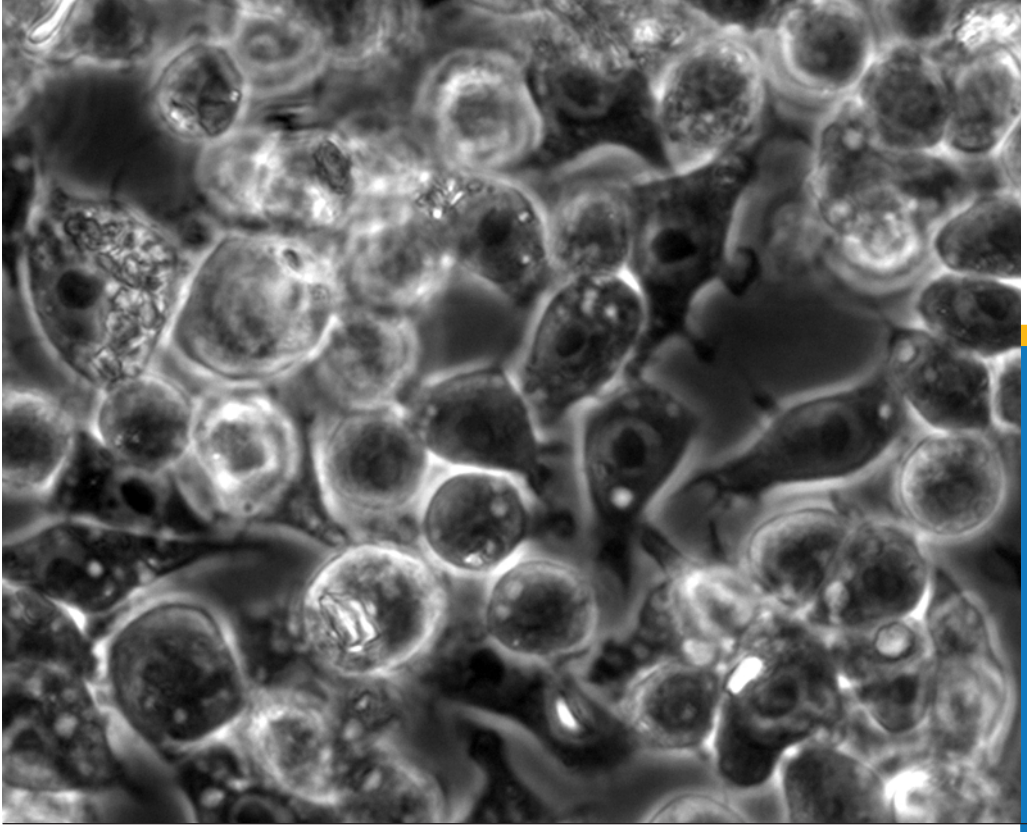

Pasi Jalava

Toxicological Characterization of Size-Segregated Urban Air Particulate Matter in Macrophage Cell Line

Effects of Chemical Composition and Sources

Publications of the National Public Health Institute A 30/2008

Department of Environmental Health
National Public Health Institute, Kuopio, Finland
and
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University of Kuopio, Finland

Helsinki, Finland 2008

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TOXICOLOGICAL CHARACTERIZATION OF SIZE-SEGREGATED URBAN AIR PARTICULATE MATTER IN MACROPHAGE CELL LINE

EFFECTS OF CHEMICAL COMPOSITION AND SOURCES

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Natural and Environmental Sciences , University of Kuopio, for public examination in auditorium, Tieto Teknia Building, on December 5th 2008, at 12 o'clock noon..

Department of Environmental Health,
National Public Health Institute, Kuopio, Finland
and
Department of Environmental Science, University of Kuopio, Finland

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Hiukkasnäytteellä altistettuja RAW 264.7 makrofageja (63 x suurennos)
RAW 264.7 macrophages, exposed to particulate sample (63x magnification)
Pasi Jalava/KTL

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Helsinki 2008

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ABSTRACT

Urban air particulate pollution is currently regarded as the most harmful environmental exposure causing premature mortality in Europe. Thoracic particles (PM_{10}; diameter <10 μm) and fine particles ($PM_{2.5}$; diameter <2.5 μm) have been consistently associated with excess mortality and morbidity among susceptible population groups e.g. individuals with chronic respiratory or cardiovascular disease. In addition to size and lung dose, the chemical composition of inhaled particles has been hypothesized as being an important determinant of the adverse health outcomes. Inflammation has been regarded as the most important biological mechanism mediating the health effects of urban air particles in subjects with cardio-respiratory diseases. The main objective of the present thesis was to improve understanding of the immunotoxic properties of urban air particles and their association with potentially harmful sources and chemical compositions.

A series of sampling campaigns were conducted in six European cities during contrasting air pollution situations in different seasons. Particulate samples for toxicological studies were collected in 7-week sampling campaigns using a high volume cascade impactor (HVCI) that consisted of four consecutive stages for coarse ($PM_{10-2.5}$), intermediate ($PM_{2.5-1}$), accumulation ($PM_{1-0.2}$) and ultrafine ($PM_{0.2}$) size-range particles. The size-segregated particulate mass, collected on polyurethane foam strips and backup filter, was extracted with methanol and pooled together according to size range and respective sampling location. In most studies, the mass in two of the size ranges, $PM_{2.5-1}$ and $PM_{1-0.2}$ was pooled together to form one fine particulate ($PM_{2.5-0.2}$) sample per campaign. Extensive inorganic and organic chemical analyses were made from the pooled HVCI samples. These were complemented with analysis results from samples collected with parallel low-volume samplers. This permitted the use of the chemical mass closure method in the characterization of the gravimetrically measured, size-segregated particulate mass of the HVCI samples used in toxicological studies. These results and well-established chemical tracers were utilized in the identification of potentially harmful particulate sources.

Inflammatory properties of the HVCI particulate samples were investigated in an immortalized mouse macrophage cell line (RAW264.7). They were assessed by measuring the production of proinflammatory cytokines (IL-6, TNFα) and chemokine (MIP-2) by macrophages exposed to the particulate samples. Nitric oxide production was also measured. Cell viability, apoptosis and the stage of the cell cycle of the macrophages were analyzed as indicators of cytotoxicity.

Toxicity profiles of the samples collected during different air pollution situations in Helsinki varied extensively. The overall toxicity of the $PM_{1-0.2}$ mass per cubic meter of air during transnational forest fire smoke episodes was estimated as being higher than the seasonal average in springtime.

The particulate samples in $PM_{10-2.5}$ size range were the most potent inducers of inflammation and cytotoxicity. However, the air pollution situation strongly affected the particle-induced responses in six European cities. There was more heterogeneity in the toxic responses in association with the $PM_{2.5-0.2}$ than the $PM_{10-2.5}$ samples. In both size ranges, the responses were mainly due to the insoluble fraction of the particulate samples with only minor effects by the water-soluble or organic solvent soluble fractions. The $PM_{0.2}$ samples did not substantially increase cytokine production, but some samples exhibited cytotoxic and apoptotic activity. This suggests that the solubility and the chemical composition of the particulate material affect the toxic potency and that the material in different particulate size ranges can activate distinct biological mechanisms.

There were a larger number of statistically significant positive or negative correlations between the chemical constituents, and the inflammatory and cytotoxic activity of the HVCI samples in the fine particulate than the coarse particulate size-range. The inflammatory activity of the $PM_{2.5-0.2}$ samples had high positive correlations with tracers of photo-oxidation of the organic material in the atmosphere (dicarboxylic acids), some transition metals (Ni, V, Fe, Cu, Cr), as well as soluble (Ca^{2+}) and insoluble (Ca, Al, Fe, Si) soil constituents. In contrast, markers of incomplete biomass combustion (monosaccharide anhydrides) and coal combustion (As), and the PAH-compounds displayed high negative correlations with the inflammatory parameters, but not with cytotoxicity. The chemical compositions of $PM_{10-2.5}$ samples were more uniform than those of the $PM_{2.5-0.2}$ samples. Possibly due to this reason, there were only

occasional high correlations between the chemical constituents, endotoxins, and the toxicological response parameters.

The present studies strengthen the concept that local emission sources, climate and season affect the toxicity of urban air particles via the chemical composition. The most important particulate sources for toxicity were incomplete biomass and coal combustion, oil combustion, resuspended road dust and the long-range transported forest fire smoke.

Keywords: Air pollution, particulate matter, macrophage, inflammation, cytotoxicity, cell cycle, particulate sources, particle size, particulate solubility, chemical composition, chemical mass closure

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TIIVISTELMÄ

Kaupunki-ilman hiukkaset lisäävät merkittävästi ennenaikaista kuolleisuutta ja niitä pidetäänkin nykyisin kaikkein haitallisimpana ympäristöaltisteena Euroopassa. Kaupunki-ilman hengitettävien hiukkasten (PM_{10}; läpimitta <10µm) ja pienhiukkasten ($PM_{2.5}$; läpimitta <2.5 µm) pitoisuudet ovat olleet yhteydessä herkkien väestöryhmien, kuten sydän- ja hengityssairaiden, lisääntyneeseen kuolleisuuteen ja sairastavuuteen. Koon ja keuhkoannoksen lisäksi hiukkasten kemiallisten ominaisuuksien on arveltu olevan tärkeä tekijä terveyshaittojen synnyssä. Tulehdusta pidetään tärkeimpänä hengitys- ja sydänsairauksien pahenemisen biologisena mekanismina. Väitöstutkimuksen päätavoite oli lisätä tietoa kaupunki-ilman hiukkasten immunotoksisista ominaisuuksista ja niiden yhteyksistä päästölähteisiin ja hiukkasten kemialliseen koostumukseen.

Hiukkaset kerättiin eri vuodenaikoina ja erilaisista ilmanlaatutilanteista kuudessa eurooppalaisessa kaupungissa. Hiukkasnäytteet toksikologisiin tutkimuksiin kerättiin 7 viikon keräysjaksojen aikana suurtehokeräimellä (HVCI) neljässä kokoluokassa: karkeat hiukkaset ($PM_{10-2.5}$), välikokoiset hiukkaset ($PM_{2.5-1}$), kertymähiukkaset ($PM_{1-0.2}$) ja ultrapienet hiukkaset ($PM_{0.2}$). Kokoluokiteltu hiukkasmassa kerättiin polyuretaanivaahtoliuskoille ja pohjasuodattimille, joista se uutettiin metanolilla. Jokaisen kaupungin näytteet yhdistettiin kokoluokittain. Useimmissa osatutkimuksissa $PM_{2.5-1}$ ja $PM_{1-0.2}$ kokoluokkien hiukkasmassat yhdistettiin yhdeksi mittauskampanjakohtaiseksi pienhiukkasnäytteeksi ($PM_{2.5-0.2}$). Yhdistetyistä HVCI-hiukkasnäytteistä tehtiin laaja epäorgaaninen ja orgaaninen kemiallinen analyysi, jota täydennettiin analyyseillä samaan aikaan pientehokeräimillä kerätyistä hiukkasnäytteistä. Tämän ansiosta kemiallisen massasulkeuman menetelmää voitiin käyttää eri yhdisteiden tunnistamisessa hiukkasnäytteistä, joita käytettiin toksikologisissa tutkimuksissa. Näitä tuloksia ja tunnettuja kemiallisia merkkiaineita käytettiin todennäköisesti haitallisten päästölähteiden tunnistamisessa.

Tässä tutkimuksessa selvitettiin HVCI-hiukkasnäytteiden tulehdusvaikutuksia hiiren makrofagisolulinjassa (RAW 264.7). Tutkimuksessa mitattiin altistettujen solujen tuottamia proinflammatorisia sytokiineja (TNFα, IL-6) sekä kemokiinia (MIP-2). Myös solujen tuottama typpioksidi mitattiin soluviljelynesteestä. Solujen elävyys, apoptoosi ja solusykli mitattiin solutoksisuuden osoittajina.

Helsingistä keväällä kerättyjen hiukkasnäytteiden toksiset ominaisuudet poikkesivat toisistaan erilaisissa ilmanlaatutilanteissa. Metsäpalosavujen kaukokulkeuman aikana ilmakuutiossa olleen $PM_{1\text{-}0.2}$ hiukkasmassan toksisuus arvioitiin suuremmaksi kuin samana vuodenaikana keskimäärin.

Kaikissa mittauskampanjoissa $PM_{10\text{-}2.5}$ hiukkaset tuottivat voimakkaimmat tulehdus- ja solutoksisuusvasteet. Kuudesta eurooppalaisesta kaupungista kerättyjen HVCI-näytteiden aiheuttamat soluvasteet vaihtelivat kuitenkin selvästi ilmanlaatutilanteen mukaan. $PM_{2.5\text{-}0.2}$ näytteiden tuottamissa soluvasteissa oli enemmän eroja keräyspaikkojen välillä kuin $PM_{10\text{-}2.5}$ näytteiden vasteissa. Molemmissa kokoluokissa HVCI-näytteiden vaikutukset makrofageihin aiheutuivat pääosin liukenemattomasta hiukkasjakeesta. Vedellä ja orgaanisella liuottimella liuotetut jakeet aiheuttivat vain pieniä, pääosin merkityksettömiä soluvasteita. $PM_{0.2}$-näytteet eivät merkittävästi lisänneet sytokiinien tuotantoa, mutta jotkut näistä näytteistä aiheuttivat merkittävää akuuttia solutoksisuutta sekä apoptoosia. Tulosten perusteella voidaan päätellä, että hiukkasmassan liukoisuus ja kemiallinen koostumus vaikuttavat toksisuuteen ja että eri kokoluokkien hiukkaset voivat aktivoida erilaisia biologisia mekanismeja.

Kemiallisilla tekijöillä oli pienhiukkaskokoluokassa selvästi useammin tilastollisesti merkitsevä korrelaatio HVCI-näytteiden tulehdus- ja solutoksisuuspotentiaalin kanssa kuin karkean kokoluokan hiukkasnäytteillä. $PM_{2.5\text{-}0.2}$ näytteiden tulehdusaktiivisuudella oli korkea positiivinen korrelaatio ilmakehän orgaanisten yhdisteiden foto-oksidaation merkkiaineiden (dikarboksyylihapot), joidenkin siirtymämetallien (Ni, V, Fe, Cu, Cr) sekä liukoisten (Ca^{2+}) ja liukenemattomien (Ca, Al, Fe, Si) maaperän aineiden kanssa. Sitä vastoin biomassan huonon polton merkkiaineilla (monosakkaridianhydridit), hiilenpolton merkkiaineella (As) ja PAH-yhdisteillä oli korkea negatiivinen korrelaatio $PM_{2.5\text{-}0.2}$-näytteiden tulehdusaktiivisuuden, mutta ei solutoksisuuden kanssa. $PM_{10\text{-}2.5}$ näytteiden kemiallinen koostumus vaihteli vähemmän keräyspaikkojen välillä kuin $PM_{2.5\text{-}0.2}$ näytteiden koostumus. Mahdollisesti tästä syystä karkeiden hiukkasten kemiallisen koostumuksen, mukaan lukien

endotoksiini, ja havaittujen toksisuusvasteiden välillä oli vain satunnaisia merkitseviä korrelaatioita.

Tämä väitöstutkimus vahvistaa käsitystä siitä, että paikalliset päästölähteet, ilmasto ja vuodenaika vaikuttavat kaupunki-ilman hiukkasten toksisiin ominaisuuksiin kemiallisen koostumuksen kautta. Tulehdus- ja solutoksisten vaikutusten kannalta tärkeimpiä hiukkaslähteitä olivat epätäydellinen biomassan ja hiilen poltto, öljynpoltto, liikenteen nostama katupöly sekä kaukokulkeutunut metsäpalosavu.

Asiasanat: Ilmansaasteet, hiukkaset, makrofagi, tulehdus, solutoksisuus, solusykli, hiukkasten lähteet, hiukkaskoko, hiukkasten liukoisuus, kemiallinen koostumus, kemiallinen massasulkeuma

CONTENTS

ABBREVIATIONS

EC	Elemental Carbon
ED-XRF	Energy Dispersive X-Ray Fluorescence
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GCMS-SIM	Gas-Chromatograph-Mass Spectrometer with Selected Ion Monitoring
HVCI	High Volume Cascade Impactor
IC	Ion Chromatograph
ICP/MS	Inductively-Coupled Plasma Mass Spectrometer
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-6	Interleukin 6
LAL	Limulus Amebocyte Lysate
LC/MS	Liquid Chromatograph Mass Spectrometer
LRT	Long Range Transport
MIP-2	Macrophage Inflammatory Protein 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NO	Nitric Oxide
OC	Organic Carbon
OE	Other Elements
PAH	Polycyclic Aromatic Hydrocarbon
PI	Propidium Iodide
PM_x	Particulate Matter with aerodynamic diameter less than x µm
PM_{x-y}	Particulate Matter with aerodynamic diameter between x and y µm
POM	Particulate Organic Matter
PUF	Polyurethane Foam
RAW 264.7	Abelson murine leukemia virus transformed mouse macrophage/monocyte cell line
SEM	Standard Error of the Mean
ΣMA	Sum of Monosaccharide Anhydrides (levoglucosan, galactosan, mannosan)
SS	Sea Salt
TNFα	Tumor Necrosis Factor α
TOA	Thermal-Optical Analyzer
TSP	Total Suspended Particulates
UM	Unidentified Matter
VI	Virtual Impactor
WIS	Water-Insoluble Soil
WSS	Water-Soluble Soil

LIST OF ORIGINAL PUBLICATIONS

I Jalava P, Salonen RO, Hälinen AI, Sillanpää M, Sandell E, Hirvonen M-R. 2005. Effects of sample preparation on chemistry, cytotoxicity and inflammatory responses induced by air particulate matter. INHALATION TOXICOLOGY 17:107-117.

II Jalava PI, Salonen RO, Hälinen AI, Penttinen P, Pennanen AS, Sillapää M, Sandell E, Hillamo R, Hirvonen M-R. 2006 *In-vitro* Inflammatory and cytotoxic effects of size-segregated particulate samples collected during long-range transport of wildfire smoke to Helsinki. TOXICOLOGY AND APPLIED PHARMACOLOGY 215: 341-353.

III Jalava PI, Salonen RO, Pennanen AS, Sillanpää M, Hälinen AI, Happo MS, Hillamo R, Brunekreef B, Katsouyanni K, Sunyer J, Hirvonen M-R. 2007. Heterogeneities in inflammatory and cytotoxic responses of RAW 264.7 macrophage cell line to urban air coarse, fine and ultrafine particles from six European sampling campaigns. INHALATION TOXICOLOGY 19 (3) 213-225.

IV Jalava PI, Salonen RO, Pennanen AS, Happo MS, Penttinen P, Hälinen AI, Sillanpää M, Hillamo R, Hirvonen M-R. 2008 Effects of solubility of urban air coarse and fine particulate samples on cytotoxic and inflammatory responses in RAW 264.7 macrophage cell line. TOXICOLOGY AND APPLIED PHARMACOLOGY 229: 146-160.

V Jalava PI, Hirvonen M-R, Sillanpää M, Pennanen AS, Happo MS, Hillamo R, Cassee, FR, Gerlofs-Nijland M, Borm PJA, Schins RPF, Janssen NAH, Salonen RO. Sources and chemical constituents responsible for the inflammatory and cytotoxic activities of urban air particles. Submitted.

1 INTRODUCTION

Particulate air pollution is one of the most important health concerns worldwide. It has been estimated that within European Union countries, particulate air pollution is responsible for up to 350 000 premature deaths annually and an average of 8 months' loss of statistical life expectancy, which is mainly due to life years lost by cardiorespiratory patients (up to 10 yrs) (EU/CAFE, Directive 2008/50).

In epidemiological studies, the focus has been on the effects associated with three size ranges: coarse ($PM_{10-2.5}$; particle diameter $10<D_p<2.5$ µm), fine ($PM_{2.5}$;, $D_p<2.5$ µm) and ultrafine particles ($D_p<0.1$ µm). The epidemiological data suggest that fine particulate material in most cases is more harmful to human health than coarse particles. However, there is recent evidence, which suggests that coarse particles can also significantly affect morbidity. Ultrafine particles have been studied to a lesser extent in epidemiology, but their health effects seem as detrimental as those of fine particles. It is noteworthy that epidemiological studies have rarely included particle collection for chemical and source analysis (WHO 2005).

Particulate air pollution can cause a large variety of health consequences, but clearly cardiac and respiratory morbidity and associated mortality are the most serious. The adverse health effects are accentuated in susceptible population groups, including subjects with chronic respiratory and cardiac diseases, children and the elderly. Furthermore, certain types of cancers are associated with particulate air pollution via genotoxicity, mutagenicity or secondary mechanisms related to inflammation and cytotoxicity. Air pollution can also be associated with impaired pregnancy outcomes and infant mortality. Inflammation and associated cytotoxicity are regarded as the main mechanisms in air pollution-related health effects in chronic respiratory and cardiovascular diseases. Several other mechanisms and organs can be involved in the particulate air pollution-associated health effects. These include loss of ciliary or surfactant functions in the lung, cell proliferation and angiogenesis, interstitial protein functions, nervous system damage etc.

In urban environments, the major local sources are traffic related air pollution, resuspension dusts and energy production. However, particulate air pollution is not simply a local problem; transboundary long-range transport is an important source of the particulate air pollution. There is increasing prevalence of air pollution episodes caused by wildfires and agricultural fires. In addition to these anthropogenic sources, also biological material (pollens, bacteria, fungi and spores) is an important source of the particulate material present in the atmosphere (Jones and Harrison 2004).

In experimental studies, coarse particles have most often dominated the responses. The discrepancy between epidemiological and experimental data may be due to different lung dosimetry and toxicokinetics of the particulate matter in experimental studies and in the real-life situation. There are many proposed harmful constituents which have been evaluated in toxicological studies, e.g. are transition metals (Ghio and Devlin 2001), soil components (Becher et al. 2001, Hetland et al. 2000), endotoxins (Schins et al. 2004; Monn and Becker 1999), organic compounds - including polycyclic aromatic hydrocarbons (PAH) (Binkova et al. 2003) - and secondary sulfates (Duvall et al. 2008).

It has been suggested that particulate chemical composition is an even more important modifier of the health outcomes than particle size. There are only few experimental studies that have utilized extensive particle characterization, including both inorganic and organic chemistry. The present set of studies aims to add knowledge of the potentially harmful chemical composition and emission sources of urban particulate air pollution.

The present studies are part of a wide systematic approach to evaluate the immunotoxicological responses and to supplement this with an extensive chemical characterization of the same or parallel particulate samples (Sillanpää et al 2005 and 2006, Pennanen et al. 2007, Saarikoski et al. 2008, Saarnio et al. 2008). The inflammatory parameters that were measured included nitric oxide (NO), tumor necrosis factor alpha (TNFa), interleukin 6 (IL-6), and macrophage inflammatory protein 2 (MIP-2). Cytotoxicity was measured via the MTT-test and flow cytometric methods. This set of studies is also part of the PAMCHAR-project that was conducted in six European cities to provide an in-depth toxicological and chemical characterization of urban air particles. The sampling sites were chosen to represent contrasting source environments and seasons of public health interest. The results of this thesis provide an overview of the potentially harmful particulate sources, size ranges and chemical compositions in the European context.

The current epidemiological data suggest that there is no unambiguous threshold value for urban air particulate matter concentrations below which no adverse health effects occur. Due to insufficient scientific data on the relative harmfulness of the chemical components and sources, current legislative regulation of particulate air pollution throughout the world is mainly based on the mass concentrations of PM_{10} and its subfraction $PM_{2.5}$. There are large gaps in our knowledge and major uncertainties with regard to the causal relationships of the sources, and especially the chemical compositions which pose serious health effects. Toxicological studies can contribute to understanding these associations at the level of tissue or cellular mechanisms. This information is needed in the risk characterization and risk assessment of the complex mixture of urban particulate air pollution.

2 REVIEW OF THE LITERATURE

2.1. Atmospheric aerosol particles

Atmospheric aerosol is formed by a complex mixture of solid and liquid components, from a wide variety of sources. Particulate size distribution in urban environments consists of four modes: nucleation mode (particle diameter D_p <0.01µm), Aitken mode (D_p<0.1) accumulation mode (0.1µm < D_p < 1µm) and coarse mode (D_p>1 µm) (Finlayson-Pitts and Pitts 2000). The main particulate sources and components of the size ranges are described in table 1.

The properties of aerosol particles have been reviewed by several authors (Seinfeld and Pandis 1998, Finlayson-Pitts and Pitts 1986, Sillanpää 2006, Salonen and Pennanen 2007). The smallest <0.1µm particles originate mostly from primary combustion sources e.g. small-scale combustion and diesel engine exhaust, but also from gas-to-particle conversion. These particles have a very limited lifespan in the atmosphere since they grow very rapidly due to coagulation with each other and with larger particles as well as condensation of water on their surfaces. Water-solubility of ultrafine particles varies with vicinity and type of specific source.

Most of the ultrafine particles gradually become enlarged in these processes in the accumulation mode. In addition, accumulation particles consist of soot from combustion and secondary organic or -inorganic particles and also from products of photochemical reactions. Accumulation particles can be transported in the atmosphere even thousands of kilometers, consequently most of the long range transported aerosol consists of this size range.

The fine particle size range contains the accumulation particles and in addition the lower end of the coarse size range. Coarse thoracic particles consist mostly of mechanically generated and windblown dusts, sea spray and biogenic material e.g. pollens and microbial components. Most of these particles are of mineral origin and therefore insoluble, they are also mostly dry deposited to ground by gravitation and they have a limited lifespan in the atmosphere.

Table 1. The main sources and compositions associated with three particulate size ranges of health interest (Sillanpää 2006; Salonen and Pennanen 2007).

	Ultrafine	Accumulation+ Fine	Coarse
Sources	Combustion Engine exhaust Nucleation Gas-to-particle conversion Natural emissions from vegetation	Combustion Biomass burning Industry Energy production Agglomeration and hygroscopic growth of ultrafine particles Photochemical transformation Secondary aerosols Resuspension	Mechanically generated dust Sea salt Resuspension Windblown dust Biogenic material
Main components	EC OC SO_4^{2-} Trace metals	SO_4^{2-} NH_4^+ NO_3^- EC OC Trace metals	Si Al Ca Fe Na Cl NO_3^- OC

EC = elemental carbon, OC = Organic carbon

OC composition is mainly hydrocarbons in ultrafine and ac+fine particles and biological material in coarse particles

Most of the particles worldwide are of natural origin. However, in urban environments, anthropogenic sources usually dominate the particulate mass. The main sources for the particulate air pollution in the urban environments are secondary organic and inorganic material from regional and long range transport, other local sources; traffic, other combustion sources, resuspended road dusts and long range transport.

2.2. Epidemiological background

Public health interest and regulations are usually based on three size ranges that do not necessarily follow the modal pattern of the urban particulate pollution: ultrafine, fine and coarse thoracic particles. There is increasing evidence about the particulate composition related effects, which now is supplementing findings based on traditionally studied mass concentrations.

2.2.1. Heterogeneities in particulate mass concentration-related health effects

The particulate mass concentration in air is not the only explanatory factor for the observed adverse health effects. Instead, in epidemiological studies, there have been source, season and geographical location-related heterogeneities associated with the adverse effects. These include regional differences in the particulate exposure-response relationships with the short-term mortality (Samoli et al., 2005) and hospital admissions (Atkinson et al., 2001) in Europe and in the United States (Dominici et al. 2006; Peng et al. 2005). A site in close proximity to some local sources has also induced a stronger than average concentration-response relationship. These sources include domestic heating with wood (Boman et al. 2003; Naeher et al. 2007) and coal (Clancy et al. 2002), fuel oil combustion (Tsai et al. 2000) and heavy traffic (Laden et al., 2000; Hoek et al. 2002; Janssen et al. 2003; Lanki et al., 2006), and poorly controlled metal industry (Ghio 2004).

2.2.2. Health effects related to particle size

There is consistent epidemiological evidence that the current levels of outdoor air PM_{10}, and in particular $PM_{2.5}$, are associated with increased respiratory and cardiovascular mortality and morbidity in urban areas worldwide (WHO 2003; USEPA 2004). There is also evidence in some cases that coarse particles can cause an even larger increase in hospital admissions than fine particles (Brunekreef and Forsberg 2005). Nonetheless, there is much poorer exposure characterization available for coarse and ultrafine particles than for fine particles. It seems likely central site monitoring being a much better proxy for true personal exposure to fine particles compared to the situation for coarse and ultrafine particles. This may be one reason for the stronger exposure-response relationships in association with fine particules in epidemiological studies. Furthermore, the role of urban air ultrafine particles should not be underestimated, since there is increasing epidemiological data that they can cause adverse effects to human health. There are indications that in subjects with cardiac diseases, ultrafine particulate pollution can have a greater impact on the heart than fine or coarse particles (Chuang et al. 2005). Ultrafine particles from combustion sources and traffic seem to play an important role in these adverse cardiovascular health effects (Delfino et al. 2005). These particles can penetrate into cells and can pass through lung epithelium to interstitium and the circulation more readily than larger particles (Geiser et al. 2005, Nemmar et al. 2006, Semmler-Behnke et al. 2007). Moreover, ultrafine particles are transported via non-active processes, such as diffusion whereas larger particles are taken up by phagocytosis (Geiser et

al. 2005). It is clear that the particle deposition in the lung is important; coarse thoracic particles are deposited in the nasal epithelium or upper airways, whereas the fine and ultrafine particles can penetrate as far as the distal airways and the alveoli.

2.2.3. Health effects related to particle sources and composition

There is still very limited knowledge of the specific sources of particulate air pollution that might be harmful to human health. However, epidemiological data from studies conducted in traffic and industrial sites where it is possible to estimate specific source contributions to provide insight to the potentially harmful sources.

After the ban of coal use in domestic heating in Dublin, Ireland there was a major reduction in the numbers of cardiovascular and respiratory daily deaths (Clancy et al. 2002). Combustion sources, especially coal combustion and traffic were associated with increased mortality in the Harvard six cities study (Laden et al. 2000). Furthermore in the follow up of the same study, the mortality rates declined when the $PM_{2.5}$ levels in the air decreased (Laden et al. 2006). Combustion sources and traffic have also been associated with an exacerbation of ischemic heart disease (Lanki et al. 2006), asthma (Penttinen et al. 2006) and stroke (Kettunen et al. 2007).

In the study of Tsai et al. (2000), oil burning, vehicular traffic, industry and sulphate aerosols were associated to daily mortality in New Jersey. It was also shown in Spokane, Washington that biomass burning contributed to respiratory hospitalizations (Schreuder et al. 2006). Residential heating with wood has also been associated with adverse health effects (Boman et al. 2003; Naeher et al. 2007). In the Netherlands, cardiopulmonary mortality (Hoek et al. 2002) and respiratory symptoms in children (Brauer et al. 2002, Janssen et al. 2003) have been claimed to be more prevalent in subjects living near to a major road. The large effect of traffic may due to the fact that residences within the vicinity of a major road have had consistently larger particle concentrations in four European cities than residences with small impact of traffic (Lianou et al. 2007).

Black carbon or elemental carbon from combustion and traffic sources is one important component of particulate air pollution. However few epidemiological studies have examined this topic. Decreased black smoke concentration in the air as a result of a ban of coal use has decreased the mortality rates (Clancy et al., 2002). Traffic derived black smoke concentrations have been associated with increased cardiopulmonary mortality (Hoek et al. 2002) and exacerbations of ischemic heart disease (Lanki et al. 2006). The levels of elemental and organic carbon have also been associated with cardiovascular emergency room visits (Metzger et al.

2004). The level of elemental and organic carbon in $PM_{2.5}$ has also been linked to increased prevalence of bronchitis in asthmatic children (McConnell et al. 2003). Polycyclic aromatic hydrocarbons (PAH) derived from all kind of combustion may contribute to the development of a variety of air pollution-related diseases (Schwarze et al. 2006). PAH-compounds are a known cause for lung cancer in occupational settings (Armstrong et al. 2004) and most probably also this occurs at levels of these compounds present in the environment (Vineis and Husgafvel-Pursiainen 2005). Air pollution has been shown to cause oxidative DNA-damage and consequently to increase the risk for lung cancers (Møller et al. 2008). There is still a lack of information on the effects of PAH-compounds on human health parameters.

There is evidence that of particulate-associated metals can exacerbate respiratory and cardiac diseases and increase mortality. The closure of the steel mill in Utah Valley decreased the overall PM pollution in the area. In line, respiratory hospital admissions decreased after closure but increased again after the refurbishment of the steel mill (Pope 1991 Pope et al. 1992, Ghio 2004). However, the decrease in hospitalizations and mortality rates was not fully explained by the decreased PM mass concentrations (Ghio 2004). Therefore it is apparent that the high metal concentrations were an explaining factor for the stronger than average effects. Moreover, in the former eastern Germany, children living in the vicinity of mining and metal-smelters have had a higher prevalence of respiratory symptoms and allergy than those in non-industrialized areas (Heinrich et al. 1999).

Secondary inorganic ions may also have a role in the observed human health effects either themselves or with co-existing particulate properties. Sulphate is associated with adverse health outcomes in epidemiology (WHO, 2003; USEPA, 2004, Dockery et al. 1993, Pope et al. 2002). However, the relevance of sulphate aerosol to human health is strongly doubted (Schlesinger and Cassee 2003). It has been proposed that the sulphate aerosol is more likely a surrogate for co-existing factors, like traffic and industrial emissions (Grahame and Schlesinger 2005).

Soil components or crustal material which are mostly detected in the coarse PM in the atmosphere, have also been connected to human respiratory health. Soil components are mostly associated with the prevalence of upper respiratory tract symptoms (Tiittanen et al. 1999) and asthma (Meister and Forsberg 2007). Moreover, respiratory hospital admissions may be even more dependent on the coarse than on fine particles (Brunekreef and Forsberg 2005). In the area with PM_{10} dominated by soil derived material, it seems that the particulate levels have increased mortality (Ostro et al. 1999). The mechanism behind the increased respiratory symptoms and mortality associated with crustal material may be different to that of fine particles and they may be possibly related to increased inflammation in the airways. This concerns mostly the soil derived compositions in the coarse particulate size range.

Table 2. Selected epidemiological studies that have characterized particulate sources by chemical constituents or other means.

Source	PM characterisation	Co-pollutants	Study population	Key findings	Reference
Traffic-related	Black smoke	NO_2, proximity	Elderly in the Netherlands	Increased mortality associated with living in vicinity of major road	Hoek et al. 2002
Combustion	$PM_{2.5}$, Elements	n.a.	Inhabitants of six U.S. cities	Increased mortality associated with traffic combustion sources	Laden et al. 2000
	PM_{15}, $PM_{2.5}$ Elements, sulphate, organics	CO	Inhabitants of three U.S. cities	Increased mortality associated with vehicular traffic	Tsai et al. 2000
	Particle number, PM_{10}	NO_2, CO, O_3	Adults in five European cities	Increased risk for myocardial infarction associated with traffic related particulate air pollution	Lanki et al. 2006
	PM_{10}, TSP, Blackness	NO_2, CO, O_3, SO_2	Inhabitants of Helsinki, Finland	Traffic related blackness of the filters was associated with mortality	Penttinen et al. 2004
	$PM_{2.5}$, Black carbon	NO_2, CO, O_3	Elderly in Boston	Increased risk for myocardial infarction associated with combustion	Zanobetti and Scwartz 2006
	$PM_{2.5}$	n.a.	Inhabitants of Worcester, MA, USA	Increased risk for myocardial infarction associated with living in vicinity of major road	Tonne et al. 2007
	$PM_{2.5}$	n.a.	Inhabitants of six U.S. cities	Stronger association of traffic derived $PM_{2.5}$ than overall $PM_{2.5}$ with daily deaths	Schwartz et al. 2002
	$PM_{2.5}$, PM_{10}	NO_2, CO, O_3, SO_2	Children in Australian and New Zealand cities	Increased respiratory hospital admissions associated with PM pollution mostly from traffic	Barnett et al. 2005
	PM_{10}	n.a.	14 U.S. cities	PM_{10} from traffic increased hospital admissions for cardiovascular and respiratory causes	Janssen et al. 2002
Coal combustion	Black smoke	n.a.	Inhabitants of Dublin, Ireland	Ban of coal sale and decreased air pollutant concentrations, decreased respiratory-,cardiac- and all-cause-mortality	Clancy et al. 2002
	$PM_{2.5}$, Elements	n.a.	Inhabitants of six U.S. cities	Increased mortality associated with coal combustion	Laden et al. 2000
	TSP	SO_2	Inhabitants of six Czech regions	Increased mortality associated with brown coal combustion smog	Jelinkova and Branis 2001
Oil combustion					
	$PM_{2.5}$, Elements	n.a.	Inhabitants of six U.S. cities	Increased mortality associated with fuel oil combustion	Laden et al. 2000
	PM_{15}, $PM_{2.5}$ Elements, sulphate, organics	CO	Inhabitants of three U.S. cities	Increased mortality associated with oil combustion	Tsai et al. 2000
	PM_{10}	n.a.	Inhabitants of Hong Kong	Limitation of sulphur in fuel oil decreased daily mortality rates	Hedley et al. 2002
	PM_{10}	n.a.	14 U.S. cities	PM10 fromoil combustion increased hospital admissions for cardiovascular and respiratory causes	Janssen et al. 2002
Small scale wood combustion	$PM_{2.5}$,	Elements, NO_3^-, TC	Inhabitants of Spokane, WA, USA	Increased cardiac and respiratory emergency visits associated with biomass burning, especially at heating season	Schreuder et al. 2006
	PM_{10}	NO_2, O_3, haze	Inhabitants of Santa Clara, CA, USA	Increased emergency visits for asthma associated with PM_{10} from residential heating with wood	Lipsett et al. 1997
	PM_{10}	NO_2, CO, O_3, SO_2	Asthmatic children in Seattle, WA, USA	Increased emergency visits associated with PM_{10} in heating season	Norris et al. 1999

Wildfire smoke	PM_{10}	n.a.	Inhabitants of Kuching region, Malaysia.	Forest fire smoke increased cardiorespiratory hospital admissions	Mott et al. 2005
	PM_{10}, visibility	n.a.	Inhabitants of Kuala Lumpur, Malaysia.	Forest fire smoke increased cardiorespiratory mortality	Sastry 2002
	PM_{10}	n.a.	Inhabitants of Brisbane, Australia	Bushfires and PM_{10}-pollution increased respiratory hospital admissions	Chen et al. 2006
	PM_{10}	NO_2, NO, NO_x,CO	Inhabitants of Vilnius, Lithuania	Forest fire smoke increased respiratory emergency visits	Ovadnevaitè et al. 2006
	TSP, PM_{10}	n.a.	Inhabitants in Californian counties	Forest fire smoke increased respiratory emergency visits	Duclos et al. 1990
	PM_{10}	n.a.	Children in Californian counties	Forest fire smoke increased respiratory hospital visits	Künzli et al. 2006
Metal industry	PM_{10}	n.a.	Inhabitants of Utah Valley	PM_{10} levels increased daily mortality	Pope et al. 1992
	PM_{10}	n.a.	Children in Utah Valley	Decreased respiratory hospital admissions after closure of steel mill	Pope 1991

It has been estimated that up to 25% atmospheric particles are of biological origin e.g. fragments of pollens and microbes and all kinds of plant and animal debris (Jones and Harrison 2004). The relevance of this material to human health is largely unknown, current knowledge is mainly limited to the gram negative bacterial endotoxins. The epidemiological studies on the effects of biological material on human health are mostly from occupational settings and from studies from indoor environments. It is known that endotoxins increase the risk for asthma development (Tavernier et al. 2005) and occupational respiratory symptoms (Douwes et al. 2003). Moreover, pollens have been associated with exacerbation of asthma (Delfino 2002), and microbial growth in damp indoor environments has been linked to respiratory symptoms and asthma (Bornehag et al. 2001; 2004). The contribution of the biological fraction of ambient air on human health certainly has some relevance, although it has not been widely evaluated. The overview of the epidemiological literature is presented in table 2.

2.3. Mechanisms of immunotoxicity

This review is based on the proposed main mechanisms of the toxicological responses induced by particles. However, particulate air pollution may also evoke a wide variety of responses in the cells of human host defence system and subsequent symptoms in living organisms. In particular, the responses in the alveolar macrophages and in lung epithelial cells play a key role in the airway responses to particulate matter. The main mechanisms proposed to be associated with particulate air pollution related health effect are presented in figure 1. Many of the mechanisms are associated with oxidative stress, which was not measured in this set of studies.

Innate immunity plays a major role in the host defence system against inhaled particles. Epithelial cells form a physical barrier against inhaled substances in the lungs. However, the epithelium in the lungs is covered by lung lining fluid, which contains chemical defence compounds e.g. antimicrobials. Phagocytic cells (macrophages, neutrophils, dendritic cells) are the primary cell types combatting particulate material. Macrophages are present in the lung lining fluid and in the interstitium of the epithelium. The main functions of the macrophages are the recognition and clearance of the foreign material from the lungs. In addition to the phagocytosing properties of the macrophages, these cells are capable of producing many inflammatory mediators e.g. nitric oxide, proinflammatory cytokines and chemokines and they act also as antigen presenting cells for the adaptive immune response.

Many of the innate immunity functions are regulated via cytokines. Phagocytosing cells and epithelium communicate via cytokines and macrophage stimulation leads to epithelial cell stimulation and vice versa. Cytokines affect many of the mechanisms needed for effective defence against inhaled particles, such as macrophage migration and neutrophil recruitment (chemokines). However, a prolonged inflammatory response has also adverse effects. Many of the airway diseases (asthma, COPD) can be traced to inappropriate inflammatory cell activation.

At the level of the whole organism, adaptive immunity plays an important role in host defence against inhaled particles. T- and B-lymphocytes are the key cell types in the adaptive immune system. They exert a rapid response against previously introduced antigens. T-cells are involved in the development of the cell-mediated immune system and they activate the antibody producing B-cells, triggering a wide variety of immune responses e.g. via production of immunoglobulins.

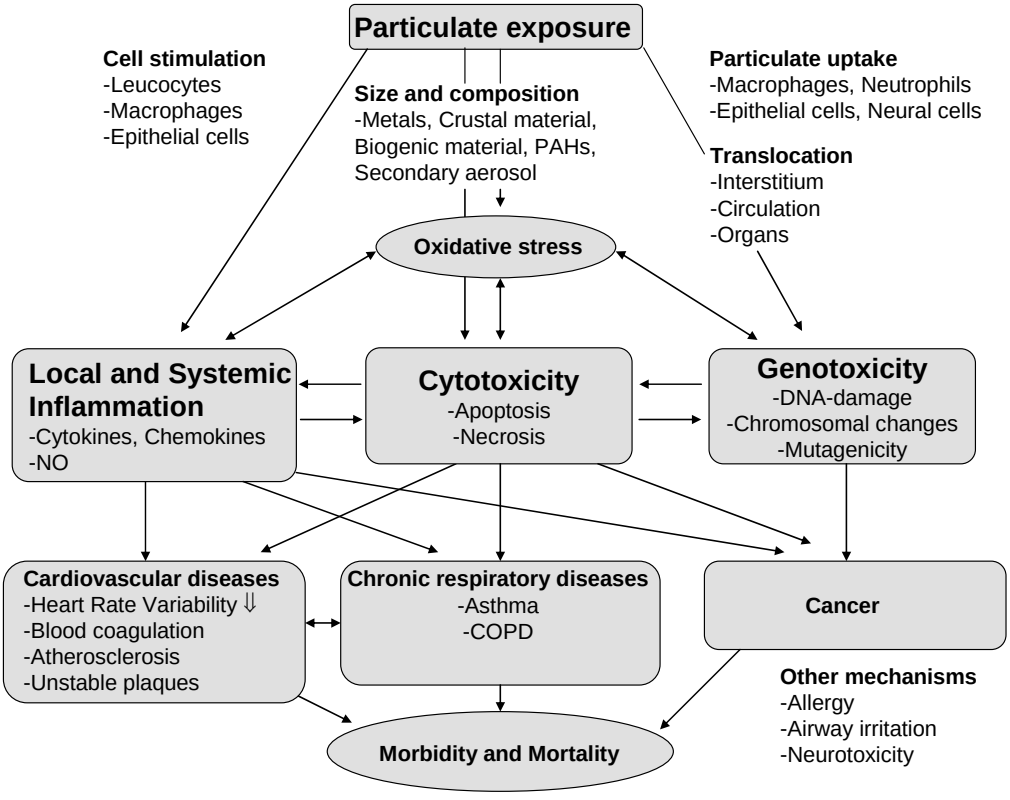

Figure 1. Main mechanisms suggested as being involved in exposure to and health effects of particulate air pollution.

2.3.1 Inflammation

Particulate-induced inflammation has been suggested to be the main mechanism causing exacerbations of air pollution related respiratory and cardiac diseases (Pope and Dockery 2006). It has been shown that particulate inhalation can lead to increased symptoms of obstructive lung diseases (COPD, asthma) that are of inflammatory origin (Frampton 2006, Holgate and Polosa 2006). There are reports of air pollution related systemic inflammation, which can cause cardiovascular effects, such as atherosclerosis, blood coagulation, decrease in heart rate variability, ST-segment depression (Nemmar et al. 2006, Godleski 2006). It has been proposed that inflammatory effects in macrophages and epithelial cells modify neutrophil recruitment from bone marrow and the release of C-reactive protein from liver (van Eeden and Hogg 2002). This cascade activates the mechanisms that trigger cardiopulmonary diseases.

In experimental setups, inflammation is the main endpoint which has been evaluated. The ability of urban air particulate samples to activate inflammatory responses in macrophages and respiratory epithelial cells has been demonstrated in several studies (Hetland et al., 2005; Becker et al., 2005; Dybing et al., 2004; Becker and Soukup, 2003 Becker et al., 2003; Pozzi et al. 2003; Becker et al., 2002; Imrich et al., 2000; Monn and Becker, 1999). Moreover, PM_{10} exposure has increased the release of procoagulant proteins from human macrophages, epithelial- and endothelial cells (Gilmour et al. 2004).

As previously stated, macrophages are the primary cell type combatting inhaled particles in the lung. Thus it was decided that a macrophage cell line was examined since these cells are the first line of defence against particulate matter.

In the present studies, TNFα, IL-6 and MIP-2 were analyzed as indicators of inflammation. TNFα is an early phase proinflammatory cytokine produced by macrophages, and it enhances the production of other cytokines and increases the phagocytic activity of the cells. This cytokine stimulates recruitment of neutrophils and monocytes to the site of inflammation and stimulates epithelial and endothelial cells. TNFα can also induce cells to undergo both apoptosis and necrosis (Barnes et al. 1998; Luster et al. 1999). IL-6 has important roles in both innate and adaptive immunity, it is produced by many different cell types and also affects the functions of many cell types. It can also stimulate the growth and differentiation of B-cells. MIP-2 was chosen in this set of studies to represent a chemotactic cytokine, i.e. chemokine. Production of these cytokines is stimulated by several factors e.g. TNFα and they play a major role in cell recruitment to sites of inflammation. MIP-2 belongs to group of CXC chemokines that have their main target on neutrophils. Moreover, it has a major role in the acute inflammatory response.

2.3.2. Cytotoxicity

Cytotoxicity is related to airway remodeling in chronic respiratory diseases and it has also possible effects on the development of cardiac diseases. Inflammation induced epithelial damage is associated with asthma pathogenesis in human lung (Holgate et al. 2003). Moreover, the cytotoxic activity of lymphocytes has affected the impairment of COPD (Chrysofakis et al. 2004) whereas cytotoxicity in natural killer cells has been associated with coronary artery disease (Jonasson et al. 2005). In addition, there is data suggesting that apoptosi plays an important role in fibrotic lung diseases (Kuwano et al. 2002).

In vivo studies have shown particulate exposure induced tissue damage in the lungs of laboratory rodents (Happo et al. 2007; Gerlofs-Nijland et al. 2007). These findings are supported by in vitro data from several experimental setups which clearly reveal the capability of particulate matter to induce cytotoxicity. The size range of particulate matter has been shown to affect significantly the associated cytotoxic properties of monocytes (Monn and Becker 1999, Osornio-Vargas et al. 2003), macrophages (Salonen et al. 2004, Monn and Becker 1999) and this size dependency has also been demonstrated in epithelial cells (Frampton et al. 1999, Hetland et al. 2004). Overall, in the *in vitro* studies, the coarse particles seem to have greater cytotoxic potential than particles in the smaller size ranges. In vitro experiments have shown that urban particulate samples (Alfaro-Moreno et al. 2002) and diesel exhaust particles (Bai et al. 2002) are able to cause cytotoxicity in endothelial cells. This is of special interest since endothelial dysfunction is known to be one of the major causes of cardiopulmonary diseases (Bai et al. 2007).

For this set of studies, the MTT-test was chosen for analysis of acute, general cytotoxicity. Cytotoxicity (necrosis, apoptosis) is an important mechanism determining the health effects induced by particulate air pollution. Necrosis usually is a result of toxicity or trauma that leads to cell lysis or destruction of cell organelles. TNFα can cause cytotoxicity, but on the other hand, the cytotoxicity itself can also evoke inflammatory responses after cells are lysed. It is known that cytotoxicity plays a major role e.g. in acute respiratory distress syndrome (Hamacher et al. 2002). Acute cytotoxicity or necrosis most often correlates with inflammation.

On the contrary, apoptosis is mostly independent of the inflammatory response. Apoptosis is a controlled procedure in cells, in which there is DNA fragmentation, shrinkage of the cells and subsequent cell death. Apoptosis occurs in many normal processes in organisms, especially in the developing tissues and organs. Apoptosis is a “normal” way for cells to die.

2.3.3. Genotoxicity

Genotoxicity has been associated to air pollution in various studies. In Prague, it was observed that high concentrations of PAH compounds in the air could cause chromosomal aberrations in exposed subjects (Sram et al. 2007). Moreover, oxidative DNA damage has been detected in subjects living in Eastern European cities (Prague, Kosiče, Sofia) in areas with high PAH-concentrations, but the genetic background has affected the severity of the responses (Singh et al. 2007).

Recent findings indicate that in human hepatoma, fibroblast and monocyte cell lines particulate matter with high genotoxic PAH contents cause DNA-damage (Sevastyanova et al. 2007). Moreover, PAH -associated genotoxicity was seen when RAW 264.7 macrophages were incubated with urban air $PM_{2.5}$ samples collected from traffic sites (Poma et al. 2006). In the study of Karlsson et al. (2006), the subway particles were the most genotoxic of all their studied samples (road traffic, wood combustion, tire wear). The workers in that study hypothesized that the genotoxicity was due to high metal concentrations in the subway-derived particles. In line, the levels of water-soluble metal and organic soluble PAHs have been associated with micronuclei formation in human epithelial cells after exposure to particulate samples collected in Mexico City (Roubicek et al. 2007).

Genotoxicity was not investigated in the present thesis. However, the main mechanisms of the particulate air pollution induced effects are strongly linked to each other. Particulate air pollution is associated also with many other adverse health effects that are not discussed here.

2.4. Toxicological findings in association with particulate characteristics

There are many similarities, but also some dissimilarities in the toxicological findings in association to particulate matter when compared to epidemiological findings. Numerous studies have examined the effects of urban air particles on various toxicological endpoints. However, caution should be observed when comparing the results with each other. There are a variety of sample collection methods, sample preparation methods and toxicological methods that can all affect the measured endpoints. There is also large amount of data available from different source particulates e.g. diesel exhaust particles (DEP), residual oil fly ash (ROFA), coal fly ash etc, but these cannot be directly compared to the results on ambient particles. The common denominator for many of the effects induced by particulate matter is

oxidative stress. This review of the literature is limited mostly to the ambient particles in urban environments and to their *in vitro* effects.

2.4.1. Immunotoxic effects related to particle size

There are a vast amount of toxicological studies that have examined the effects of different ambient particulate size fractions. There is substantial evidence that in *in vitro* toxicological setups, coarse ambient particulate samples induce greater inflammatory responses than fine particulate samples (Hetland et al. 2005, Soukup and Becker 2003, Becker et al. 2003, 2002, Monn and Becker 1999). Moreover, the responses to the fine ambient particulate samples have been greater than those of the ultrafine particulate samples (Becker et al. 2003, 2005). The same pattern applies in most cases also to cytotoxicity (Monn and Becker 1999, Osornio-Vargas et al. 2003). In contrast, fine particulate samples have induced more cells to undergo apoptosis than coarse particulate samples (Hetland et al. 2004). There are however also opposite findings related to model particles (e.g. polystyrene, titanium dioxide, carbon black). The responses to ultrafine model particles seem to be dependent on the large relative surface area of these particles (Brown et al. 2001; Hohr et al. 2002; Oberdörster et al. 2005). Ultrafine particles can even translocate to extrapulmonary tissues and cause adverse effects in these sites (Elder et al. 2006, Oberdorster et al. 2004). Generally, the studies indicate that particulate composition may be more important than the particulate size per se as reviewed by Schwarze et al. (2006).

2.4.2. Toxic effects related to particle sources and composition

Little is known about how different sources contribute to the toxicological responses. Few studies have aimed to separate the effects related to different emission sources. However, there has been heterogeneity in toxicological responses estimated on an equal mass basis depending on the sampling location. This emphasizes the importance of particulate composition in the induced responses. An overview of the toxicological studies on particulate air pollution is summarized in table 3.

There has been spatial variation in the inflammatory and cytotoxic responses evoked by PM_{10} sample in different areas of Mexico City (Alfaro-Moreno et al. 2002). In that study, inflammatory responses in human lung epithelial cells were most severe with the samples

collected from an area dominated with traffic, whereas cytotoxicity endpoints were greatest in an area with industrial emissions. It has been also observed that soil components are worse than combustion sources in their ability to evoke inflammation in rat alveolar macrophages (Hetland et al. 2004). It was shown in the six U.S. cities study that wood combustion sources have a stronger association with IL-8 gene expression in human airway epithelial cells than coal combustion and traffic (Graff et al. 2007). It has also been observed that PM_{10} with different mineral compositions are not equally potent inducers of IL-8 in human lung epithelial cells (Schwarze et al. 2007). In a study with particulate samples from wood combustion, tire wear, street traffic and subway particles, it was noted that the traffic particles had strongest inflammatory potential (Karlsson et al. 2006). An oil combustion source has been implicated in the NF-κB induction in human bronchial epithelial cells (Maciejczyk and Chen 2005). A seasonal variation in the same location has affected the inflammatory responses and oxidative stress caused by urban particulate samples (Becker et al. 2005, Monn et al. 2003). There is some discrepancy in the toxicological findings associated to sources, depending on the cell lines, size ranges and endpoints. However, it does seem that different local sources are important modifiers of the toxicological responses, with the amount of traffic, combustion sources and soil composition being the key factors.

Transition metals have been shown to affect the inflammatory as well as the cytotoxic responses in vitro (Pagan et al. 2003; Frampton et al. 1999). Moreover, metal toxicity in particulate matter seems to be dependent on the metal mixture and the oxidation state of the metals (Merolla and Richards 2005; Pagan et al. 2003). Particulate samples with high metal contents have evoked inflammatory responses in human alveolar macrophages (Schwarze et. al. 2007) and in human epithelial cells (Frampton et al. 1999). Some of the metals have been associated to distinct sources, e.g. Ni and V with oil combustion. These two metals were found to increase the inflammatory responses of human bronchial epithelial cells (Maciejczyk and Chen 2005). Metal chelators have been used in toxicological studies to remove the effects of the metals in the particulate matter. It has been observed that treatment with a chelator can reduce their inflammatory properties in human epithelial cells (Molinelli et al. 2002) confirming the important contribution of metals. However, the use of antagonists always includes the risk for non-specific effects and therefore misinterpretation.

Although there is some epidemiological evidence for an association between secondary inorganic ions (SO_4^{2-}, NO_3^- and NH_4^+) and adverse health effects, they have not been confirmed by available toxicological data in following reviews (Schlesinger and Cassee 2003; Grahame and Schlesinger 2005; Schlesinger et al. 2006). Thus it is likely that these compounds are surrogates of other more reactive compounds, originating from the same

emission sources and the secondary ion cause effect only at levels far higher than those relevant in the environment (Schwarze et al. 2007).

Organic compounds as assessed with organic carbon content affected the particulate induced toxicity in the rat lung (Kodavanti et al. 2005). Organic compounds potentially play a significant role in the cytotoxic mechanisms associated with particulate matter (Molinelli et al. 2006). It has been observed that emission sources with potentially high PAH concentrations may be associated with markers of asthma and allergy in mice (Steerenberg et al. 2006) Moreover, PAH compounds can cause genotoxicity and oxidative stress (Binkova et al. 2003, Farmer et al. 2003, Xia et al. 2004), and DNA-adducts and strand breaks (Gabelova et al. 2007). PAH-compounds have also elicited apoptotic or anti-apoptotic signals in Hepa1c1c7 (Solhaug et al. 2004). Moreover, combustion processes release semiquinoid substances, which may release hydroxyl radicals in biological systems through quinoid redox cycling (Squadrito et al. 2001). Particulate matter with potentially high PAH-contents has caused apoptosis, oxidative stress and mitochondrial dysfunctions (Xia et al. 2004, Hiura et al. 1999). The soluble organic compounds in the particulate samples have been claimed to be associated with cytotoxic and genotoxic properties (Schwarze et al. 2007).

In addition, soil composition affects the particulate induced responses. The toxicological studies on environmental mineral particles are mostly limited to road dusts or windblown desert dusts. Particles with different mineral compositions have caused different responses *in vitro* (Hetland et al. 2000). Moreover, the shape of the mineral particles has affected the *in vitro* inflammatory responses (Holopainen et al. 2004). The rock type used in the pavement material has also affected the *in vitro* responses evoked by the mechanically generated particles (Lindblom et al. 2006). Moreover, it has been postulated that higher proinflammatory potential of PM_{10} samples collected during spring dust episodes in Finland was due to particle bound endotoxins (Salonen et al. 2004).

The studies on toxicological relevance of biological material in the particulate matter are mostly limited to gram negative bacterial endotoxins (Schlesinger et al. 2006). Biological material is more prevalent in the coarse size range than in fine size range particulates (Schwarze et al. 2007). There have been consistently larger endotoxin concentrations detected in coarse PM than the corresponding fine PM (Heinrich et al. 2003; De Vizcaya-Ruiz et al. 2006). The endotoxin content in particulate matter has induced inflammatory responses in vitro (Monn and Becker 1999; Long et al. 2001). Biogenic air pollution in urban environment is by far the least studied component. Some insight could be acquired from following the examples of studies into indoor air and occupational settings, where the microbes and biogenic dusts have caused inflammatory effects. (Bornehag et al. 2000).

Table 3. Selected toxicological studies that have included size-segregated chemical characterization of outdoor air particles.

Cell type	Specification	species	PM size ranges	Chemical characterization	Key findings	Reference
Macrophage	RAW 264.7	mouse	$PM_{2.5}$, $PM_{10\text{-}2.5}$	elements, C, Ca, Si	$PM_{2.5}$ was more active in NO-production and cytotoxicity per surface area and weight independent on concentration	Diociaiuti et al. 2001
Macrophage	J774A.1	mouse	PM_{10}	Metals	Metal chelation decreased TNFα responses in cells	Hutchison et al. 2005
Epithelial	A549	human	$PM_{2.5}$, $PM_{10\text{-}2.5}$	Metals, endotoxin, microbes	Location, season, composition and size-range affected the cytotoxic responses	De Vizcaya-Ruiz et al. 2006
Epithelial	A549	human	$PM_{2.35\text{-}0.12}$, $PM_{8.5\text{-}2.35}$	Elements, Ions, PAHs endotoxin	Inflammation, toxicity and allergy sensitization were dependent on composition and size but independent on each other	Steerenberg et al. 2006
Macrophage	primary alveolar	rat				
Epithelial	primary alveolar	rat				
Epithelial	BEAS-2B	human	$PM_{2.5}$	Elements, EC, OC endotoxin	Chemical composition affected the inflammatory responses	Veranth et al. 2005
Macrophage	J774A.1	mouse	PM_{10}	Elements, EC, OC endotoxin	Chemical composition and sampling location affected the inflammatory and cytotoxic responses	Perez et al. 2007
Macrophage	RAW 264.7	mouse	PM_{10}	Elements, Ions, PAHs	Sampling season and composition affected the inflammatory and cytotoxic responses	Salonen et al. 2004
Macrophage	RAW 264.7	mouse	$PM_{2.5}$, $PM_{10\text{-}2.5}$	Metals, C, Ca, Si endotoxin	Chemical composition and collecting season affected the macrophage responses	Pozzi et al. 2005
Epithelial	NHBE	human	PM_{10}	Metals	Possible cytotoxic role of Ni, V, As ans Cu	Molinelli et al. 2006
	BEAS-2B	human				
Monocyte	THP-1	human	24-31 nm, PM_{10}	PAHs, OC, tot organics	Different inflammatory responses for wood smoke, road dust and diesel emission	Kocbach et al. 2008
Epithelial	A549	human	$PM_{2.5}$, PM_{10}	Metals, mineral types	Different mineral types affected the cellular responses	Øvrevik et al. 2008
	SAEC	human				
T	Primary T2	rat				
Epithelial	primary bronchial	human	$PM_{0.1,}$ $PM_{2.5}$	Elements	Positive associations between cytokines and Fe, Si and Cr	Becker et al. 2005
Macrophage	primary alveolar	human	$PM_{10\text{-}2.5}$			
Epithelial	BEAS-2B	Human	PM_{10}	Metals	Metal concentration affected the IL6 and IL-8 induction	Frampton et al. 1999

2.5 Research needs for future regulation of particulate air pollution

Particle size is mainly used as the basis for current outdoor air quality regulations and legislation. The size-ranges of the particles usually regulated and measured on a mass basis by the authorities are all thoracic particles (PM_{10}) and, more recently, $PM_{2.5}$subfraction. There has not been enough scientific collected data on the relative harmfulness of the chemical components and particle sources to allow a more targeted regulation, although there is increasing evidence on the importance of particulate composition (Grahame and Schlesinger 2007). Toxicological studies can provide important additional information on the causative association between measured biological endpoints and the chemical composition of particulate matter. This would complement the findings from epidemiological studies and provide a sound basis for improved risk characterization in with the field of particulate air pollution.

3 AIMS OF THE STUDY

1. To validate the particulate sample procedures, cell exposure methods and time-response relationships in a macrophage cell line using well-characterized reference particulate samples. (I)
2. To investigate the dose-relationships of responses to size-segregated particulate samples, collected from different sampling sites and different air pollution situations. (II, III)
3. To investigate the dependency of the inflammatory and cytotoxic responses to urban air fine and coarse particulate samples on the subfractions of varying solubility. (IV)
4. To identify the potentially causative chemical compositions and sources of urban air fine and coarse particulate samples to the inflammatory and cytotoxic responses. (IV, V)

4 MATERIALS AND METHODS

4.1 PM sampling (I-V)

4.1.1 Tested samples

All the tested samples are summarized in Table 4. Four different air PM samples, including total suspended reference particles, fractionated fine PM sample and diesel PM were chosen to Study I to best represent PM samples which would be different in their composition. The urban dust standard reference material SRM1649a collected as total suspended particles (TSP) from Washington DC, and the diesel PM standard reference material SRM1650, were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). The Ottawa dust EHC-93, collected as TSP but filtered to correspond to the fine fraction as described by Vincent et al. (1997), was from the Environmental Health Centre, Ottawa, Canada. In addition, one ambient air $PM_{2.5}$ sample (HFP-00) from Helsinki, was used. It was collected on PUF with a single-phase, high volume, low cutoff inertial impactor (Salonen et al. 2000).

For Study II on long-range transport of wildfire smoke aerosol, size-segregated ambient air particulate samples were collected in four size ranges ($PM_{10-2.5}$, $PM_{2.5-1}$, $PM_{1-0.2}$ and $PM_{0.2}$) in Helsinki. The samples were as follows: seasonal average, wildfire episode and mixed episode.

For Studies III-V, the particulate samples for toxicological studies were collected in 7-week sampling campaigns in six European cities: Duisburg (Oct 4 - Nov 21, 2002), Prague (Nov 29 – Jan 16, 2003), Amsterdam (Jan 24 – Mar 19, 2003), Helsinki (Mar 21 – May 12, 2003), Barcelona (Mar 28 – May 19, 2003) and Athens (Jun 02 – Jul 21, 2003). These samples were collected similarly to those in study II, but the $PM_{2.5-1}$ and $PM_{1-0.2}$ samples were pooled together to form the fine size range.

4.1.2 Particulate samplers

In particulate samplings, a modified Harvard HVCI with a flow volume of 51 m^3/h was used (Sillanpää et al. 2003, Pennanen et al. 2007). In the toxicological studies, the $PM_{10-2.5}$, $PM_{2.5-1}$ and $PM_{1-0.2}$ samples were collected on polyurethane foam (PUF) (Antistatic

polyurethane foam 87035K13, McMaster-Carr, New Brunswick, NJ, USA) and the $PM_{0.2}$ samples were collected on glass fiber filters (Munktell MGA, Munktell Filter AB, Grycksbo, Sweden). Three similar virtual impactors (VI) were used in parallel to the HVCI for collection of reference low-volume particulate samples in two size ranges: fine ($PM_{2.5}$; D_p<2.5 μm) and coarse ($PM_{10-2.5}$; 2.5 μm<D_p<10 μm). The VI samples were collected on polytetrafluoroethylene (PTFE) filters (FS, Millipore, Ireland) at an air flow of 16.7 l/min. These samples were utilized to complement the relatively narrow characterization of the chemical composition and sources of particles that was possible due to methodological reasons, to be obtained from the HVCI samples.

Table 4. Particulate samples, size ranges and doses, and the toxicological endpoints used in the five studies.

Study	PM sample origin	Size ranges	Dose µg/ml	Timepoints (h)	Endpoints	Specification
I	SRM1649a SRM1650 EHC-93 HFP-00	TSP[1] Total emitted PM $PM_{2.5}$ $PM_{2.5}$	15, 50 ,150 ,300	2, 4, 8, 16, 24	IL-1, IL-6, IL-10 TNFα, MTT, NO	Samples treated with methanol in different sonication timepoints Time course of the responses
II	Seasonal average Mixed episode Wildfire episode	$PM_{10-2.5,}$ $PM_{2.5-1}$ $PM_{1-0.2,}$ $PM_{0.2}$	15, 50 ,150 ,300	24	IL-6, TNFα, MIP-2 MTT, NO PI-cellcycle, apoptosis	Dose response of samples of different LRT Episodes and seasonal average (spring) air pollution in Helsinki
III	Duisburg Prague Amsterdam Helsinki Barcelona Athens	$PM_{10-2.5}$	15, 50 ,150 ,300	24	IL-6, TNFα, MIP-2 MTT, NO PI-cellcycle Apoptosis	Dose response of samples from different locations in Europe
IV	Duisburg Prague Amsterdam Helsinki Barcelona Athens	$PM_{10-2.5}$ $PM_{2.5-0.2}$	150	24	IL-6, TNFα, MIP-2 MTT, NO PI-cellcycle Apoptosis	The effects of water-soluble, organic solvent soluble and corresponding insoluble PM fractions and total PM suspensions Correlations with mass closure components
V	Duisburg Prague Amsterdam Helsinki Barcelona Athens	$PM_{10-2.5}$ $PM_{2.5-0.2}$	150	24	IL-6, TNFα, MIP-2 MTT, NO	Correlations of the toxic responses with inorganic soluble and insoluble constituents and with organic constituents

[1]TSP = total suspended particles
[2]LRT = long range transport

4.2 Description of sampling sites (II-V)

In Study II, particulate sampling was conducted at an urban background site that was 8 km east of central Helsinki. In studies III-V, the samplings were conducted in six European cities as presented in Table 5.

Table 5. Background information on the sampling campaigns in Studies II-V, including major emission sources, proximity to traffic and mean temperature during particulate sampling.

Sampling site	Sampling periods (dd.mm.yyyy)	Major local PM sources	Distance (m) to nearest busy road	Vehicle density	T (°C)
Helsinki (II)	23.8.2002-23.9.2002	Traffic, Harbor, Sea	50	5500	15
Duisburg (III-V)	4.10.2002-21.11.2002	Traffic, Metal industry	280	n.a	9
Prague (III-V)	29.11.2002-16.1.2003	Traffic, Heavy use of solid fuels	150	5000	-2
Amsterdam (III-V)	24.1.2003-13.3.2003	Traffic, Sea	50	10000	4
Helsinki (III-V)	21.3.2003-12.5.2003	Traffic, Harbor, Sea	300	30700	4
Barcelona (III-V)	28.3.2003-19.5.2003	Traffic, Harbor, Sea, Metal industry	100	17000	15
Athens (III-V)	2.6.2003-21.7.2003	Traffic	100	30000	29

4.3 PM sample preparation methods (I)

For Study I, the SRM1649a, EHC-93 and SRM1650 samples were treated with methanol in a water bath sonicator with sonication power 300W and frequency 30kHz 2×30 min, dried in nitrogen flow and stored at -20°C. This was exactly the same treatment as used for the extraction of $PM_{2.5}$ mass from the collection substrate, PUF, to form the pooled sample of HFP-00. Additionally, the effect of the sonication time during sample preparation prior to testing the inflammatory and cytotoxic effects of the samples, was tested. Sample preparation methods for the following studies were chosen on the basis of study I.

For Studies II-V, the PUF sample collection substrates and glassfiber filters were weighed using an analytical balance (Mettler Toledo AG 285, Mettler Instrumente AG, Zurich, Switzerland) before and after the sampling. The frozen samples were allowed to stabilize in the weighing room for 4 h in closed containers and for 16-18 h in open containers before weighing. The relative humidity (15-24 %) and temperature (21-22 ºC) in the weighing room were recorded. The electrostatic charges of filter and

substrate materials were eliminated with a high-voltage ionizer (HAUG Static Line ENSL, Leinfelden-Echterdingen, Germany). After weighing, the samples were stored at -20°C until extraction for the chemical analyses and toxicological studies.

The sampled particles on PUF or glass fiber filters were extracted and pooled together according to size range and campaign period (Studies II-V). Detailed descriptions of the sample preparation methods are presented in Studies I and II. The methanol suspension containing $PM_{0.2}$ particles was filtered in order to remove glass fibers derived from filters. The methanol-particulate suspensions were divided on a particulate mass basis into 10-ml glass tubes, dried under nitrogen flow and stored at -20°C. These dry samples were used for toxicological and chemical analyses. An extraction procedure similar to the actual particulate samples was used also for the field blanks. The blank samples for the chemical analyses and toxicological studies were prepared by particulate size range from blank PUF-strips or glass fiber filters that had gone through the same procedures as the sampling substrates of the actual samples.

4.4 Chemical and source characterization of particulate samples (I-V)

Extensive chemical characterization was made from the samples. The analyzing methods for particulate composition are presented in Table 6.

Table 6. Chemical analysis methods and constituents in the analysis of particulate samples.

Method	Abbreviation	Analytes
Ion chromatography	IC[1,2]	Anions: Cl^-, NO_3^-, SO_4^{2-} succinate, malonate, oxalate Cations: Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}
Inductively coupled plasma mass spectroscopy	ICP/MS[1,2]	Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn
Energy dispersive X-ray fluorescence	ED-XRF[2]	Al, Ca, Cl, Cu, Fe, K, Mn, Ni, Pb, Si, V, Zn
Liquid chromatography/mass spectrometry	LC/MS[2]	Sum of levoglucosan, galactosan, mannosan (ΣMA)
Thermal optical carbon analyser	TOA[2]	EC, OC
Gas chromatograph mass spectrometry - selected ion monitoring	GCMS-SIM[1]	total of 31 PAH-compounds *
Limulus amebocyte lysate-assay	LAL-assay[2]	Endotoxin

Analyses from: [1]HVCI-samples, [2]VI-samples. *For detailed comparison with cellular responses, the PAH compounds suggested to be monitored according to Directive 2004/107/EC, were chosen.

In addition, the chemical mass closure method was used to provide better characterize the different particulate sources in the sampling campaigns. The definitions of mass closure components are presented in table 7.

Table7. Components of the HVCI chemical mass closure, their abbreviations and calculation formulas.

Mass closure component	Abbreviation	Formula
Non sea salt-sulphate[a]	nss-SO_4^{2-}	[Nss-SO_4^{2-}] = [SO_4^{2-}] - 0.246 x [Na^+]
Nitrate	NO_3^-	
Ammonium	NH_4^+	
Sea salt[a]	SS	[SS] = 3.248 x [Na^+]
Water-soluble soil[b]	WSS	[WSS] = [Fe_2O_3] + [Al_2O_3] + [CaO] + [K_2O]
Water-insoluble soil[c]	WIS	[WIS] = [Fe_2O_3] + [SiO_2] + [Al_2O_3] + [CaO] (+ [$CaCO_3$]) + [K_2O] - [WSS]
Other elements	OE	[OE] = [As]+[Cd]+[Co]+[Cr]+[Cu]+[Ni]+[V] +[Mn]+[Pb]+[Zn]
Elemental carbon	EC[d]	
Particulate organic matter	POM[d]	[POM] = 1.4 * OC
Unidentified matter	UM	[UM] = [gravimetric PM_x] - [Σ identified components of PM_x]

[a]Brewer (1975).

[b]WSS is based on the IC and ICP-MS data.

[c]WIS is based on the WSS data and the insoluble-to-soluble ratios of constituents in the reference (VI) data.

[d]The relative (percentage) values of EC and POM were taken directly from the VI data of Sillanpää et al. (2006).

In addition, several constituents were utilized as tracers of certain particulate sources: Ni and V for fuel oil combustion, As for coal combustion, ΣMA for biomass combustion, DA for photochemical transformation, Al, Ca and Si (and WSS and WIS) for soil components and Endotoxin, OC (and POM) for organic or biological fraction. Mass closure components are presented in parenthesis.

4.5 Study design for cell experiments (I-V)

The mouse macrophage/monocyte cell line RAW 264.7 was obtained from American type culture collection (ATCC, Rockville, MD, USA). The cells are Abelson murine leukemia virus transformed, immortalized cell line. The cells were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin. Cells were cultured at 37°C and in 5% CO_2 atmosphere.

In the experiments, the cell suspension was diluted to 5×10^5 cells/ml. The cells were cultured for 24 h before the experiments and one hour before the experiments, 1 ml of fresh medium (37°C) was changed on the 6-well plates.

The macrophage cell line was exposed to the samples at four doses of 15, 50, 150 and 300 µg/ml in studies I-III, whereas in studies IV and V a single dose (150µg/ml) was used. On the basis of study I where there were five timepoints (2-24h), 24h timepoint was chosen for Studies II-V. In all studies, three independent experiments were run in duplicate. All experiments were conducted on six-well plates, in a volume of 2 ml in each well. To be sure that the particulate collection materials were inert and to exclude other artifacts, each plate had also an untreated cell control and a PUF or filter blank.

After exposing the macrophages to the particulate samples for 24 h, the cells were resuspended into cell culture medium by scraping. The viability of the cells was measured with the MTT-test from the cell suspension (2×100 µl) of each well. The remaining cell suspension was centrifuged (5min, 8000rpm, +4°C) to separate the cells and the medium. Nitric oxide analysis from supernatant was conducted directly after the exposures. The remaining supernatants were stored at -80°C for cytokine analysis (Jalava et al., 2005). The cell pellet was washed, suspended to PBS and fixed in 70% (v/v) ethanol for subsequent propidium iodide staining (Penttinen et al., 2005).

4.6 Biochemical analysis (I-V)

Toxicological endpoints in different studies are summarized in Table 4. There were several inflammatory parameters evaluated: nitric oxide (NO) was measured with Griess-method (Green et al. 1998), proinflammatory cytokines IL-1, IL-6, TNFα, anti-inflammatory IL-10, and chemokine MIP-2 were assayed from the cell culture medium after the cells were exposed to particulate samples according to manufacturer's instruction, that were slightly modified (R&D systems, MN, USA). Cytotoxicity after the exposures was assessed with the MTT-test from the cell suspension (Mossmann 1993). Detailed descriptions of the above mentioned methods are presented in study I. The DNA content was analyzed by propidium iodide (PI) staining of permeabilized cells. (Penttinen et al. 2005) This method was used in assessing the cell cycles of the macrophages as well as in assessing the proportion of the apoptotic cells in the cultures (Darzynkiewicz et al. 1992).

4.7 Statistical methods (I-V)

Statistical methods used in studies I-V are presented in Table 8. All statistical analysis was made with SPSS software versions 10-15 in Studies I-V, respectively.

Table 8. Statistical methods used in the five studies.

Study	Analysis	Method
All	Equality of variances	Levene's test
I	Dose response	Dunnett's test
	Time course	
	Effect of methanol treatment	
II	Dose response	Dunnett's test
	Comparison between induced	Tukey's test &
	Responses	Dunnett's C
	Dose response trends	Equation fit
III	Dose response	Dunnett's test
	Comparison between samples	Tukey's test &
		Dunnett's C
	Dose response trends	Equation fit
	Correlations between toxic	
	Endpoints	Pearson's correlation
IV	Comparisons between treatments	Dunnett's test
	Cell cycle analysis	Kruskall-Wallis
	Correlations between responses	Spearman's rank
	and mass closure components	correlation
V	Correlation between responses	Spearman's rank
	and chemical composition	correlation
	Trends between TNFα and	
	selected source indicators	Equation fit

5 RESULTS

5.1. Time-courses of response parameters (I)

The time-courses of the responses were tested in Study I for the maximum response of the cytokines, TNFα and IL-6, was found at 24 hrs (2, 4, 8, 16 and 24 hrs were tested). In the assessment of cytotoxicity and MIP-2 production (data not shown), the maximum responses occurred earlier, at 16 and 8 hrs, respectively. However, these responses remained relatively stable for up to 24 hrs. Thus, in the four subsequent studies, 24 hrs was selected as the optimal timepoint of response recording.

5.2. Sample treatment (I)

The possibility that the sonication and methanol extraction could have altered the particulate-induced responses was tested in Study I using reference particulate samples with and without methanol extraction. On basis of this dataset, a sonication time of 30 min was selected from the tested timepoints (5, 10, 15, 20 and 30 min) for optimal sample preparation, i.e. it did not substantially modify the particulate-induced responses, and ensured an efficient extraction of the particulate mass from the collection substrates and a homogenous suspension for the cell exposures.

5.3. Inflammatory and cytotoxic responses to particulate samples (I-IV)

There were large differences in the abilities of the samples to induce inflammatory mediator production or cytotoxicity. The concentrations of inflammatory mediators after the exposures were dependent on the parameter, i.e. IL-6 revealed the smallest and MIP-2 displayed the largest changes in the levels. The differences between the inflammatory responses to the samples were larger in the $PM_{1-0.2}$, $PM_{2.5-1}$ and $PM_{2.5-0.2}$ size-ranges than in $PM_{10-2.5}$. The inflammatory mediators were sensitive parameters in separating the responses within smaller size ranges. Cytotoxicity parameters, especially apoptosis, were sensitive response indicators in all the size-ranges.

The $PM_{10-2.5}$ size range samples showed the greatest inflammatory potency. There was also a systematic inverse dependency between particle size and the magnitude of the inflammatory responses in the macrophages. However, the differences between the responses in cytotoxic and apoptotic potency were not as large between the particulate samples of different size- ranges. The results for Studies I-V are summarized in table Table 9 as percentile values across the studies.

Table 9. The inflammatory and cytotoxic responses to the particulate samples ranked as percentiles across the five studies. Explanations are given in the footnote.

Study			Inflammation				Cytotoxicity	
	Size-range	Sample	NO	IL-6	TNFα	MIP-2	Viability	Apoptosis
I	TSP	SRM1649a	bcl	+	+	nm	+	nm
	TSP(<2.5 μm)	EHC93	+	++	+	nm	bcl	nm
	$PM_{2.5}$	PPF04	(+)	bcl	(+)	nm	(+)	nm
II	$PM_{10-2.5}$	Seasonal	++	+++	+++	+++	+++	++
		Wildfire	++	++	++	++	+++	(+)
		Mixed	++	++	++	++	++	+
	$PM_{2.5-1}$	Seasonal	(+)	+	++	++	+++	+++
		Wildfire	(+)	(+)	+	(+)	++	(+)
		Mixed	++	+++	++	++	+++	(+)
	$PM_{1-0.2}$	Seasonal	++	bcl	(+)	(+)	++	+++
		Wildfire	+	bcl	(+)	(+)	+	++
		Mixed	+	bcl	bcl	(+)	+	++
III-V	$PM_{10-2.5}$	Dui	+++	+++	+++	+	+	++
		Pra	+++	+++	+++	+++	++	(+)
		Ams	+++	++	+++	+++	+	++
		Hel	+++	++	++	++	+	+
		Bar	+++	+++	+++	+++	+++	++
		Ath	+++	+++	+++	+++	+++	+++
	$PM_{2.5-0.2}$	Dui	+	+	+	+	(+)	++
		Pra	(+)	bcl	(+)	(+)	(+)	+
		Ams	(+)	+	(+)	+	bcl	+
		Hel	+	bcl	+	+	bcl	+
		Bar	+	++	++	+	+	(+)
		Ath	++	+	+	++	+	+++

nm	not measured
bcl	below control level
(+)	statistically significant, below 25th percentile of response magnitude
+	between 25th and 50th percentiles of response magnitude
++	between 50th and 75th percentiles of response magnitude
+++	over 75th percentile of response magnitude

5.4. Heterogeneities in inflammatory and cytotoxic responses (II-V)

TNFα correlated best with the other response parameters and was, therefore, selected to represent inflammation. Cytotoxicity was independent of the inflammatory responses. MTT-test results were chosen to represent the cytotoxicity, although other parameters such as apoptosis did not, in most cases correlate with cell viability measured with MTT. The long-range transport episodes were studied using four particulate size-ranges, since the long range transport most clearly affected the accumulation size range ($PM_{1-0.2}$). Three size-ranges were used in the toxicological characterization of samples from six European cities

5.4.1. Effects of air pollution episodes in Helsinki (II)

There were major differences in the inflammatory responses induced by different size-range particulate samples, collected in Helsinki during air pollution episodes. Inflammatory responses caused by $PM_{0.2}$ and $PM_{1-0.2}$ were minor compared to the $PM_{2.5-1}$ and $PM_{10-2.5}$ samples. Interestingly, the mixed episode $PM_{2.5-1}$ possessed a greater inflammatory potency than the respective $PM_{10-2.5}$ samples. In general, the air pollution situation led to clear heterogeneity in the inflammatory potency of the size-segregated particulate samples (Figure 2), with the largest variation being seen in the $PM_{2.5-1}$ size-range. Data for $PM_{0.2}$ are not shown due to their negligible inflammatory responses.

$PM_{1-0.2}$ samples were the least cytotoxic, and the $PM_{2.5-1}$ and $PM_{10-2.5}$ samples had stronger, almost equal, cytotoxic potencies, depending on the air pollution situation. Interestingly, $PM_{0.2}$ samples were somewhat more cytotoxic at the largest mass doses than $PM_{1-0.2}$ samples, but the heterogeneity between samples in the former size-range was smaller (data not shown). The air pollution situation had the largest effect on the responses to $PM_{1-0.2}$ samples, followed by the $PM_{2.5-1}$ and $PM_{10-2.5}$ samples (Figure 3).

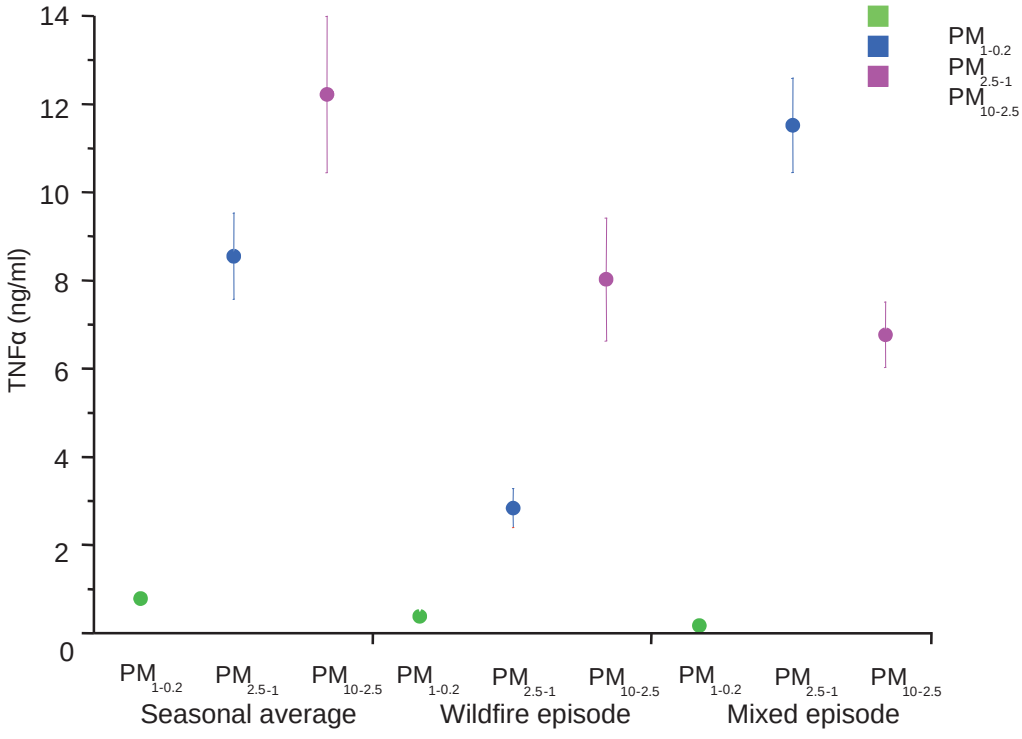

Figure 2. TNFα concentrations (ng/ml) produced by mouse RAW 264.7 macrophages in response to a 24-h incubation with the $PM_{1\text{-}0.2}$, $PM_{2.5\text{-}1}$ and $PM_{10\text{-}2.5}$ samples (150 µg/ml) of the seasonal average, wildfire episode and mixed episode air pollution situations. The dots represent the arithmetic mean and the whiskers are the standard error of the mean (SEM) (n=6).

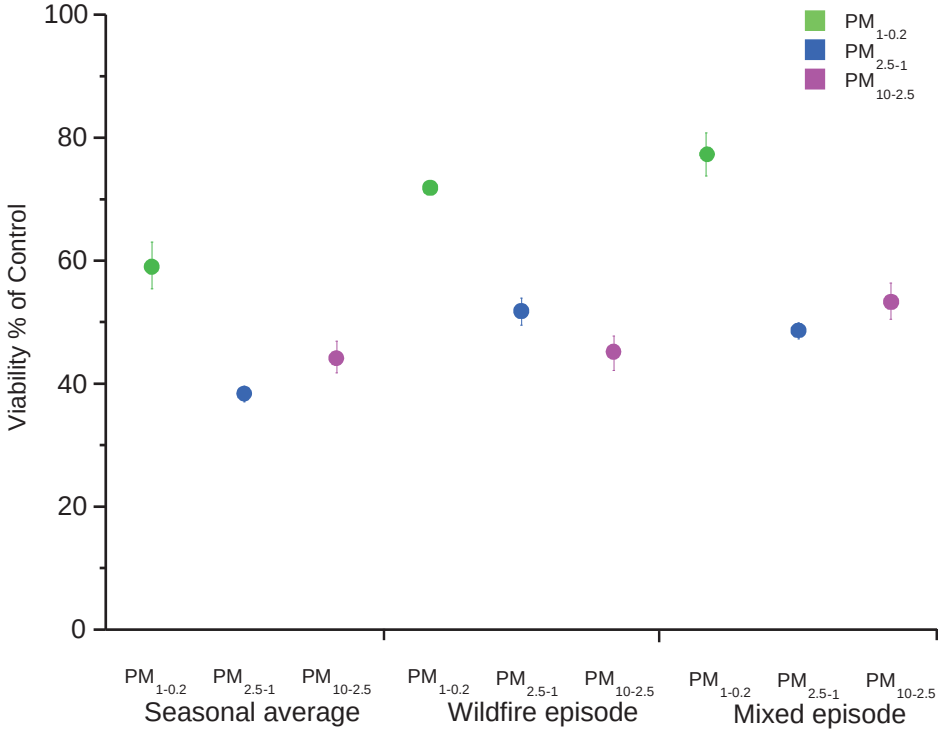

Figure 3. Cell viability (% of unexposed control) of RAW 264.7 macrophages assessed using the MTT-test after a 24-h exposure to the $PM_{1\text{-}0.2}$, $PM_{2.5\text{-}1}$ and $PM_{10\text{-}2.5}$ samples (150 µg/ml) of the seasonal average, wildfire episode and mixed episode air pollution situations. The dots represent the arithmetic mean and whiskers the SEM (n=6).

5.4.2. Effects of air pollution situation in Europe (III-V)

The $PM_{10-2.5}$ samples were consistently more potent inducers of inflammation than the $PM_{2.5-0.2}$ samples. However, there was a greater heterogeneity in the $PM_{2.5-0.2}$ than the $PM_{10-2.5}$-related inflammatory potency. The responses to particulate samples in these two size ranges were not dependent on each other. In $PM_{2.5-0.2}$ size range, the Mediterranean spring- and summertime samples were the most potent, whereas the Prague wintertime sample was the least potent inducer of inflammatory responses. In $PM_{10-2.5}$, the Duisburg and Helsinki samples exhibited somewhat lower inflammatory potencies than the other samples in the same size-range (Figure 4).

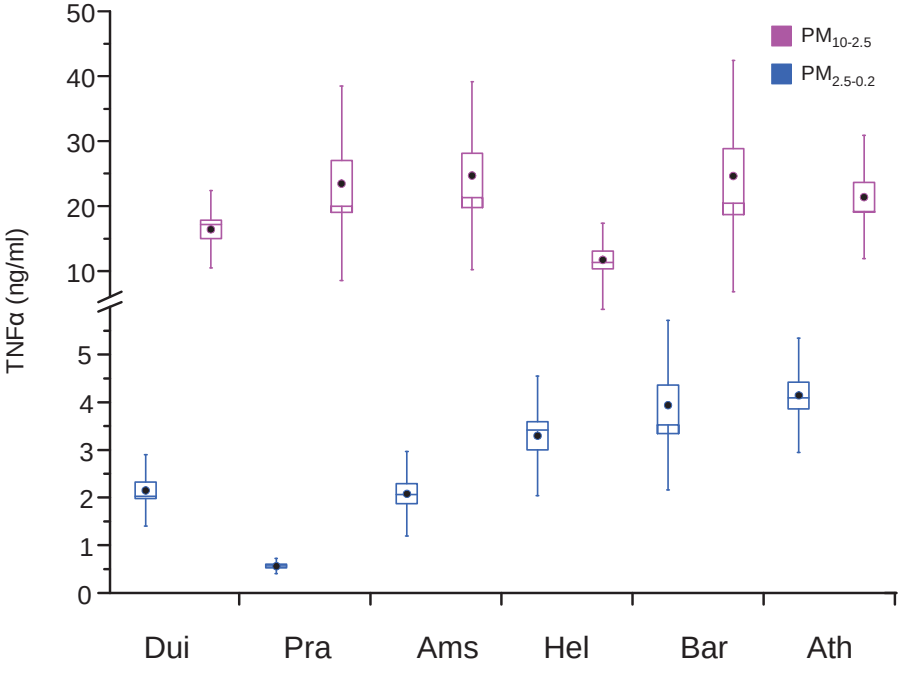

Figure 4. TNFα –concentrations (ng/ml) produced by mouse RAW 264.7 macrophages in response to a 24-h incubation with the $PM_{2.5-0.2}$ and $PM_{10-2.5}$ samples (150 µg/ml) from Duisburg (Dui), Prague (Pra), Amsterdam (Ams), Helsinki (Hel), Barcelona (Bar) and Athens (Ath) sampling campaigns. The dot is the arithmetic mean response (n=18 from Studies III - V), and the vertical line shows the median, box the standard error (SEM), and the whiskers 5th and 95th percentiles of the response. Note the different scale for responses to $PM_{10-2.5}$ samples.

All $PM_{10-2.5}$ samples had higher cytotoxic potencies than the $PM_{2.5-0.2}$ samples. However, there was more heterogeneity in the potency of the $PM_{2.5-0.2}$ than the $PM_{10-2.5}$ samples. In $PM_{10-2.5}$ size range, the Prague sample was the most potent, whereas in $PM_{2.5-0.2}$ the Athens sample had the highest cytotoxic potency (Figure 5).

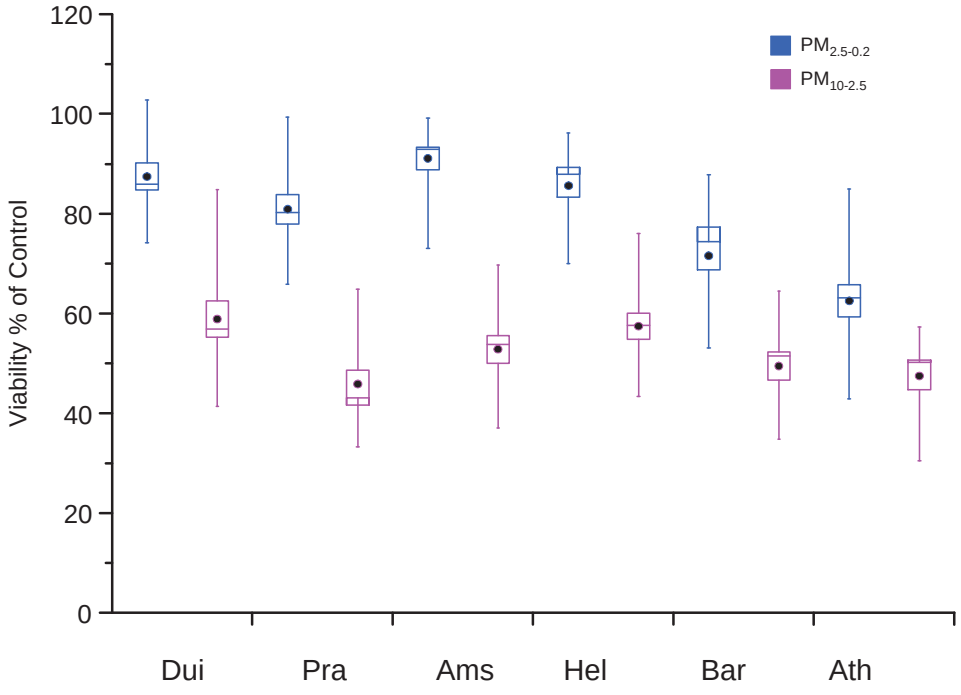

Figure 5. Cell viability (% of unexposed control) of RAW 264.7 macrophages assessed using the MTT-test after a 24-h exposure to the $PM_{2.5-0.2}$ and $PM_{10-2.5}$ samples (150 µg/ml) of Duisburg (Dui), Prague (Pra), Amsterdam (Ams), Helsinki (Hel), Barcelona (Bar) and Athens (Ath) sampling campaigns. The dot is the arithmetic mean response (n=18 from Studies III - V), and the vertical line shows the median, box the standard error (SEM) and the whiskers 5th and 95th percentiles.

$PM_{0.2}$ samples from the six European cities induced negligible inflammatory responses. However, the largest dose (300 µg/ml) of the samples in this size range did induce significant cytotoxicity. This dose of the Prague wintertime $PM_{0.2}$ sample induced the largest cytotoxic response of all the studied particulate samples in all size ranges (data not shown) (III).

5.4.3. Effects of solubility (IV)

The inflammatory and cytotoxic activities of the water-soluble and -insoluble as well as organic-solvent-soluble and –insoluble fractions of the urban air $PM_{2.5-0.2}$ and $PM_{10-2.5}$ particulate samples exhibited great diversity (Table 10). Generally, the responses to the soluble fractions were rather minor and the effects were mainly caused by the insoluble fractions. There was, however, some inflammatory activity associated with the soluble fractions of the Prague wintertime $PM_{2.5-0.2}$ sample. It was remarkable that in many cases removal of either of the two soluble fractions from the particulate samples increased the responses to the remaining non-soluble fractions. However, dichloromethane extraction decreased the response to the Prague $PM_{2.5-0.2}$ sample.

Table 10. The relative inflammatory (TNFα) and cytotoxic activities of particulate fractions of varying solubility prepared from the six-city fine and coarse size range samples at a fixed dose of 150 µg/ml. The value 1 is given for total suspension (TS) of the sample within each sampling campaign and size range. The responses to the water-soluble (WS)/ organic-solvent-soluble (DS) and the respective non-soluble fractions (NWS/NDS) are calculated as ratios to the respective TS response.

		$PM_{2.5-0.2}$						$PM_{10-2.5}$					
Endpoint	Fraction	Dui	Pra	Ams	Hel	Bar	Ath	Dui	Pra	Ams	Hel	Bar	Ath
Inflammation	TS	1	1	1	1	1	1	1	1	1	1	1	1
	WS	0.07	0.37	0.07	0.03	0.04	0.07	0.04	0.02	0.02	0.03	0.03	0.03
	NWS	1.30	1.38	1.55	1.04	1.42	1.25	0.86	0.99	1.14	0.93	1.32	0.93
	DS	0.11	0.30	0.07	0.08	0.07	0.07	0.06	0.02	0.03	0.03	0.02	0.03
	NDS	1.60	1.26	0.62	0.58	1.07	0.64	0.44	1.35	1.01	1.16	0.81	0.94
Cytotoxicity	TS	1	1	1	1	1	1	1	1	1	1	1	1
	WS	0.18	0.01	2.55	nd	nd	0.01	nd	0.03	0.03	nd	nd	0.04
	NWS	1.44	1.61	11.22	2.92	1.35	1.40	1.12	1.18	1.38	1.22	1.03	1.09
	DS	1.12	nd	0.78	nd	nd	0.47	nd	nd	nd	nd	0.01	0.19
	NDS	1.59	0.26	1.40	1.15	1.58	1.92	1.00	1.29	0.96	0.74	1.16	1.14

nd = no detectable cytotoxicity

5.4.4. Effects of chemical composition (IV-V)

There were frequently more associations of the inflammatory and cytotoxic responses with the chemical composition in the $PM_{2.5-0.2}$ than the $PM_{10-2.5}$ size range. The chemical composition also varied more between the sampling campaigns with respect to the $PM_{2.5-0.2}$ samples than the $PM_{10-2.5}$ samples.

All the correlation coefficient values between the chemical constituents in $PM_{2.5-0.2}$ and the TNFα or cytotoxic response are shown in Figure 6. Several transition metals were in the quarter of positive correlations with both inflammation and cytotoxicity, as were the markers of photo-oxidation of the organics in the atmosphere (oxalate, malonate, succinate). Also the soil components, Ca^{2+}, and insoluble Ca Fe, Al and Si were located in the same quarter.

An opposite effect of negative correlations with both inflammation and cytotoxicity was seen with markers of incomplete combustion of solid fuels. Wood combustion indicators (ΣMA, K^{+}) and a coal combustion indicator (As) were placed in the lower left corner with regard to both response parameters. PAH-compounds displayed a negative correlation with inflammation but a positive correlation with cytotoxicity (Figure 6).

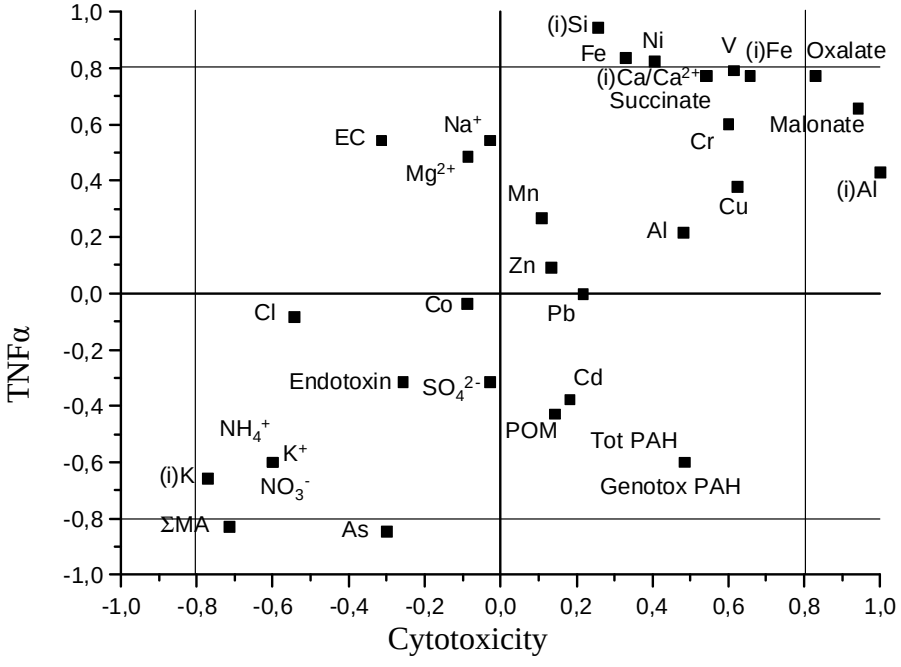

Figure 6. Scatterplot of Spearman's correlation coefficients (ρ) between the chemical constituents and cellular responses to the $PM_{2.5-0.2}$ samples from six European sampling campaigns. X-axis shows the negative and positive correlations between the constituents and cytotoxicity, and Y-axis those between the constituents and TNFα concentrations. Thin lines show the level of statistical significance (0.8/-0.8). (i) means modeled insoluble fraction of some constituents.

In the $PM_{10-2.5}$ size range, there were much fewer significant or nearly significant correlations between the chemical constituents and the inflammatory and cytotoxic responses than in $PM_{2.5-0.2}$. Furthermore, no consistent groups of

chemical constituents representing some specific particulate source could be identified (Figure 7). The vast majority of the correlations were relatively weak.

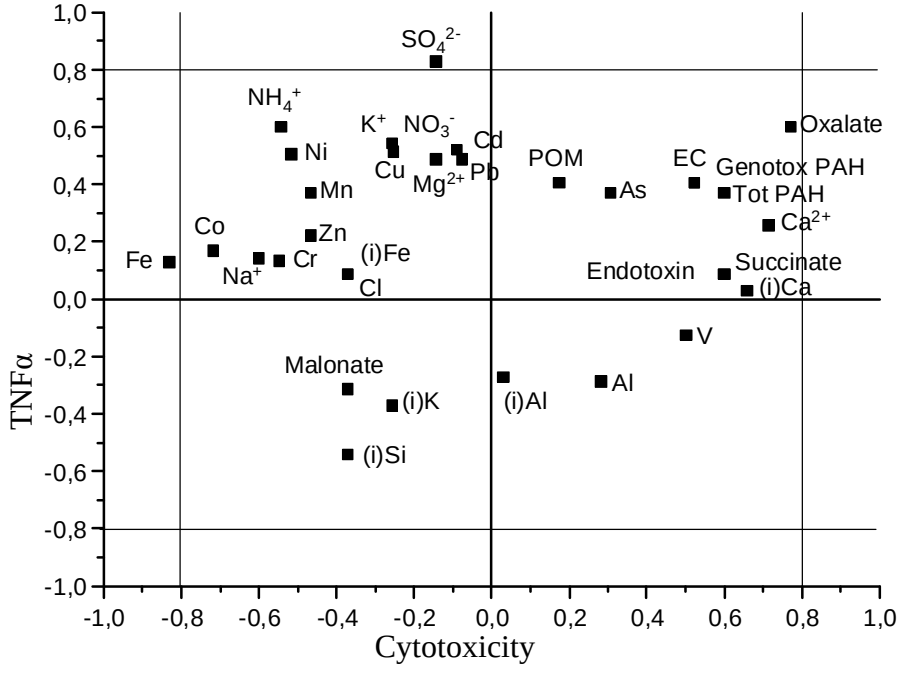

Figure 7. Scatterplot of Spearman's correlation coefficients (ρ) between the chemical constituents and the cellular responses to the $PM_{10-2.5}$ samples from six European sampling campaings. X-axis shows the negative and positive correlations between the constituents and cytotoxicity, and Y-axis those between the constituents and TNFα concentration, respectively. Thin lines show the levels of statistical significances (0.8/-0.8). (i) means modeled insoluble fraction of some constituents.

5.4.5. Relative toxic activity of particulate samples (II, III)

Most of the results shown in the present studies on the comparison of the toxic activities of the fine and coarse particulate samples within each size range were made at the same fixed mass dose as used in macrophage exposures. This method was independent of the actual particulate mass concentration in the outdoor air during the sampling campaigns. In Studies II and III, the toxic activities from the macrophage exposures were weighted with the campaign-mean mass concentration of the respective size-range particles in cubic meters of outdoor air (Table 11).

Table 11. Size-segregated particulate mass concentrations in urban air during the sampling campaigns and the relative inflammatory and cytotoxic activities of particulate samples in the respective size-ranges.

	Sampling	PM mass concentration	Relative response per µg of PM mass | per m^3 of air									
	Campaign		NO		IL-6		TNFα		MIP-2		Cytotoxicity	
$PM_{0.2}$	Seasonal	1.9	**1.6**	1.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.7	1.0
	Wildfire	**5.4**	1.0	**1.8**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.0	1.6
	Mixed	3.3	1.2	1.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**1.8**	**1.8**
$PM_{1-0.2}$	Seasonal	3.4	**1.7**	1.0	**5.5**	1.0	**4.5**	1.3	**10.1**	3.0	**1.8**	1.0
	Wildfire	**16.2**	1.0	**2.8**	1.1	**1.9**	2.2	**3.1**	2.6	**3.7**	1.2	**3.3**
	Mixed	11.5	1.0	2.0	1.0	1.3	1.0	1.0	1.0	1.0	1.0	1.9
$PM_{2.5-1}$	Seasonal	1.5	1.3	1.0	5.0	1.0	3.0	1.0	5.1	1.0	**1.3**	1.0
	Wildfire	**7.3**	1.0	3.6	1.0	1.0	1.0	1.6	1.0	1.0	1.0	**3.8**
	Mixed	5.5	**1.9**	**5.3**	**19.1**	**14.4**	**4.1**	**4.9**	**7.3**	**5.5**	1.1	3.1
$PM_{10-2.5}$	Seasonal	5.3	**1.5**	1.0	**1.9**	1.2	**1.8**	1.1	**1.4**	1.0	**1.2**	1.0
	Wildfire	**10.3**	1.2	**1.5**	1.4	**1.6**	1.2	**1.4**	1.0	**1.4**	1.2	**1.9**
	Mixed	8.8	1.0	1.1	1.0	1.0	1.0	1.0	1.0	1.2	1.0	1.4
$PM_{0.2}$	Dui	2.8	1.2	1.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	15.5	22.5
	Pra	4.7	**1.7**	**2.4**	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**32.8**	**47.7**
	Ams	3.8	1.1	1.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.4	7.8
	Hel	2.7	1.5	1.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.0	1.0
	Bar	4.5	1.0	1.3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4.6	6.7
	Ath	**6.7**	1.2	2.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.1	1.0
$PM_{2.5-0.2}$	Dui	15.8	1.0	1.9	6.7	4.2	3.7	2.3	2.2	1.4	1.0	1.1
	Pra	**25.1**	**1.4**	**4.2**	1.0	1.0	1.0	1.0	1.0	1.0	2.2	3.8
	Ams	22.8	1.0	2.8	5.9	5.3	3.4	3.1	1.8	1.6	1.3	2.0
	Hel	8.3	1.0	1.0	3.3	1.1	5.0	1.6	4.3	1.4	1.8	1.0
	Bar	14.3	1.1	1.9	17.6	10.0	7.8	4.4	3.5	2.0	2.5	2.4
	Ath	18.9	1.2	2.8	**17.6**	**13.2**	**7.8**	**5.9**	**9.0**	**6.8**	**3.7**	**4.8**
$PM_{10-2.5}$	Dui	7.6	1.3	1.2	2.0	1.2	1.0	1.0	1.0	1.0	1.2	1.1
	Pra	5.9	1.4	1.0	2.7	1.3	2.3	1.8	**3.1**	2.4	**1.4**	1.0
	Ams	9.8	1.9	2.2	**3.5**	2.6	**2.5**	3.2	2.6	3.3	1.2	1.5
	Hel	12.8	1.0	1.5	1.0	1.0	1.0	1.7	1.2	2.0	1.0	1.6
	Bar	22.9	1.9	5.2	3.1	5.6	2.4	**7.1**	2.3	6.9	1.2	3.3
	Ath	**29.6**	**1.9**	**6.8**	2.8	**6.5**	1.8	6.9	2.4	**9.3**	1.1	**4.1**

In the left column for each studied parameter, the value 1 is given to the smallest response to an equal mass dose (150 μg/ml) of coarse ($PM_{10-2.5}$), fine ($PM_{2.5-0.2}$ or $PM_{2.5-1}$), accumulation ($PM_{1-0.2}$) and ultrafine ($PM_{0.2}$) particulate samples. In the right column for each parameter, a similar comparison of toxic activities is made for particulate mass per cubic meter of urban air in the respective air pollution situation. Further, n.a means not applicable for this comparison due to negligible toxic activity. Values in boldface are the largest response within each studied parameter and particulate size range.The values are comparable only within each study, i.e. Study II in grey background and Studies III-V in white background.

6 DISCUSSION

There were major differences in the immunotoxic responses of particulate samples from different air pollution situations. The greatest inflammatory responses to the samples were observed with the coarse particulate samples, followed by fine samples, whereas the ultrafine particles induced negligible responses. Acute cytotoxic and apoptotic responses were not dependent on each other in the different particulate size ranges. Most of the responses were associated with the insoluble particulate fraction. The local sources of incomplete combustion and traffic related resuspension dust were the most harmful sources for particulate air pollution associated with the detected responses. This study did not attempt to identify any single chemical components present in the particulate matter that are responsible for the effects. Instead, some groups of components, originating from the same source or acting simultaneously in certain air pollution situations, were identified.

6.1 Validation of high volume particulate sample treatment and toxicological methods (I)

Several previous studies have used water (Gilmour et al. 2007; Monn and Becker 1999), water/methanol (Hetland et al. 2004; Janssen et al. 2008) or water/ethanol (Becker et al. 2003; Duvall et al. 2008) combination as an extraction method from the sampling substrates and are, therefore, not fully comparable with the present study. During water extraction, in particular the extraction of lipid-soluble organic compounds was poor. Methanol, unlike water, wets thoroughly the PUF sampling substrate and allows for a high extraction efficiency of the particulate mass. It gives rise however to the question, whether

the sample composition and consequent biological responses could be modified by methanol. The present results showed that the modifying effect was very minor, though there was some increase in the recovery of water-soluble metals from the sampling substrates by methanol extraction. Furthermore, some modification of the biological responses could be seen after exposure to emission particulate samples, rich in organic compounds. The responses to urban air samples were not modified to any significant extent. This, and the gained high extraction efficiency, emphasized the usefulness of methanol extraction from the HVCI sampling substrates.

Likewise, it was important to use a suspension vehicle that did not substantially change the chemical composition of the collected and extracted particulate sample. Several previous studies have used saline or cell culture medium for the sample suspension (Hetland et al. 2004; Pozzi et al. 2003; Imrich et al. 2000; Monn and Becker 1999). There is, however, a possibility that liquids containing salts, may interact with the similar types of compounds in the samples. In this set of studies, water was used for the sample suspension. However, sonication is needed to attain a homogenous suspension of the particulate samples in water. This is extremely important with samples rich in poorly water-soluble organic compositions. The sonication time used, 30 min, provided an effective suspension of urban air particulate samples, but did not modify their cellular effects.

It is also important to be aware of the appropriate time-points for each study endpoint in order to be able to obtain reliable results on the toxic properties of particulate samples, collected at different locations and times. Study I clearly highlighted the importance of using appropriate timepoints in the analysis of toxicological responses. A similar finding has also been made in the *in vivo* study of Happo et al. (2007) investigating the inflammatory effects of the present

size-segregated particulate samples from six European cities on the mouse lung. By using relevant timepoints and the same response parameters, it was possible to obtain remarkably similar results in both the cell and animal studies.

6.2 Heterogeneities in urban air particulate induced responses (II-V)

6.2.1 Effect of particle size

Considerable heterogeneities between particulate size-ranges were found both in the Helsinki study (II) and the European six-city study (III). In most cases, the inflammatory activity increased with particulate size. Thus, $PM_{0.2}$ samples were the least potent and $PM_{10-2.5}$ samples the most potent inducers of inflammatory responses. A similar pattern has been noted also in several of previous studies (e.g. Hetland et al., 2005; Dybing et al., 2004; Becker et al., 2003). In addition, acute cytotoxicity was somewhat particle size-dependent, but no such systematic effect was seen with apoptosis. Previous studies (Monn and Becker 1999; Osornio-Vargas et al. 2003) have also shown greater cytotoxicity with coarse than fine particles. Moreover, in the study of Hetland et al. (2004), urban air coarse and ultrafine particles were more cytotoxic than fine particles. In the present study, a similar pattern was seen with respect to apoptosis.

6.2.2 Effect of air pollution situation

The air pollution situation had a major effect on the reponses of the macrophage. Long-range transport episodes of air pollution were associated with lower inflammatory potency of particulate samples than the seasonal average samples collected from Helsinki. Nonetheless, it was remarkable that the episode

samples were associated with enhanced inflammatory and cytotoxic activity per cubic meter of outdoor air due to the increased mass concentrations. Consequently, this finding may have public health implications. Indeed, agricultural fires and uncontrolled wildfires are an increasing problem that are predicted to have public health consequences on a global scale (Naeher et al. 2007). This is also challenging for policy-makers, since transboundary air pollution can pose health risks to large populations living even thousands of kilometers distant from the fires.

The results from the six European cities showed that the prevailing sources of urban air particulate pollution were associated with clear heterogeneities in the inflammatory and cytotoxic activities of especially the urban air $PM_{2.5-0.2}$ samples. It should be noted, however, that the results are from samples taken during different seasons in the sampling campaigns. Therefore, they cannot be generalized and do not represent an overall air pollution situation of any specific city, but rather different mixtures of particulate matter originating from different emission sources and atmospheric transformation related to the selected seasons.

Samples from springtime Barcelona and summertime Athens $PM_{2.5-0.2}$ and $PM_{10-2.5}$ were associated with the highest inflammatory potency. In these two campaigns, there was the largest impact of photo-oxidative transformation of the organic compounds in the atmosphere and this may have enhanced the toxic activities of the particulate samples, e.g. via increased oxidative stress in exposed tissues. The Prague samples, that were determined to reflect the heavy use of solid fuels (coal, biomass) in domestic heating, displayed a distinctive toxicity profile when compared to samples from the other sampling campaigns. These samples showed a high cytotoxic and apoptotic potency, and a low inflammatory potency. Furthermore, the ultrafine particulate samples from Prague were unique, in that they were able to cause cell cycle arrest in

macrophages. According to these data, it is obvious that emission sources play an important role in the heterogeneity observed in the particulate-induced response in different locations and seasons. Previously, distinct sources and chemical composition have also been associated with varying *in vitro* and *in vivo* inflammatory, cytotoxic and allergy adjuvant responses to particulate samples, collected across Europe (Steerenberg et al. 2006). Moreover, local traffic sources have been found to modify several inflammatory and other toxicity enpoints in the rat lung (Gerlofs-Nijland et al. 2007). Temporal differences in the activity of certain particulate sources have also partly explained the observed seasonal variation seen in previous toxicological studies (Becker et al. 2005; Dybing et al. 2004; Monn et al. 2003) and epidemiological studies in the United States (Peng et al. 2005) and Europe (Samoli et al. 2001).

Weighing of the results with respect to toxic activity at one representative mass dose with particulate mass concentration in the outdoor air was conducted in order to achieve a more realistic and real-life consideration of their significance. This approach changed considerably the results on the experiments with fixed mass. Episodic events of long-range transport increased the mass concentration of particles present in the outdoor air. These events most likely pose an increased health risk despite the fact that the toxic potency of particulate mass per mass unit may well have been reduced due to aerosol aging. Moreover, smaller size-range particles may have larger toxic potential than coarse particulate material in the real-life situation due to their considerably larger mass concentrations in the air in most sampling sites.

6.2.3 Effect of solubility

Data on the inflammatory and cytotoxic effects of the water-soluble, organic-solvent-soluble and insoluble particulate fractions in the macrophages clearly indicated that urban air particulate fractions of different solubilities displayed large differences in their toxic activities. The responses were dominated by the water-insoluble and organic-solvent-insoluble particulate fractions prepared from the $PM_{2.5-0.2}$ and $PM_{10-2.5}$ samples. In previous studies, there have been similar findings, showing that the insoluble particle components are mostly responsible for the inflammatory mediator production in the human (Soukup and Becker 2001; Huang et al. 2004), mouse (Ning et al. 2000) and rat (Imrich et al. 2000; 2007) alveolar macrophages However, macrophages are only one cell type in the defence system though these cells do specialize in the uptake and clearance of the larger fine particles and coarse thoracic particles. Consequently, responses to the soluble particulate fractions in other cell types cannot be excluded. Furthermore, it is possible that insoluble particles act as carriers, especially for lipid-soluble compounds attached on their surfaces.

6.3 Potentially harmful source environments and compositions (IV, V)

6.3.1 Ultrafine particulate matter

Ultrafine particles originate mostly from local combustion sources in urban environments and their lifetime in the atmosphere is limited due to their fast growth into accumulation size-range. Consequently, ultrafine particles may have

a strong local impact on human exposure and health effects, although there is still a lack of convincing evidence for that assumption. Overall, the inflammatory responses to $PM_{0.2}$ samples were minimal in the present studies, which can be partly explained by the macrophage cellular properties which are not conducive to phagocyting these small sized particles but also be methodological issues – the $PM_{0.2}$ extracts were filtered to remove glassfibre fragments. However, ultrafine particles caused a similar magnitude or even larger cytotoxic and apoptotic responses compared to fine particulate matter. This provides some insight into the different mechanisms related to ultrafine particulate exposures and effects. Correlations of the responses with the chemical composition of the ultrafine particles were not calculated, due to their negligible inflammatory responses.

$PM_{0.2}$ samples from Prague had a high apoptotic activity, and this seemed to be coupled with their ability to cause cell cycle arrest in macrophages. Coal combustion is a known source of PAH-compounds. The observed immunosuppressive effects, combined with cell cycle effects are well in line with previous findings on PAHs (Binkova et al 2003). Moreover, it is known that PAH compounds can evoke a significant cellular oxygen radical formation if they are present in ultrafine particles (Li et al. 2003). Moreover, ambient ultrafine particles induced oxidative stress related DNA-damage (Bräuner et al. 2007). When combined with genotoxicity, this kind of effect may lead to increased risk of cancer. The Prague samples in all size-ranges had the highest contribution of PAH compounds to particulate mass found in the sampling campaigns, which was clearly associated with the known heavy use of coal and solid biomass in domestic heating.

6.3.2. Fine particulate matter

The chemical composition of particulate mass in the $PM_{2.5-0.2}$ size range displayed the largest differences between sampling campaigns. Most of the mass in this size range are accumulation particles, but there are also lower end coarse particles present. Thus, this size-range contains a variable mixture of local source as well as regional and long-range transported particles.

Some potentially harmful constituent combinations or sources were discovered in the present studies, mostly in association with $PM_{2.5-0.2}$. These sources were mostly identified with known tracers of the emission sources. Biomass combustion (ΣMA) contributed clearly to the sampled particles both in Prague and Helsinki. Distant wildfires increased episodically the fine particulate mass concentration in Helsinki, and consequently the toxicity of outdoor air. In fact, as reviewed by Naeher et al. (2007), biomass combustion from residential use and wildfires is a major emission source, affecting the air quality of large populations all over the world. However, the relative toxicity of these particles has not been clarified in comparison to other emission sources, e.g. diesel exhaust particles. The present results on ΣMA and PAHs agree with previous findings that have suggested diminished inflammatory and increased cytotoxic responses to appear when there is a large contribution of particles derived from small-scale biomass combustion (Happo et al. 2007; Seagrave et al. 2006). The observations point to an immunosuppressive effect.

In Prague, also local small-scale coal combustion (As, PAHs) affected the air quality and the $PM_{2.5-0.2}$ sample was associated with responses which were different from the respective samples of all the other sampling campaigns. It has been previously observed in Dublin that small-scale coal combustion has an

association with both mortality and morbidity (Clancy et al. 2001). In Prague, there was a heavy use of both coal and biomass in domestic heating during the sampling campaign. Due to that fact, that the highest concentrations of PAHs and Arsenic were measured in the samples of the Prague campaign, i.e. the role of coal use was obvious. The present results indicate that poorly controlled small-scale combustion of solid fuels may lead to increased toxicity in the inhaled particles, which may substantially increase the risk of adverse health effects in susceptible population groups.

Fuel oil combustion (Ni, V) was one of the most prominent sources that emerged from different sampling campaigns. Its contribution seemed to be largest in warm seasons and associated with the photo-oxidation of the organics (DA). Together with Ni and V the DAs (oxalate, succinate and malonate) formed a group of components that had the most consistent high positive correlations with the inflammatory activity of $PM_{2.5-0.2}$ samples. This mechanism may well be the main explanation for the high inflammatory activity of $PM_{2.5-0.2}$ samples from the Mediterranean sampling campaigns.

Photochemical transformation of organic compounds may lead to highly active compounds, e.g. formation of quinoid substances from the PAHs (WHO-IPCS 1998). Quinones are hypothesized to be efficient producers of reactive oxygen species in particle-exposed cells (Squadrito et al. 2001). The observed negative associations between the PAH content and induced inflammatory responses suggest that PAH-compounds can inhibit macrophage function as previously reported (Van Grevenyngche et al. 2004).

It is likely that the high metal concentrations in the Duisburg $PM_{2.5-0.2}$ samples originated from long-term industrial metal emissions. However, these samples were by no means exceptionally toxic. Some possibly metal-induced effects were detected when the effect of particulate fractions with different

solubilities were studied. When combined with the fact that there was a trend for some metals (Fe, Cu, Cr) to be positively associated with both cytotoxicity and inflammation, the role of water-soluble transition metals from industrial sources, could not overruled. Another potential source of metals is traffic. For example. Zn, Cu, Fe and Cd can originate from car tires, brakes and clutches (Hjortenkarns et al. 2007; Yli-Tuomi et al. 2005).

Both traffic-related combustion and resuspension dust are important sources of particulate pollution in urban areas. Traffic-related combustion releases large amounts of carbonaceous material, but also is a source for metals, PAHs and gaseous pollutants. In Study V, EC was associated with increased production of inflammatory mediators. Traffic-related combustion has shown stronger correlation with health and mortality in epidemiological studies. Moreover, in the recent study of Gerlofs-Nijland et al. (2007), traffic sources contributed substantially to the inflammatory, cytotoxic and blood coagulation parameters when samples were administrated to rats. Moreover, Fe and especially Cu have a strong Fenton-reactivity (Shi et al. 2003), which indicates their oxidative properties.

Soil components (Ca, Al, Fe, Si) in $PM_{2.5-0.2}$ samples may originate from traffic-related resuspension dust. These soil components were associated with increased inflammatory activity. In addition to traffic-related resuspension, soil components can enter to the atmosphere as windblown dust from non-vegetated areas, construction sites etc. Consequently, local soil composition may make a substantial contribution to the particulate material present in the outdoor air. The results on soil components agree with the *in vivo* findings of Happo et al (2008) as well as with those from some other previous studies (Steerenberg et al. 2006; Veranth et al. 2006). A low-level presence of soil-derived components in the fine particulate size range is a common feature in urban atmospheres (Putaud et

al. 2004; Vallius et al. 2005), this kind of solid material can partly explain the detected toxic properties of the dry season (spring, summer) $PM_{2.5-0.2}$ samples.

6.3.3. Coarse particulate matter

Coarse particles are mostly of local origin. The chemical composition of the $PM_{10-2.5}$ samples in the present study was the most uniform of all of the size ranges. Soil minerals and biogenic material make large contributions to particulate mass in this size range. Since the composition of coarse particles and the toxic responses varied less than with the $PM_{2.5-0.2}$ samples, there were also fewer significant or nearly significant correlations between the chemical constituents and the response parameters in this size range.

There were much fewer high correlations of the inorganic water-soluble or insoluble constituents with the toxicological responses than with the $PM_{2.5-0.2}$ samples. Moreover, the detected correlations were not consistent with respect to all the toxicological endpoints. A similar observation has recently been reported by Duvall et al. (2008). However, the inflammatory potency of the $PM_{10-2.5}$ samples was generally much higher per unit of mass than that of the $PM_{2.5-0.2}$ samples (Study IV). This may be due to the fact that the contribution of insoluble soil-derived particulate material to $PM_{10-2.5}$ has been much higher (Sillanpää et al. 2006; Study IV).

Despite the small amount of significant correlations between constituents and toxic responses, the role of urban air coarse particles should not be underestimated. These particles were associated with high inflammatory, cytotoxic and apoptotic properties. In fact, there is epidemiological evidence that

urban air coarse particles may provoke exacerbations of respiratory disease even more strongly than fine particles (Brunekreef and Forsberg 2005).

Gram-negative bacterial endotoxin has been proposed as one of the most harmful compounds present in outdoor air particulate matter. Endotoxin has been more abundant in coarse particles compared to fine particles (Heinrich et al. 2003). However, endotoxin did not emerge in the present results as a highly potent component, which is in line with recent findings of Duvall et al. (2007). The LAL-assay is an indirect method and its specificity has not been fully confirmed. Gram-negative bacterial endotoxin is only one biological component of the large (up to 25%) amount of biological material which contributes to the coarse particulate matter (Jones and Harrison 2004). A large proportion of the $PM_{10-2.5}$ mass (14-49%) remained unidentified in the present studies. This may, at least partially, be due to the non-identification of the biological fraction in the particulate mass.

6.4 Methodological considerations in toxicology (I-V)

As previously stated, macrophages are the primary cells defending against particulate exposure. Although, cell studies will never fully correspond or be completely analogous to animal studies, these bioassays can have clear relevance when predicting responses in the whole organism. Macrophages are an important cell type in the primary inflammatory responses especially in innate immunity and this justifies their use in studies on urban air particles. Inflammation is regarded as the main mechanism mediating the impairment of cardiorespiratory diseases in association with exposure to particulate pollution.

Other cell types, like respiratory epithelial cells, are also important in maintaining inflammatory responses. However, it is believed that to a large

extent these cells make a secondary contribution to the overall inflammatory response of the lungs, which is initiated by other cells. Epithelial cells are also widely used in cell studies on particulate air pollution, and they may be more sensitive to some soluble constituents present in particulate samples.

One approach is to use primary cells in the *in vitro* studies. This, however, always requires the use of animals or human subjects for cell collection. The primary cells would most probably be more sensitive and require to be exposed to less particulate mass. However, studies in primary cells do not have as good repeatability as studies conducted on cell lines due to the variable exposure histories of the donors. Moreover, primary cells may lose their viability during experimentation sooner than a secondary cell line. Overall, there is a risk with primary cells that the responses to particulate samples will display a larger variation than that seen with cell lines. The above mentioned issues were considered when choosing macrophage cell line for the present studies.

The results are only from one cell type and they do not therefore necessarily represent the situation in the whole organism. However, the present results emphasizing the proinflammatory activity of the fine particulate samples from six European cities, and the responsible sources and constituents, agree well with the parallel studies of Happo et al. (2007; 2008) in the mouse lung, where there are interactions between different cell types as well as more effective up and down regulation of the various responses.

Apoptotic responses in this study were detected in a flow cytometric cell cycle analysis with PI-staining. This is an indirect method that has been used successfully in the determination of the proportion of the apoptotic cells in some previous studies (Darzynkiewicz et al. 1992; Penttinen et al. 2005). There are also direct methods, such as caspase analysis, which can be used for the investigation of apoptosis (Hughes and Mehmet 2003). This would have

required additional experiments and more particulate mass and, thus, they were not feasible in the present set of studies.

One important factor in many of the responses associated with particulate air pollution is oxidative stress. Additional experiments into this topic would have required more particulate mass and for this reason they could not be included in this present set of studies. Moreover, particulate material may give rise to strong autofluorescence, which would most probably have modified the results from both flow cytometric and fluorometric ROS-measurements. In addition to cellular ROS production, some particulate constituents are capable of intrinsically generating oxygen radicals (Øvrevik et al. 2006). However, reactive oxygen species may play an important role in many mechanistic pathways and should be measured in the future studies.

The largest particulate doses in the present study were high when compared to average doses in the human lungs under normal exposure conditions in urban air. However, surface doses of particles within the lungs can exhibit thousand-fold variations in respiratory patients due to the uneven particle deposition. According to Phalen et al. (2006), these variations can result in even larger maximal surface doses in the human lungs than the largest dose used in these cell culture experiments. Moreover, also other factors, like exercise, can increase the particle dose penetrating into the lungs.

One confounding factor to consider is the dose of $PM_{0.2}$ in the present cell studies. The masses of $PM_{0.2}$ samples were calculated from the net filter weights but in fact they represented mostly the water-soluble and lipid-soluble fractions, since it was necessary to include filtering step in the particulate sample preparation after extraction from glass-fibre filter.

7 CONCLUSIONS

The present thesis aimed at adding to our knowledge of the immunotoxic properties of urban air particles and their association with potentially harmful sources and their chemical compositions. On basis of this work, the following conclusions can be made:

1. A systematic approach was developed for the utilization of the size-segregated HVCI particulate samples in cell toxicology studies. A high extraction efficiency from the sampling substrates was achieved without any significant modification of the toxic properties of the urban air particles. The response parameters, indicated that 24 hrs was the optimal time for exposure of the RAW264.7 macrophages to the particulate samples. However, it is crucial that the optimal time-points for different response parameters are always tested in each new experimental setup. (I)

2. Dose-relationships of different shapes were observed for the inflammatory and cytotoxic responses to the urban air fine and coarse particulate samples collected from different air pollution situations. Coarse particulate samples had the highest inflammatory potency, followed by fine particles. Ultrafine particulate samples had negligible inflammatory activity, but there were occasional effects on cell viability as well as evidence of apoptosis and interruption of the macrophage cell cycle. The coarse and fine particulate samples from the Mediterranean spring and summer campaigns showed higher inflammatory potencies that the respective samples from other sampling sites. The fine and ultrafine particulate samples from wintertime Prague were highly cytotoxic and immunosuppressive. Cellular responses to fine particulate

samples displayed the largest heterogeneity in the dose-response relationships between the different air pollution situations. (II, III)

3. The insoluble fraction of both the fine and coarse particulate samples was responsible for most of the immunotoxic responses in macrophages. When tested separately, the water-soluble or organic-solvent-soluble fractions induced most often only minor inflammatory and cytotoxic responses. However, the role of soluble components in the whole lung and in real-life exposure situations should not be underestimated, since especially lipid-soluble organic compounds tend to be bound to solid carbon particles. Moreover, macrophages are specialized in the uptake and clearance of the larger fine particles and coarse thoracic particles, whereas other cell types such as epithelial and endothelial cells may also be involved in the maintenance of chronic inflammation occurring with long-term exposures. (IV)

4. The immunotoxic properties of the particulate samples were highly dependent on the chemical composition and prevailing sources. Photo-oxidized organic compounds and transition metals (most clearly Ni and V) originating from fuel oil combustion, had consistent positive associations with the inflammatory activity of fine particulate samples in the macrophages. PAH-compounds from incomplete biomass and coal combustion were primarily associated with cytotoxicity. The present results suggest that local sources of incomplete combustion of biomass and coal, local fuel oil combustion especially in warm seasons, traffic-related resuspension dust, and long-range transport of forest fire smoke particles are important contributors to the toxic properties of the particulate material all over Europe. However, they may exert their toxic effects via a wide variety of mechanisms. (II, IV, V)

Overall, the extensive toxicological and chemical characterization of the complex urban air particulate mixture revealed interesting new findings on the role of different emission sources and atmospheric processes in particulate composition and immunotoxic effects. This information may well be useful in the evaluation of health risks associated with urban particulate air pollution. Moreover, the variations in particulate composition and toxic responses may help to explain some of the heterogeneities in adverse health effects reported in epidemiological studies. The potentially harmful compositions and sources of urban air particles should be considered when planning future legislation and effective abatement measures to improve urban air quality. Local traffic and incomplete combustion sources should be given special attention in efforts to reduce harmful particulate air pollution.

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