for particulate matter. Volume 1 Taken from http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=58003.

Flecha B. (2004): Oxidant mechanisms in response to ambient particles. Molecular aspects of medicine;25:169-82.

Fujii T et al. (2002) Interaction of alveolar macrophages and airway epithelial cells following exposure to particulate matter produces mediators that stimulate the bone marrow. Am J Respir Cell Mol Biol.;27:34-41.

Gehring U et al. (2006) Long-term exposure to ambient air pollution and cardiopulmonary mortality in women. Epidemiology;17:545-551.

Gilmour PS. et al. (2003): Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. Am J Physiol Lung Cell Mol Physiol;284:L533-40.

Halonen J. et al. (2008). Urban air pollution, ad asthma and COPD hospital emergency room visit. Thorax; 63:635-641.

Hollingsworth JW et al. (2004) The role of Tool-like receptor 4 in environmental airway injury in mice. Am J Respir Crit Care Med 2004;170:126-132.

Jimenez LA. et al. (2000): Activation of NF-kappaB by PM10 occurs via an iron-mediated mechanism in the absence of IkappaB degradation. Toxicol Appl Pharmacol;166:101-10.

Katsouyanni K. et al.(2001): Confounding and effect modification in the short-term effects of ambient particles on total mortality: results from 29 European cities within the APHEA2 Project. Epidemiology;12:521–531.

Kundu Sk et al. (1995) Description of an in vitro platelet function analyzer—PFA-100. Semin Thromb Hemost;21(Suppl 2):106–112.

Laden F. et al. (2006) Reduction in fine particulate air pollution and mortality: extended follow-up of the Harvard Six Cities Study. Am J Respir Crit Care Med;173:667-672.

Larssen, S., State of air quality in Europe 1990–2002. , in European environmental outlook 2005: background document air quality 1990−2030, E.H.e. al., Editor. 2005, European Topic Centre on Air and Climate Change: Bilthoven.

Li XY. et al. (1996): Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. Thorax;51:1216-22.

Lindmark E. et al. (2001): Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. JAMA;286:2107–2113.

Lippmann M. (1994): Particle deposition and accumulation in human lungs. In: Dungworth D.L. et al. Toxic and carcinogenic effects of solid particles in the respiratory tract., Washington DC, ILSI Press; 291-306 (ILSI Monographs).

MacNee W., Donaldson K. (2003): Mechanism of lung injury caused by PM10 and ultrafine particles with special reference to COPD. Eur Respir J;21:Suppl.40,47s-51s.

Mammen EF et al. (1995) Preliminary data from a field trial of the PFA-100 system. Semin Thromb Hemost;21(Suppl 2):113–121.

Mazzera DM et al. (2001)Sources of PM10 and sulphate aerosol at McMurdo Station, Antarctica. Chemosphere;45:347-356.

Miller KA et al. (2007) Long-term exposure to air pollution and incidence of cardiovascular events in women. N Engl J Med.;356:447-458.

Mills N. et al. (2005) Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation;112:3930-3936.

Mills N. et al. (2009) Adverse cardiovascular effects of air pollution. Nature clinical practice;6(1):36-44.

Morawska, L. et al (2003) Desktop literature review and analysis of health impacts of ultrafine particles, Australian Department of Environment and Heritage: Canberra.

Møller P et al. (2010) Role of oxidative damage in toxicity of particulates. Free Radc Res ;44:1-46.

Naess Ø et al. (2007) Relation between concentration of air pollution and cause specific mortality: four-years exposure to nitrogen dioxide and particulate matter pollutants in 470 neighbourhoods in Oslo, Norway. Am J Epidemiol;165:435-443.

Nordenhem A. et al. (1998) Tissue palsminogen activator (tPA) anitigen in plasma: correlation with different tPA/inhibitor complexes. Scand J Clin Lab Invest; 58:457-483.

O'Neill MS. et al. (2005). Diabetes enhances vulnerability to particulate air pollution-associated impairment in vascular reactivity and endothelial function. Circulation; 111:2913–2290.

Oberdoster G. et al. (2005) Principles for characterizing the potential human effects from exposure to nanomaterials: elements of a screening strategy. Particle and Fibre Toxicology ;2:8.

Ostro BD et al. (2008) The impact of components of fine particulate matter on cardiovascular mortality in susceptible subpopulations. Occup Environ Med;65:750-756.

Pauwels, R.A. et al. (2001) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. Am J Respir Crit Care Med; 163(5):1256-76.

Pauwels, R.A. et al. (2001) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. Respir Care;46(8):798-825.

Peters A. et al. (1997): Increased plasma viscosity during an air pollution episode: a link to mortality? Lancet; 349(9065):1582-7.

Peters A. et al. (2001): Particulate air pollution is associated with an acute phase response in men. Results from the MONICA-Augsburg Study. European Heart Journal; 22:1198-1204.

Pinkerton K. et al. (2000) Distribution of particulate matter and tissue remodeling in the human lung. Environ. Health Perspect.; 108:1063–1069.

Pope CA et al. (1995) Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. Am J Respir Crit Care Med;151:669-674.

Pope CA. et al. (2000) Epidemiology of fine particulate air pollution and human health: biologic mechanisms and who's at risk? Environ health Perspect; 108(Suppl):713-723.

Pope CA et al. (2002) Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. JAMA; 287:1132-1141.

Pope C.A. et al. (2004) Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. Circulation;109:71-77.

Pope C.A. et al. (2004b): Ambient Particulate Air Pollution, Heart Rate Variability, and Blood Markers of Inflammation in a Panel of Elderly Subjects Environ Health Perspect 112:339–345.

Puett RC et al. (2000) Chronic particulate exposure, mortality and coronary heart disease in the nurses' health study. Am J Epidemiol.;168:1161-1168.

Ridker PM. et al. (2000): Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation.;101:1767–1772.

Riediker M. et al. (2004): Particulate matter exposure in cars is associated with cardiovascular effects in healthy young men. Am J Respir Crit Care Med.; Vol 169. pp 934–940.

Rotko, T. et al. (2002) Determinants of perceived air pollution annoyance and association between annoyance scores and air pollution (PM2.5, NO2) concentrations in the European EXPOLIS study Atmospheric Environment; 36(29):4593-4602.

Ruckerl R. et al. (2006) Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. Am J Respir Crit Care Med;173:432-441.

Rudex G. et al. (2009) Effects of ambient air pollution on hemostasis and inflammation. Environ Health Perspect;117:995-1001.

Sakai M. et al. (2004) Effects of relocationg to areas of reduced atmospheric particulate matter levels on the human circulating leukocytes count. J Appl Physiol;97:1774-1780.

Samoli E et al. (2008) Acute effects of ambient particulate matter on mortality in Europe and North America: results from the APHENA study. Environ Health Perspect.;116:1480-1486.

Samet JM et al. (2000): Fine particulate air pollution and mortality in 20 U.S. cities, 1987–1994. N Engl J Med;343:1742–1749.

Schwartz J. (1994): What are people dying of on air pollution days? Environmental Reseach;64:26-35.

Schwartz J. et al. (1996) Is daily mortality associated specifically with fine particles? Journal of the Air and Waste Management Association; 46:927-939.

Seaton A. et al. (1999): Particulate air pollution and the blood. Thorax; 54:1027–1032.

Sen CK. et al. (1996): Involvement of intracellular Ca++ in oxidant-induced NF-kB activation. FEBS Lett; 385:58-62.

Squadrito GL. et al. (2001): Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled airborne particulate matter. Free Radical Biology & Medicine; 31(9): 1132–1138.

Stafoggia M. et al. (2009) Inquinamento atmosferico e mortalità in dieci città italiane. Risultati del progetto EpiAir. Epidemiol Prev ;33(6) suppl1: 65-67.

Standards of medical care in diabetes-2007. Diabetes Care, 2007. 30 Suppl 1: p. S4-S41.

Sullivan JH. et al. (2005). Association between short term exposure to fine particulate matter and heart rate variability in older subjects with and without heart disease. Thorax; 60:462–466.

Tan WC et al. (2000) The human bone marrow response to acute air pollution caused by forest fire. Am J Respir Crit Care Med;161:1213-1217.

Van Eeden SF. et al. (2001): Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM10). Am J Respir Crit Care Med.;164:826–830.

Volpato S. et al. (2001): Cardiovascular disease, interleukin-6, and risk of mortality in older women: the Women's Health and Aging Study. Circulation;103:947–953.

WHO (2003): Health aspects of air pollution with particulate matter, ozone and nitrogen dioxide. Report on a WHO Working Group. Bonn, Germany 13-15 January, 2003. WHO 2003 EUR/03/5042688.

WHO (2004): Health aspects Of air pollution Results from The who project "systematic review Of health aspects Of air pollution In Europe" June 2004 Http://www.euro.who.int/document/E83080.pdf

Willerson JT et al. (2002) Systemic and local inflammation in patients with unstable artherosclerotic plaques. Prog Cardiovasc Dis; 44:469-478.

Wong CM et al. (2008a) The effects of air pollution on mortality in socially deprived urban areas of Hong Kong, China . Environ Health Perspect;116:1189-1194.

Wong CM et al. (2008b) Public health and Air Pollution in Asia (PAPA): a multicity study of short-term effects of air pollution on mortality. Environ Health Perspect; 116:1195-1202.

World Health Organization. World Health Report 2002. Geneva, Switzerland: World Health Organization; 2002.

WHO Air Quality Guidelines (AQG) for Europe. 2005, WHO - Regional Office for Europe.

WHO, 2009. Cardiovascular diseases fact sheet n. 317. http://www.who.int/mediacentre/factsheets/fs317/en/index.html

Yeh ET. et al. (2001) C-reactive protein: linking inflammation to cardiovascular complications. Circulation;104:974-975.

Zanobetti A. et al. (2000) Are there sensitive subgroups for the effects of airborne particles? Environ Health Perspect; 108:841-5.

Zanobetti A. et al. (2003) The temporal pattern of respiratory and heart disease mortality in response to air pollution. Environ Health Perspect;111:1188–1193.

Zeka A. et al. (2006) Individual-level modifiers of the effects of particulate matter on daily mortality. Am J Epidemiol;163:849-859.

8 APPENDIX

Table 6 Differences of blood parameters during the heating and no-heating period in the three groups and differences among groups. **Data shown as median 25th-75th; * for p<0.05; [a] in vitro after stimulation with LPS; [b] in vitro after stimulation with PHA**

	Differences between no-heating and heating period									Dif. among groups	
	"Healthy" Group		Test	Heart Group		Test	Lung Group		Test	No-heating	heating
	No-heating	Heating		No-heating	Heating		No-heating	Heating			
Subjects [n]	**27**	**27**		**34**	**34**		**20**	**20**			
Eritrocytes [10^6/µL]	4.58 4.29-5.07	4.77 4.43-5.17	*	4.58 4.29-4.88	4.63 4.44-5.00	*	4.86 4.54-5.06	4.80 4.54-5.06	*		
Platelets [10^3/µL]	242 212-270	249 218-290	*	197 169-226	211 175-229		244 198-290	260 187-309		*	*
Haemoglobin [g/dL]	14.20 13.10-15.40	14.30 13.20-15.50		14.40 13.15-15.05	14.25 13.30-15.32	*	14.15 13.20-15.12	14.30 13.18-15.20			
Haematocrit [%]	41.80 39.10-45.20	42.50 39.60-46.10		41.80 39.20-44.50	43.00 40.40-45.90	*	43.60 40.90-44.50	43.35 39.43-45.65			
Leukocytes [10^3/µL]	5.71 5.06-6.94	5.80 4.97-6.90		6.52 5.80-7.21	6.59 5.97-7.49	*	6.92 5.94-8.41	6.41 5.73-7.77		*	*
Neutrophils [10^3/µL]	2.98 2.57-4.09	3.01 2.66-3.84		3.88 3.12-4.49	3.76 3.34-4.87		3.84 3.12-4.75	3.67 2.95-4.90		*	*
Lymphocytes [103/µL]	1.86 1.53-2.27	2.04 1.76-2.29		1.93 1.48-2.42	1.92 1.47-2.51		2.12 1.72-2.39	1.98 1.67-2.42			
Monocytes [10^3/µL]	0.37 0.33-0.51	0.42 0.38-0.50		0.49 0.39-0.56	0.53 0.44-0.69	*	0.47 0.42-0.62	0.53 0.39-0.66		*	*
Eosinophils [10^3/µL]	0.11 0.09-0.17	0.14 0.09-0.20		0.15 0.07-0.22	0.15 0.08-0.29	*	0.23 0.17-0.39	0.20 0.14-0.29	*	*	
Basophils [10^3/µL]	0.02 0.02-0.03	0.02 0.01-0.03		0.02 0.02-0.03	0.03 0.02-0.04		0.03 0.03-0.48	0.03 0.02-0.03			

sRI-TNFα [pg/mL]	552 498-614	332 297-412	*	515 300-732	394 312-514		455 328-577	322 265-367	*		*
sRIITNFα [pg/mL]	361 333-428	1295 411-1789	*	458 376-2095	1598.5 1211-2053	*	439 340-2140	1117 412-1970		*	
TNFα [pg/mL][a]	565 282-755	670 330-1468		516 225-894	1135 641-2346	*	724 430-856	725 369-1793			
IL8 [pg/mL][a]	4760 3670-8180	9387.5 6645-18675	*	4850 3187-8845	8817.5 5494-24593	*	5400 2080-10700	13325 8563-21263	*		
IL10 [pg/mL][b]	24.0 8.0-67.0	28.5 13.8-58.0		20.5 0.5-60.0	42.0 24.0-119.5		0.5 0.5-38.0	15.0 1.1-33.8			*
D-dimer [ng/mL]	126 75-170	146 90-215	*	176 98-286	330 155-534	*	164 117-192	179 128-285			*
PFA-100 C-EPI CT [s]	127 111-163	114 104-130	*	301 170-301	173 118-301	*	117.5 106-143	123.5 99-161		*	*
PFA-100 C-ADP CT [s]	91 80-104	81 72-93	*	83 72-108	77 68-98		82 70-97	84 75-98			
vWF [%]	112 89-146	112 81-141		141 92-208	122 95-193	*	118 80-141	106.5 76-134			
F1+ 2 [pmol/L]	150 127-200	118 108-141	*	150 119-193	113 96-158	*	140 120-193	139 106-220			
tPA [ng/mL]	6 3-9	4 4-7		6 5-9	7 4-9		6 4-9	5 4-8			
Fibrinogen [mg/dL]	393 342-461	395 342-443		408 372-544	389 357-481	*	480 413-543	442 373-504			
INR	1.0 0.9-1.0	1.1 1.0-1.1	*	1.1 1.0-1.1	1.1 1.1-1.2	*	1.0 1.0-1.1	1.1 1.0-1.2	*	*	
aPTT [s]	29.60 27.20-32.20	29.30 27.20-31.10		31.10 28.40-33.70	30.65 27.75-32.52		30.70 28.60-32.70	30.50 28.92-32.72			

Table 7 Changes in monocytes number per an interquartile range increase in 24 hours average PM exposure (model adjusted for: age, gender, BMI, statins, FANS, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	0,61	-11,63	12,86	0,92	**7,90**	-0,96	16,76	**0,08**	**-17,29**	-29,92	-4,67	**0,01**
PM1	-0,33	-12,11	11,45	0,95	**7,85**	-0,71	16,41	**0,07**	**-14,94**	-25,69	-4,20	**0,01**
PM2, 5	0,33	-11,09	11,75	0,95	**7,55**	-0,57	15,66	**0,07**	**-14,17**	-24,40	-3,94	**0,01**
PM 10	1,11	-11,78	13,99	0,86	**7,90**	-0,47	16,28	**0,06**	**-14,73**	-25,85	-3,62	**0,01**
UFP 0,02-1	-2,34	-15,55	10,86	0,72	1,94	-6,55	10,43	0,65	1,01	-20,20	22,23	0,92
FP 0,3-0,5	-3,32	-14,17	7,52	0,54	5,42	-2,85	13,69	0,19	**-14,45**	-25,84	-3,05	**0,02**
FP 0,5-1	-2,94	-9,75	3,87	0,38	5,03	-1,36	11,42	0,12	**-15,09**	-24,13	-6,06	**0,00**
FP 1-2,5	0,33	-10,67	11,33	0,95	3,29	-3,94	10,53	0,36	**-18,06**	-25,19	-10,94	**0,00**
CP 2,5-5	-0,84	-13,66	11,98	0,89	2,60	-4,66	9,86	0,48	-4,58	-15,65	6,48	0,40
CP 5-10	-3,47	-12,76	5,82	0,45	2,88	-4,04	9,80	0,41	4,79	-4,47	14,05	0,29

Table 8 Changes in lymphocytes number per an interquartile range increase in 24 hours average PM exposure (model adjusted for: age, gender, BMI, statins, FANS, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	**9,02**	3.58	14.46	**0,00**	-2,48	-9.41	4.44	0,47	-5,06	-11.90	1.78	0,14
PM1	**9,08**	3.92	14.23	**0,00**	-2,09	-8.86	4.68	0,54	-1,25	-8.01	5.51	0,70
PM2, 5	**8,55**	3.47	13.64	**0,00**	-1,51	-7.95	4.93	0,64	-1,77	-8.15	4.62	0,57
PM 10	**8,71**	2.63	14.80	**0,01**	-0,96	-7.64	5.72	0,77	-2,44	-9.25	4.37	0,46
UFP 0,02-1	**5,68**	-0.67	12.03	**0,08**	0,95	-9.41	4.44	0,76	2,54	-11.90	1.78	0,61
FP 0,3-0,5	**5.68**	2.62	12.66	**0,00**	0.95	-7.57	5.57	0,76	**-2.54**	-11.48	-0.36	**0,04**
FP 0,5-1	**7.64**	1.69	7.85	**0,00**	-1.00	-5.52	5.07	0,93	-5.92	-8.61	1.50	0,16
FP 1-2,5	4.77	-3.59	7.78	0,46	-0.22	-8.14	2.39	0,27	**-3.56**	-9.91	-0.90	**0,02**
CP 2,5-5	2.10	-12.13	1.05	0,10	-2.87	-8.82	2.55	0,27	-5.40	-8.05	1.72	0,19
CP 5-10	-5.54	-8.38	1.56	0,17	-3.13	-4.44	6.38	0,72	-3.16	-3.13	5.56	0,56

Table 9 Changes in erythrocytes number per an interquartile range increase in 24 hours average PM exposure (model adjusted for: age, gender, BMI, FANS, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	-0.09	-2.19	2.00	0.93	1.42	-0.65	3.49	0.17	-0.89	-3.42	1.65	0.48
PM1	0.16	-1.85	2.17	0.87	1.31	-0.71	3.33	0.20	-1.54	-3.49	0.42	0.12
PM2, 5	0.25	-1.70	2.19	0.80	1.15	-0.76	3.07	0.23	-1.30	-3.17	0.58	0.17
PM 10	0.33	-1.90	2.57	0.76	0.85	-1.17	2.87	0.40	-1.14	-3.17	0.88	0.25
UFP 0,02-1	-1.26	-3.49	0.96	0.25	0.47	-1.50	2.43	0.63	1.35	-2.50	5.20	0.47
FP 0,3-0,5	-0.09	-1.96	1.77	0.92	**2.14**	0.31	3.97	**0.02**	-0.51	-2.74	1.73	0.64
FP 0,5-1	0.21	-0.92	1.35	0.70	1.02	-0.50	2.55	0.18	-0.78	-2.72	1.16	0.41
FP 1-2,5	1.45	-0.31	3.22	0.10	-0.54	-2.21	1.12	0.51	**-1.59**	-3.34	0.16	**0.07**
CP 2,5-5	-1.34	-3.52	0.84	0.22	-0.61	-2.39	1.16	0.49	0.74	-0.97	2.46	0.37
CP 5-10	-1.28	-2.89	0.34	0.12	**1.90**	0.48	3.31	**0.01**	1.15	-0.28	2.59	0.11

Table 10 Changes in platelets number per an interquartile range increase in 24 hours average PM exposure (model adjusted for: age, gender, BMI, anti-clotting therapy, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	-1.80	-6.47	2.87	0.43	**6.77**	-0.84	14.38	**0.08**	-1.50	-8.23	5.22	0.65
PM1	-1.42	-5.90	3.06	0.52	**6.25**	-1.18	13.69	**0.10**	-2.06	-7.55	3.42	0.44
PM2, 5	-1.52	-5.87	2.83	0.48	5.51	-1.59	12.61	0.12	-1.62	-6.80	3.56	0.52
PM 10	-2.38	-7.36	2.60	0.34	5.29	-2.07	12.65	0.15	-1.28	-6.71	4.16	0.63
UFP 0,02-1	-1.93	-6.86	2.99	0.43	5.19	-1.83	12.20	0.14	**-8.01**	-16.72	0.69	**0.07**
FP 0,3-0,5	-0.40	-4.58	3.79	0.85	5.61	-1.52	12.75	0.12	0.12	-5.64	5.87	0.97
FP 0,5-1	0.52	-2.01	3.06	0.67	2.60	-3.19	8.39	0.37	0.13	-4.87	5.13	0.96
FP 1-2,5	0.36	-3.63	4.34	0.86	0.11	-6.10	6.33	0.97	0.88	-3.86	5.62	0.70
CP 2,5-5	-3.36	-8.26	1.54	0.17	2.01	-4.36	8.37	0.53	2.29	-1.52	6.09	0.22
CP 5-10	-1.36	-5.01	2.28	0.45	**5.19**	-0.63	11.01	**0.08**	0.47	-3.01	3.95	0.78

Table 11 Changes closure time PFA 100 C-EPI per an interquartile range increase in 24 hours average PM exposure (model adjusted for: for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	**-9.37**	-19.17	1.61	**0.09**	**-17.85**	-30.29	-3.20	**0.02**	-1.10	-15.81	16.19	0.89
PM1	**-10.04**	-19.30	0.27	**0.06**	**-17.84**	-29.84	-3.79	**0.02**	2.33	-10.78	17.36	0.73
PM2, 5	**-9.45**	-18.51	0.61	**0.06**	**-16.69**	-28.33	-3.18	**0.02**	2.18	-10.12	16.18	0.73
PM 10	-6.40	-17.05	5.61	0.27	**-16.76**	-28.80	-2.68	**0.02**	2.77	-10.11	17.49	0.68
UFP 0,02-1	-2.86	-14.76	10.70	0.66	-6.34	-19.73	9.29	0.40	15.09	-7.13	42.63	0.19
FP 0,3-0,5	**-9.63**	-18.24	-0.11	**0.05**	**-18.90**	-29.83	-6.25	**0.01**	-2.56	-16.83	14.17	0.74
FP 0,5-1	**-6.81**	-12.61	-0.62	**0.03**	**-14.98**	-23.70	-5.27	**0.00**	0.44	-12.45	15.23	0.95
FP 1-2,5	-6.01	-15.39	4.40	0.24	**-18.94**	-28.87	-7.62	**0.00**	-5.53	-17.31	7.93	0.39
CP 2,5-5	**-14.71**	-29.10	-1.92	**0.02**	**-12.15**	-22.62	-0.25	**0.05**	-1.14	-12.10	11.19	0.84
CP 5-10	**-11.26**	-21.19	-2.14	**0.02**	-5.86	-17.27	7.12	0.35	1.85	-8.31	13.12	0.72

Table 12 Changes closure time tPA per an interquartile range increase in 24 hours average PM exposure (model adjusted for: for sex, age, body mass index, anti-clotting therapy, temperature and relative humidity)

	Healthy (n.27)				Heart (n.34)				Lung (n.21)			
	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value	% change estimated regresison coefficient	95% C.I.		p-Value
PM 0, 5	**22.59**	-3,20	55,25	**0.09**	7.32	-10,60	28,84	0.44	-0.76	-20,13	23,30	0.94
PM1	**22.47**	-2,12	53,24	**0.07**	5.79	-10,03	24,38	0.49	0.50	-17,21	21,99	0.96
PM2, 5	**20.85**	-2,71	50,11	**0.08**	5.31	-9,70	22,83	0.50	1.13	-15,71	21,33	0.90
PM 10	**23.29**	-3,37	57,31	**0.09**	6.26	-9,58	24,87	0.45	2.48	-15,36	24,07	0.80
UFP 0,02-1	**24.42**	-3,19	59,91	**0.09**	3.59	-11,58	21,37	0.66	-5.42	-30,42	28,56	0.71
FP 0,3-0,5	**22.38**	-0,45	50,45	**0.05**	5.74	-8,77	22,56	0.45	3.96	-14,92	27,02	0.69
FP 0,5-1	**12.83**	-1,06	28,66	**0.07**	9.08	-1,76	21,12	0.10	5.34	-11,40	25,25	0.54
FP 1-2,5	5.28	-15,07	30,49	0.63	6.53	-8,38	23,85	0.40	-11.19	-24,65	4,68	0.15
CP 2,5-5	-16.83	-35,54	7,30	0.15	-2.11	-14,15	11,63	0.75	6.58	-8,21	23,75	0.38
CP 5-10	-11.96	-27,06	6,26	0.18	-1.58	-13,00	11,35	0.80	**16.00**	3,43	30,09	**0.01**

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