Miriam Nijland

TRAFFIC-RELATED AIR POLLUTION – THE HEALTH EFFECTS SCRUTINIZED

Traffic-related air pollution – the health effects scrutinized

Miriam Nijland

M.E. Nijland
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Traffic-related air pollution – the health effects scrutinized

Gezondheidseffecten en luchtverontreiniging – het kan verkeren
(met een samenvatting in het Nederlands)

Proefschrift

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door Miriam Elisabeth Nijland

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Promotoren: Prof. dr. F.R. Cassee
Prof. dr. ir. B. Brunekreef

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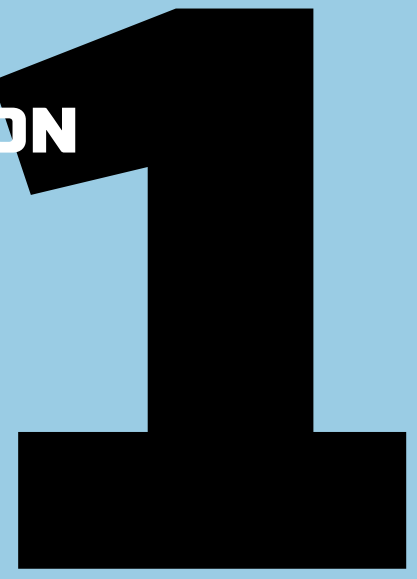
CHAPTER 1

GENERAL INTRODUCTION



Air pollution and air quality

Air pollution is a complex mixture of particulate and gaseous components originating from both natural and anthropogenic sources. The evidence for adverse health effects of airborne particles is well established and even observed at low pollution levels. The risk of air-pollution-related health effects is small compared to, for example, smoking. However, we all breathe the air and therefore entire populations are affected and on global scale air pollution is still a problem of major concern. Protection of citizen's health against the harmful effects of air pollution is one of the main rationales to develop air pollution policy.

Substantial research has been performed in the field of air pollution and although a lot of progress has been made, a number of key questions remain unanswered. On the other hand, our understanding of the air pollution mixture is increasing. Air pollution consists of a wide range of pollutants, but can be simply classified as gaseous and particulate constituents. Airborne particulate matter (PM) is a major component of the air pollution mixture due to its recognised health impacts. Even as recent as 2010 exposure to ambient PM remains ranked as one of the top ten risk factors for ill-health globally (Lim et al., 2012). Furthermore, PM has been associated with the highest burden of disease (6,000 to 10,000 Disability Adjusted Life Years (DALYs) per million people) in six European cities among nine selected environmental stressors examined including exposure to second-hand smoke (Hänninen and Knol, 2011). As air pollution pose a clear health risk, air quality is routinely monitored by measuring a few air pollutants as indicators for the whole air pollution mixture. PM is one of such pollutant and both the mass of PM_{10} (particles with an aerodynamic diameter <10 μm) and $PM_{2.5}$ (fine PM; particles with an aerodynamic diameter <2.5 μm) are monitored. However, PM mass is only a crude indicator that does not account for the complex mixture of solid particles and droplets arising from multiple sources or formed in the air from precursor gases. Major contributors to the mass of PM are sulphate, nitrate, ammonia, sodium chloride, and mineral dust. However, from a toxicological perspective, these are not considered as the most harmful compounds of the PM mixture. Nevertheless, PM mass is used as a relatively simple metric that can be measured in air quality networks on a routine basis. In general, most, if not all, air-pollution-related health effects

can be controlled by using the present indicators, including PM, in air quality monitoring although other unmeasured pollutants may also be relevant in terms of health effects.

The World Health Organisation (WHO) has published air quality guidelines (AQGs; Table 1) for some specific air pollutants. These AQGs are designed to give global guidance to reduce the health impacts of air pollution, by providing policymakers with a useful tool for air pollution abatement policy (WHO, 2006). While AQGs are not legally binding standards, they are established through expert evaluation of scientific evidence (Krzyzanowski and Cohen, 2008). The EU Air Quality Directive, of which the Directive 2008/50/EC is the most recent, has set legally binding limits for atmospheric concentrations for specific pollutants. It is intended that these air quality limits (AQLs; Table 1) will lead to improved air quality and protect human health.

Table 1. WHO air quality guidelines (AQGs) and EU air quality limits (AQLs) set for different fractions of particulate matter (PM)

PM size	Period	Value (μg/m^3)	Type	Remark
PM_{10}	daily mean	50	AQL	≤35 exceedances/year
	daily mean	50	AQG	
	annual mean	40	AQL	
	annual mean	20	AQG	
$PM_{2.5}$	annual mean	25	AQL	to be met by 2015
	annual mean	20	AQL	to be met by 2020
	annual mean	10	AQG	
	daily mean	25	AQG	

PM_{10} – particles with an aerodynamic diameter <10 μm
$PM_{2.5}$ – particles with an aerodynamic diameter <2.5 μm

Air-pollution-related health effects

Both short- and long-term exposure to air pollution is associated with significant effects on public health. Increases in respiratory and cardiovascular mortality and morbidity are prominent outcomes of air pollution exposure, although the underlying mechanisms are not completely understood (Anderson et al., 2012; Brook et al., 2010). Both gaseous and particulate pollutants in the air pollution mixture contribute to the adverse health effects though the strongest associations are observed with PM (Miller et al., 2012). The type and severity of health effects associated with PM depend on several factors including the size and composition of the particles, the level and duration of exposure, and the gender, age and sensitivity of the exposed individual (Figure 1). Current scientific evidence cannot establish a threshold below which no health effects are seen (Brunekreef and Holgate, 2002; Pope and Dockery, 2006; WHO, 2006). Importantly, the positive associations

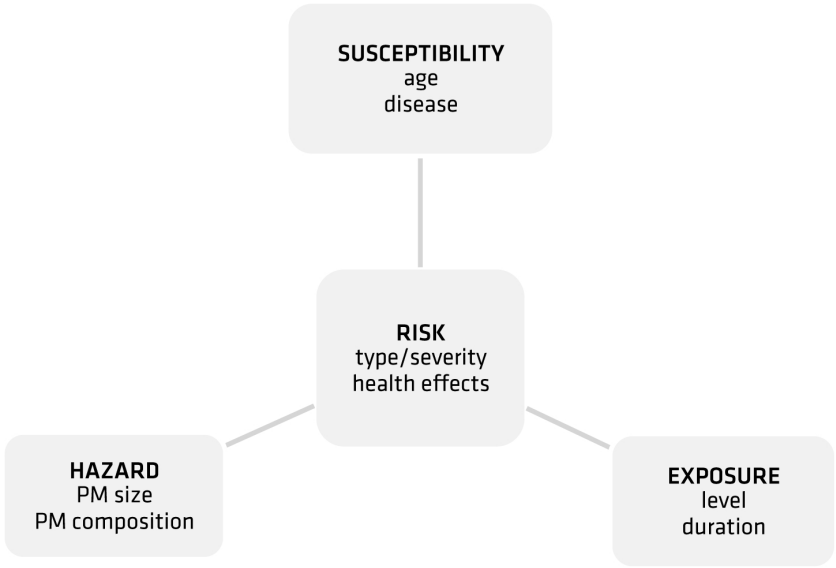


Figure 1. Factors influencing the risk of particulate matter (PM) within air pollution

shown to persist when PM levels are well below the current daily and annual ambient air quality limit values, suggesting that more stringent or alternative measures are required to further reduce the negative impact on human health, especially for susceptible individuals e.g. those with existing cardiopulmonary diseases (Wellenius et al., 2012). Accordingly, any reduction in air pollution is likely to benefit human health, in the same way that even low concentrations of ambient air pollution might pose a risk. In the past few decades, important improvements in air quality have been reached, yet it is important to maintain awareness of air-pollution-related health effects to diminish air pollution and related health risks. Continued media coverage has helped to keep air pollution an important topic for the general population, although it is arguable as to whether this awareness has translated into health benefits.

Ambient PM has been associated with a variety of respiratory outcomes such as asthma, impaired lung function, lung cancer and chronic obstructive pulmonary disease (Anderson et al., 2011; Curtis et al., 2006; HEI, 2010; WHO, 2006). Although these adverse pulmonary responses may be the most conspicuous effects of PM, in terms of mortality increasing evidence suggests that the cardiovascular effects of PM might represent the most significant PM-related public health burden (Brook et al., 2010). While initially surprising, the marked effects of PM on cardiovascular mortality are more readily understandable by simply considering the higher prevalence of cardiovascular disease in the population compared to respiratory disease (Miller et al., 2012). Both short- and long-term exposure to air pollutants has been associated to the occurrence of cardiovascular events (Brook et al., 2010; Franchini and Mannucci, 2012). For example, an increase in PM concentration of 10-20 $\mu g/m^3$ is associated with an increase of 0.5-2% in cardiovascular mortality (Miller et al., 2012), which could represent in the region of 12,600 number of premature deaths in the Netherlands. It is generally assumed that air pollution itself is not the cause of mortality but an underlying disease that might be worsen by air pollution exposure is. Thus while the immediate risk of air pollution might be small for a single individual, the pervasive nature of air pollution and high prevalence of cardiovascular disease represents a large burden on the population as a whole (Mateen and Brook, 2011).

A major shortcoming in this area of research is that outcomes are based on the "whole PM" mixture. In other words, this prevents policies directed

to the sources that emit the most toxic components of PM. Furthermore, focusing on PM alone will neglect the contribution of gaseous pollutants to the total adverse health effects. As implied above, it is highly unlikely that all PM components are equally toxic on a per mass basis. A cost-effective abatement strategy would benefit from knowing which fraction or source of emission contributes most to the observed health effects, allowing implementation of source specific measures or strategies to lower the toxicity of PM emissions. Source apportionment is also important for the elucidation of the transformation of chemical species within PM that occurs from generation to biological target; this is a vital step in bridging the gap from air pollution to source-related health effects (Solomon et al, 2012). Motorised traffic has been suggested as an especially important source of PM emissions and a major cause of air-pollution-related health effects. The evidence for this relationship is based on available source composition and the observed associations, e.g. black carbon, nitrogen dioxide and PM, and their associations with stroke (Wellenius et al., 2012) and a range of other health effects. However, other sources of PM emission should not be overlooked e.g. industrial processes and wood smoke.

Traffic-related air pollutants and health effects

Traffic is a major source of PM emission, especially of urban PM. Even ambient levels of PM in urban environments across the world can be harmful; however, the precise relationships involved have yet to be elucidated. Besides PM, combustion emissions contains carbon dioxide (CO_2), carbon monoxide (CO), hydrocarbons (HC), nitrogen oxides (NOx), as well as substances known as mobile source air toxics (MSATs), such as benzene, formaldehyde, acetaldehyde, and 1,3-butadiene. Both particulates and gaseous components of engine exhaust contain many mutagens, carcinogens and chemical species known for their toxic effects at low exposure levels. To what extent these pollutants are emitted by motor vehicles depends on many factors such as vehicle type and age, operating conditions, engine emission control technology, and the fuel and lubricant oil used.

Vehicular emissions are the predominant source of fine particulate matter in the urban environment (Robinson et al., 2010) and air pollution from traffic is clearly associated with the occurrence and exacerbation of respiratory disease (Laumbach and Kipen, 2012). The smaller, ultrafine, particles (diameter <0.1 μm) with PM appear to exert the greatest effects in terms of traffic-related cardiovascular effects (Peters et al., 2004; Tonne et al., 2007). For example, Lucking et al. (2011) show that the particulate fraction of diesel engine exhaust, a rich source of combustion-derived ultrafine or so-called nanoparticles, is especially relevant to thrombotic and vascular alterations.

Controlled human studies have greatly improved our knowledge of the role of traffic in causing adverse health effects. Short-term exposure (1-2 hours) to diesel engine exhaust in healthy volunteers causes inflammation in the airways (Behndig et al., 2006; Nordenhäll et al., 2000), induces an endothelial response leading to vasoconstriction (Peretz et al., 2008), and an impairment of vascular function (Barath et al., 2010; Mills et al., 2005). Altered vascular function seems to be a consistent property of diesel engine exhaust as similar impairment is seen after exposure of exhaust from either idling or city-cycling engines, despite the marked differences in chemical composition (Barath et al., 2010). Vascular dysfunction is diminished by

removing the particulates from the exhaust (Lucking et al., 2011; Mills and Miller et al., 2011) suggesting a role for the particulate fraction in the detrimental cardiovascular effects. The role of traffic-emission, and combustion-derived particles in general, in exerting adverse effects has been shown by the absence of adverse effects on vascular function following controlled human exposure to a predominantly non-carbon based ambient PM (Mills et al., 2008). Recently, other studies have indicated that it is not the particles per se, but the components absorbed onto the particle surface that play an important role in exerting these effects (Mills et al., 2011; Totlandsdal et al., 2012). Nevertheless, the role of composition still needs to be further elucidated. In particular, there is a need to compare the health effects caused by emissions from old and new engines, where the latter shows an improved combustion process and reduction in overall PM emission. Comparisons such as this are likely to strengthen our knowledge on the influence of composition, as well as having a clear relevance to public health, where it is expected, but not proved, that exhaust from new engines will have a diminished, or at least different toxicity (Hesterberg et al., 2011; McClellan et al., 2012). Finally, non-combustion-related emissions should also be taken into account such as wear emissions from the road, tyres and brakes. These non-exhaust emissions, including the resuspension of road dust, contribute significantly to the mass of PM emissions in urban areas and should be considered by policymakers that impose air quality limits (Denier van der Gon et al., 2012). In addition, the metal content of PM from wear emission is significant, with a high potential to exert redox-related toxicity, and may therefore pose an increased health risk (Denier van der Gon et al., 2012).

Air-pollution-based policy

Reduction in health risk is an important aspect of the air-pollution-based policy; clean air is after all one of the basic requirements of human health and well-being. Meeting the European air quality limits is still the key driver for air-pollution-related abatement strategies. During the past few years, the focus of policymakers has been on limiting traffic-exhaust to improve the air quality. Advances in engine and exhaust after-treatment technology, such as particle traps, for on-road vehicles have made it possible to reduce PM emissions substantially. However, apart from a decrease in emission of the overall mass of PM, these technologies will modify the physical and chemical properties of PM (Maricq, 2007). At present, very little data exist on the implications of the toxicity of the emissions, which may or may not be less dangerous on a per gram basis. It has been shown that particulate traps for diesel engine exhaust decreases both the PM emissions as well as weakens the PM-related cardiovascular health effects (Lucking et al., 2011), although the evidence is still limited.

New engine and exhaust after-treatment technologies have been developed in order to reach the more stringent vehicle emission standards that are introduced as a policy measure to reduce traffic-related PM. Another example of a traffic-related policy measure to reduce emission is the introduction of low emission zones (LEZ) for heavy-duty vehicles. In general, the launch of LEZ as a policy measure is believed to be too modest to observe a significant reduction in overall traffic-related air pollution, and a drop in actual traffic intensity is likely to be more effective (Boogaard et al., 2012). In the Netherlands, speed limits have been lowered from 100 to 80 kilometers per hour on motorways near some urbanised areas. This policy measure resulted in improved air quality near motorways and reduced PM_{10} emissions by 5-12% (Dijkema et al., 2008; Keuken et al., 2010). However, due to other political considerations, the speed limit was recently increased, for which the fact that current AQLs were not exceeded was just as a justification. Air pollution control tends to weaken once limit values are met, which results in a false sense of security as meeting the limit does not imply absence of detrimental effects (Brunekreef et al., 2012). The absence of a threshold concentration below which no health effects occur suggests that further reduction of the levels of PM, as well as other pollutants, will lead to improvement of public health. However, if abatement strategies

predominantly result in the reduction of relative low potent material, like e.g. sulfates are seen from a toxicological perspective (Schlesinger and Cassee, 2003), there is a risk that levels of the more harmful components will increase until the AQL is met (Figure 2; Rohr and Wyzga, 2012). Because negative health risks are still observed when AQLs are met, air-pollution-based policy should still be considered essential (Chen et al., 2008). This has been recognised by the European Union that introduced exposure reduction targets for $PM_{2.5}$ in the latest Directive, targeting the exposure of the population to fine particles. Whereas a limit value is legally binding from the date it enters into force, a target value is to be attained where possible over a given period and less strict. Hence, policymakers are being encouraged to weigh-up the different aspects of air pollution control, as it is a delicate balance with many considerations including both human social and economic views.

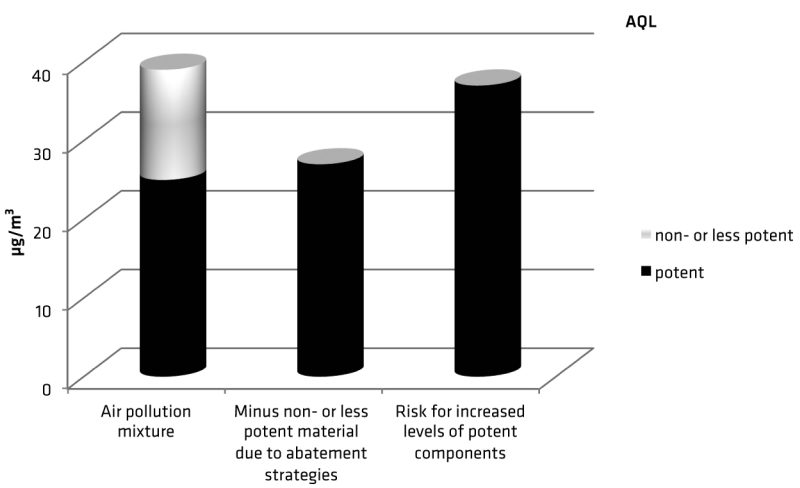


Figure 2. Possible scenario of the toxicity of the air pollution mixture after abatement strategies

The relative burden of PM emissions from non-traffic sources (e.g. road/tyre/brake wear emissions or wood smoke) could become of greater concern when PM from tailpipe exhaust is diminished. For air pollution policy it is important to identify the type of pollutants, the physiochemical characteristics and the sources that are most significant for inducing health effects (Solomon et al., 2012). The problem with air pollution exposure is the difficulty in disentangling the complexity of real-world exposures with that of the observed health effects in different populations. This complexity is due to the diversity of the pollutants present in the mixture, the broad range of air pollution levels over a given time, the variety of adverse effects and the vast number of people at risk. Considerable attention is now being placed on unravelling air pollution/health effect scenarios, the outcomes of which will provide and support policymakers with the information needed to assess the benefits and costs of control-policies.

Outline of this thesis

This thesis is based on the research of two European Commission funded projects: the Health Effects of Particles from Motor Engine exhaust and Ambient Pollution (HEPMEAP) and the Chemical and biological characterisation of ambient air coarse, fine and ultrafine particles for human health risk assessment in Europe (PAMCHAR), as well as two projects funded by the Ministry of Infrastructure and the Environment (Air Pollution and Health and Engine Emission and Health) conducted at the Dutch National Institute for Public Health and the Environment (RIVM). The overall objective of these projects is to examine the health effects of air pollution and disentangle which components/sources of pollution contribute, and to what degree, to adverse health effects.

Within the research described in Chapter 2, we assessed the adverse respiratory and cardiovascular effects in rats, a commonly used species for inhalation toxicology, of PM from sites with contrasting traffic contributions. Since the chemical composition of traffic-related PM material was determined, the influence of composition was examined as well as the role of non-exhaust traffic emissions/sources. The specific role of metals and polycyclic aromatic hydrocarbons (PAHs) was looked at in more depth in the subsequent studies (Chapter 3). The majority of the published observational and toxicological studies have focused on health effects of short-term PM exposure (hours to a few days). Yet, estimates of the biological effects resulting from chronic, cumulative exposures are needed to assess the full burden of PM-induced health effects. Hence, we examined the adverse pulmonary and cardiovascular effect of prolonged exposure (4 weeks) to traffic-derived PM in healthy rats (Chapter 4). As is usual in particle toxicology investigations, the lung, blood and other major organs were investigated for PM-related toxicity, but we also considered the central nervous system, as this system is gaining attention as a novel target for PM-induced toxicity (Chapter 5). In Chapter 6, we considered the consequences of measures that can reduce the mass of PM in traffic emissions (e.g. introduction of a particulate filter) or those that change the composition of the exhaust (e.g. introduction of biofuels). Finally, an overview of the implications of the conducted research described in this thesis is discussed in Chapter 7.

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TOXICITY OF COARSE AND FINE PARTICULATE MATTER FROM SITES WITH CONTRASTING TRAFFIC PROFILES

Miriam E. Gerlofs-Nijland, Jan A.M.A. Dormans, Henk J.T. Bloemen, Daan L.A.C. Leseman, A. John F. Boere, Frank J. Kelly, Ian S. Mudway, Al A. Jimenez, Ken Donaldson, Cecilia Guastadisegni, Nicole A.H. Janssen, Bert Brunekreef, Thomas Sandström, Leendert van Bree, Flemming R. Cassee

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Abstract

Residence in urban areas with much traffic has been associated with various negative health effects. However, the contribution of traffic emissions to these adverse health effects has not been fully determined. Therefore, the objective of this *in vivo* study is to compare the pulmonary and systemic responses of rats exposed to particulate matter (PM) obtained from various locations with contrasting traffic profiles. Samples of coarse (2.5 μm–10 μm) and fine (0.1 μm–2.5 μm) PM were simultaneously collected at nine sites across Europe with a high-volume cascade impactor. Six PM samples from various locations were selected on the basis of contrast in *in vitro* analysis, chemical composition, and traffic profiles. We exposed spontaneously hypertensive (SH) rats to a single dose (3 mg PM/kg body weight or 10 mg PM/kg body weight) of either coarse or fine PM by intratracheal instillation. We assessed changes in biochemical markers, cell differentials, and histopathological changes in the lungs and blood 24 h postexposure. The dose-related adverse effects that both coarse and fine PM induced in the lungs and vascular system were mainly related to cytotoxicity, inflammation, and blood viscosity. We observed clear differences in the extent of these responses to PM from the various locations at equivalent dose levels. There was a trend that suggests that samples from high-traffic sites were the most toxic. It is likely that the toxicological responses of SH rats were associated with specific PM components derived from brake wear (copper and barium), tire wear (zinc), and wood smoke (potassium).

Introduction

Epidemiological studies in large cities have consistently found associations between the daily changes in concentrations of ambient particulate matter (PM) and morbidity and mortality, particularly among individuals with respiratory and cardiovascular diseases (Dockery et al., 1993; Samet et al., 2000; Zanobetti et al., 2000). Hoek et al. (2002) show that cardiopulmonary mortality is associated with residential proximity to major roads. Numerous studies have shown that living in urban areas with high traffic densities is positively associated with reported symptoms of respiratory complaints (Gauderman et al., 2005; Janssen et al., 2003; Nicolai et al., 2003; Schikowski et al., 2005; Sekine et al., 2004). In addition, toxicity studies of animals and humans exposed to urban air find that more adverse health effects occur at locations with higher traffic densities, which likely have greater concentrations of air pollution (Lai et al., 2005; Lemos et al. 1994; Saldiva et al., 1992; Seagrave et al., 2006).

Current and proposed legislation recognizes that the sizes of PM can be separated by means of the behavior of the particles in the air and on the basis of the sources and processes from which the particles originate. Nonetheless, not much is known about source–composition–effect relationships of PM, and, in light of effective emission control strategies, it would be useful to learn more about the contribution of road traffic. Even traffic emissions can be separated into tailpipe, brake, and tire abrasion emissions. This study focuses on the toxicity of the PM fractions collected at sites with various traffic profiles and examines the pulmonary and systemic responses of rats to PM 24 h after intratracheal instillation. The advantage of this method is that the exact quantity and the composition of the PM are known since the physicochemical property of the PM is determined after its removal from the collection substrate. This provides a good opportunity to explore the toxicity in the whole animal, as well as an opportunity for establishing causal PM factors for the toxic endpoints observed.

The current study took place within the scope of a project funded by the European Union and entitled "Health Effects of Particles from Motor Engine Exhaust and Ambient Pollution (HEPMEAP)" (QLRT-1999-01582, www.hepmeap.org). The primary objectives of this research project are (1) to assess the heterogeneity of toxic and inflammatory potential of ambient suspended particles collected at various sites throughout Europe with

contrasting intensities and types of traffic and (2) to identify a possible role of traffic PM in toxicity and health effects. The objectives of the *in vivo* study are to compare the biological and pathological effects of the PM samples on the lungs and the vascular system of spontaneously hypertensive (SH) rats and to determine the relationship of the effects with the chemical composition; this is to be done with particular attention to components from road traffic. We hypothesized that the toxicity of the PM is a function not simply of the total mass of the dose, but also of the chemical composition, so that it was likely to differ between locations and between size fractions.

Methods

Study design

This is a randomized, controlled, *in vivo* animal study. Spontaneously hypertensive rats were chosen to receive the intratracheal instillations since this strain mimics the symptoms of a supposedly susceptible group (Kodavanti et al., 2000). In this study, we exposed each rat to one of the two optimal doses (3 mg PM/kg of body weight and 10 mg PM/kg of body weight) of the coarse (aerodynamic diameter 2.5 μm–10 μm) or fine (aerodynamic diameter 0.1 μm–2.5 μm) PM. These doses were chosen based on our previous study (Gerlofs-Nijland et al., 2005) in which we evaluated the appropriate doses and times for *in vivo* comparative toxicity screening studies in which samples from different origin can be compared for their toxicity. These doses do not reflect ambient air concentrations although they allow comparison of biological responses including toxicity of PM samples at a fixed dose level. The tested PM samples described in this article were from six locations. To study the biological effects of PM exposure on SH rats, at necropsy 24 h after the challenge, blood was collected and bronchoalveolar lavage was performed on the right lung. The left lung was used for histopathology and quantification of cell proliferation.

Sampling and characterization of particles

PM was collected at various European locations with contrasting traffic intensities and types (Bloemen et al., 2005). Samples used in the HEPMEAP project were taken at four sites in the Netherlands and two in Germany in connection with the previous epidemiological International Studies of

Asthma and Allergic disease in Childhood (ISAAC)-II. At each of these sites, four or five 2-wk samples were collected in a 1-yr period. Three sites were added to increase the variation of the source, composition, and toxicological bioactivity. The sites were a Dutch freeway tunnel that is used mainly by heavy diesel trucks (two 2-wk samples), an urban area in Rome (two 2-wk samples), and the small rural town of Lycksele in northern Sweden with particles dominated by wood smoke (one 4-wk and three 2-wk samples). The samples were collected on polyurethane foam (PUF) with a high-volume cascade impactor, which facilitated the simultaneous collection of separate coarse and fine PM fractions. All PUFs were extensively cleaned before use by sonication for 30–60 min in an excessive amount of respectively water and 100% methanol and dried to avoid interference of PUF particles. Before and after PM collection PUFs were placed for 24 h in a conditioned room with steady temperature and humidity to weigh the samples on an analytical balance. Clean PUFs were stored in closed tubes in the dark at room temperature until use. Collected PM samples were stored at –20°C for a few months before extraction. The collected PM was extracted from the PUF with methanol as described by Bloemen et al. (2005) with an efficiency >90%, and dry PM was stored at –20°C until analysis. The chemical composition of all samples was carefully determined after its extraction from foam, and we paid special attention to indicators of traffic emission (Bloemen et al., 2005).

Procedure for selecting PM samples for *in vivo* toxicity screening

The 6 samples used for the PM instillations in the current study were selected from 32 candidate samples collected at the sites just described as part of a wider PM sampling campaign (Bloemen et al., 2005). In total, 82 PM samples were collected at the various locations, but only 32 candidate samples were available at the time of selection. The six samples for *in vivo* toxicity screening were chosen to represent differences in toxicological bioactivity, composition, and sources, irrespective of the spatial and temporal characteristics. The selection of samples was based on their composition and sampling site characteristics, as well as on their activity profiles in a range of *in vitro* assays that determined the oxidative capacity (ascorbate depletion from a synthetic respiratory tract lining fluid and DNA damage) and pro-inflammatory capacity (arachidonic acid and interleukin-6 release from

alveolar macrophages). Details of these *in vitro* screening studies will be published separately in the HEPMEAP project papers. We used the chemical characterization to determine the traffic contribution in the PM samples by ranking the amounts of hopanes, steranes, vanadium, and benzo[a]pyrene. The amounts of nickel, copper, and zinc were used as measures for assessing the contribution of high-temperature burning processes. Each endpoint was ranked and based on these ranking positions the PM samples were divided into the following categories:

1. The most reactive components in both the fine and coarse fractions.
2. The least reactive components in both the fine and coarse fractions.
3. The components most greatly differing between fractions, with the fine fraction most reactive.
4. The components most greatly differing between fractions, with the coarse fraction most reactive.

This procedure resulted in one to three PM samples being selected in each category for each specific test. Then the samples were ranked according to their presence in all categories for all *in vitro* tests irrespective of the selection category, and further samples were selected. Finally, the ranking outcomes with respect to traffic contribution and burning processes, and the site selection criteria (Bloemen et al., 2005) were applied. The fine and coarse fractions from a specific PM sample were tested separately to provide the broadest information possible.

Samples of particulate matter for *in vivo* toxicity screening

The following samples were selected according to the selection procedure just described, and they are ordered on the basis of traffic density (high, moderate, and low) at or close to the site of collection. In addition, the sampling periods (start and finish) are given for all samples.

1. Germany, Munich Ost Bahnhof (MOB)—high; 2 August 2002 to 16 August 2002.
2. Netherlands, Hendrik-Ido-Ambacht (HIA)—high; 4 September 2002 to 18 September 2002.
3. Italy, Rome (ROM)—high; 10 April 2002 to 24 April 2002.
4. Netherlands, Dordrecht (DOR)—moderate; 16 April 2002 to 29 April 2002.

5. Germany, Munich Grosshadern Hospital (MGH)—low; 28 June 2002 to 12 July 2002.
6. Sweden, Lycksele (LYC);—low; 28 February 2002 to 27 March 2002.

Animals

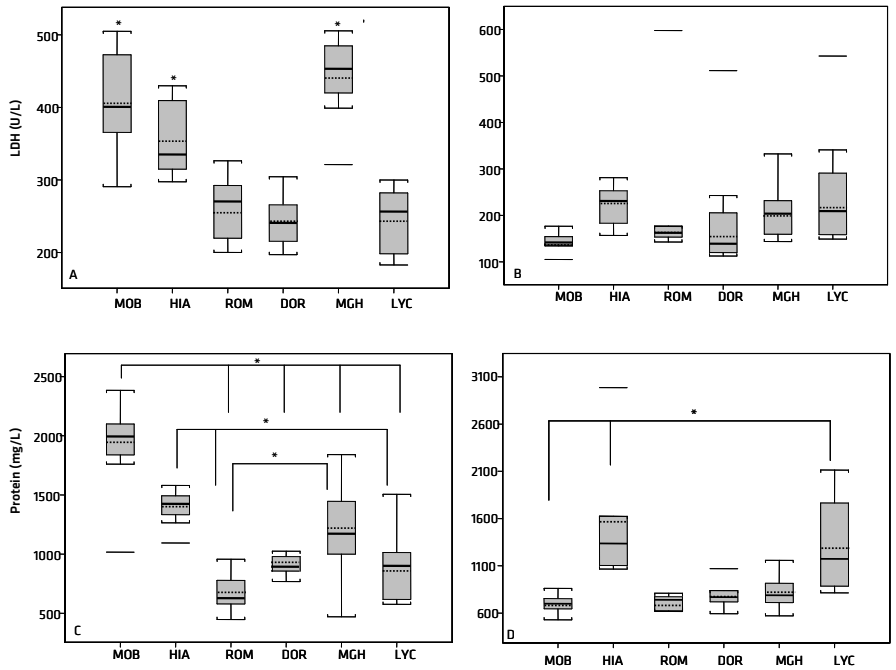
Spontaneously hypertensive male rats (SHR/NHsd) aged 12 wk and weighing 250 g–350 g were obtained from the breeding colony at Charles River (Sulzfeld, Germany). Housing of the animals and the supply of food and drink were the same as those used previously (Gerlofs-Nijland et al., 2005). Immediately after arrival, the animals were weighed and randomly allocated to the various experimental groups. The experiments started after an acclimatization period of at least 7 d. The Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI) approved the experiments.

Exposure by intratracheal instillation

The PM from each selected location was used for intratracheal instillation in rats after suspension in saline with a concentration of 1.5 mg PM/ml or 5 mg PM/ml. The rats were instilled according to the previously described procedure (Gerlofs-Nijland et al., 2005) with a volume of 2 ml/kg body weight resulting in exposure to 3 mg PM/kg body weight or 10 mg PM/kg body weight (n = 8/group). All PM samples were thoroughly mixed right before intratracheal instillation to avoid particles to settle and differences in dose. Immediately after instillation, each animal was injected subcutaneously with 40 mg/kg body weight of 5-bromo-2-deoxyuridine (20 mg/ml BrdU; Sigma-Aldrich, Zwijndrecht, the Nehterlands) for cell proliferation detection purposes.

Necropsy

Necropsy was performed 24 h after exposure. The rats were anesthetized with a mixture of ketamine/Rompun [1 ml/kg body weight ip of a 10:4 mix of 100 mg/ml ketamine (Aesculaap, Boxtel, the Netherlands) and 20 mg/ml Rompun (Bayer, Leverkusen, Germany)] and sacrificed by exsanguination via the abdominal aorta. To obtain bronchoalveolar lavage fluid (BALF), the right lung was lavaged (three in-and-out lavages with the same fluid) with a volume of saline corresponding to 27 ml/kg of body weight at 37°C after ligation of the left bronchus. The left lung was dissected, weighed, and preserved for histopathology after fixation with 10% phosphate-buffered formalin for 1 h under a constant pressure of 20 cm H_2O.



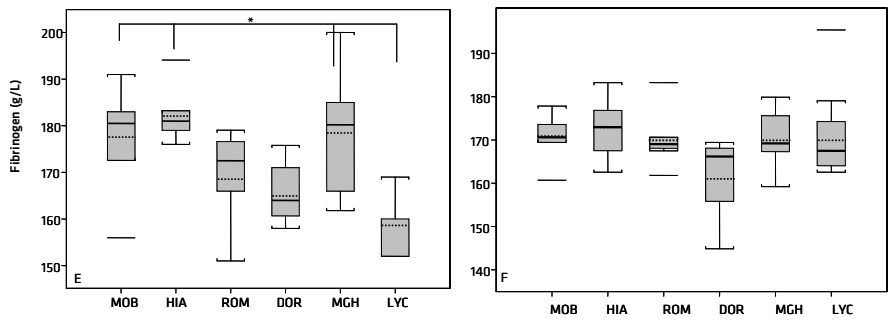


Figure 1. Location-related differences for distinct biological effect markers. Spontaneously hypertensive rats were exposed to 10 mg/kg of body weight coarse particulate matter (A, C, E) or fine particulate matter (B, D, F). The whiskers represent the minimum and maximum data values; the lower edge of the box indicates the 25th percentile and the upper hinge indicates the 75th percentile; the dashed lines represent the arithmetic mean; the horizontal lines inside the boxes represent the median; and outliers are presented as horizontal lines outside the boxes. Asterisk indicates significant difference at $p < .05$; DOR, Dordrecht; HIA, Hendrik-Ido-Ambacht; LDH, lactate dehydrogenase; LYC, Lycksele; MGH, Munich Grosshadern Hospital; MOB, Munich Ost Bahnhof; ROM, Rome.

Bronchoalveolar lavage analysis

After centrifugation of the BALF (400 × g, 4°C, 10 min), the cell-free fluid was used for measurements of cellular toxicity, inflammation, and oxidative stress. The pellet was resuspended in 1 ml saline and used for total cell counts and preparation of cytospins for differential cell counts.

Biochemistry

We determined the amounts of lactate dehydrogenase (LDH; marker for cytotoxicity), and uric acid (marker of oxidative stress) with a reagent kit from Roche Nederland (Almere, The Netherlands). Total protein (indicator for increased permeability of the alveolar–capillary barrier) was determined with a reagent kit from Pierce (Etten-Leur, The Netherlands). We determined the BALF ascorbate and urate concentrations using reversephase high-performance liquid chromatography with electrochemical detection at 400 mV and a current sensitivity of 0.2 μA according to standard protocols (Mudway et al., 2006). We measured the inflammatory mediators tumor necrosis factor α (TNF-α) and macrophage inflammatory protein-2 (MIP-2) using the enzyme-linked immunosorbent assay (ELISA) kit from Biosource (Etten-Leur, The Netherlands).

Cell counts and differentials

We mixed 0.5 ml of the cell suspension with 9.5 ml Isoton II (Beckman Coulter, Mijdrecht, The Netherlands) and counted the total number of cells in a Coulter Counter Z1 and/or Z2 (Beckman Coulter, Mijdrecht, The Netherlands). Cytospin slides were made in duplicate for differential cell counts and stained according to the May–Grünwald and Giemsa method. One cytospin slide was used for counting 300 cells per exposure, and the proportion of each cell type (macrophages, neutrophilic granulocytes, and lymphocytes) was calculated on the basis of the total number of cells per BALF sample.

Blood analysis

Fibrinogen, a marker of the coagulation response, was determined in citrate plasma (DiaFibrinogen kit catalog number 305100; DiaMed Benelux, Turnhout, Belgium).

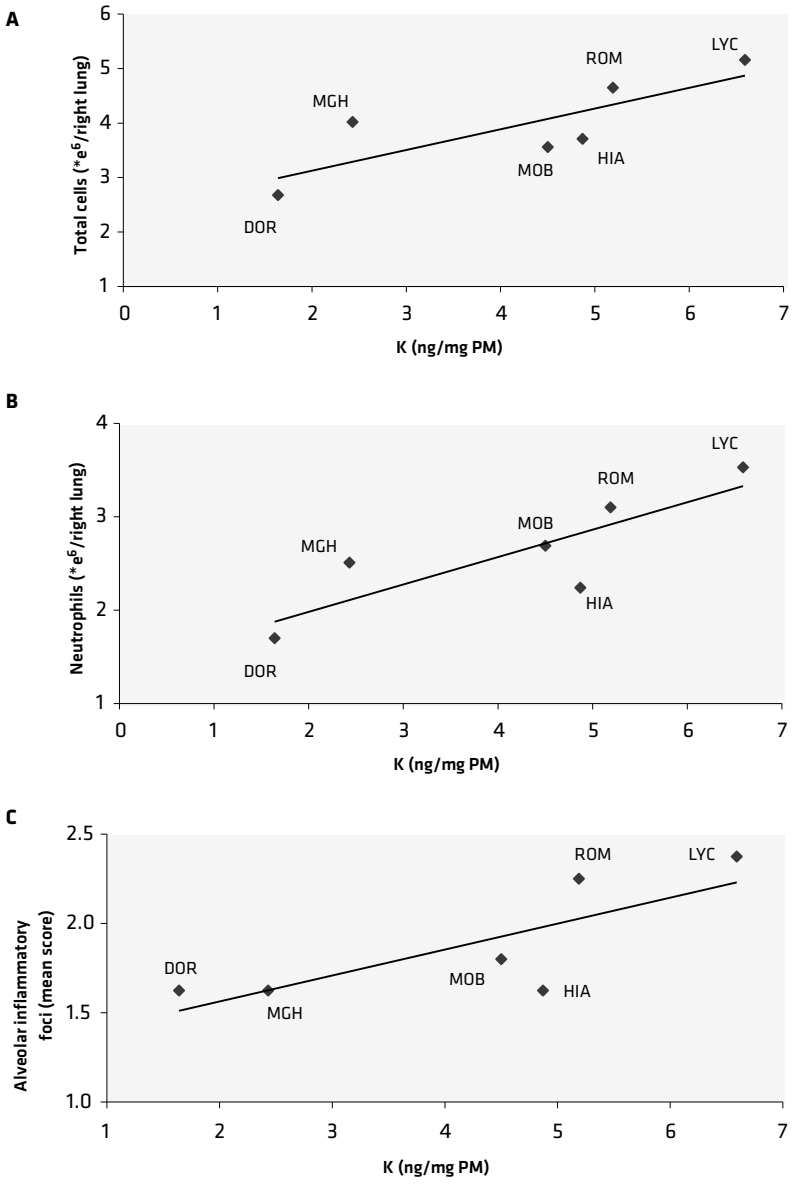


Figure 2. Correlations between potassium and distinct biological and histopathological effect markers in lungs of spontaneously hypertensive rats: total cells (A); neutrophils (B); alveolar inflammatory foci (C). DOR, Dordrecht; HIA, Hendrik-Ido-Ambacht; K, potassium; LYC, Lycksele; MGH, Munich Grosshadern Hospital; MOB, Munich Ost Bahnhof; PM, particulate matter; ROM, Rome.

Pathology

Histopathology. Within 24 h after fixation, the left lung was embedded in paraplast. Lung sections 5 µm thick were stained with hematoxylin and eosin (H&E) and examined light microscopically. Inflammation in the lung (alveolar inflammatory foci, alveolitis, and bronchiolitis) was semiquantitatively and blindly scored as absent (0), minimal (1), slight (2), moderate (3), marked (4), and strong (5). The mean score and standard deviation (SD) of these lesions was calculated (n = 8/dose; Table 3). Alveolar inflammatory foci contain only inflammatory cells and in situations of alveolitis these inflammatory cells are accompanied by leakage of proteins and sometimes also damage to alveoli. Bronchiolotis is defined as an inflammation in the lumen or epithelium of the bronchi.

Morphometry. Cumulative cell proliferation was examined by immunohistochemical staining with a peroxidase-labeled anti-BrdU antibody (Boehringer, Mannheim, Germany). Background BrdU labeling in airways and alveolar septa in lungs of control SH rats was evaluated as zero. Additionally, the number and size of BrdU-labeled alveolar inflammatory foci were semiquantitatively scored as minimal (1), slight (2), moderate (3), marked (4), and strong (5). The mean score and SD of this labeling was determined (n = 7–8/dose; Table 3).

Statistical analysis

All biological effect parameters were log-transformed, and a two-way analysis of variance (ANOVA) was performed. Two-way ANOVA techniques were used to assess differences due to PM exposure with Bonferroni post hoc analyses (PM effect: Tables 1 and 2). Cell differential data are not normally distributed; therefore, the Kruskal–Wallis nonparametric test was used to find differences for a specific parameter. The Wilcoxon rank test was used to expose differences between specific groups. We used a two-way ANOVA to examine a dose-response effect and roughly indicate location differences from data exclusive of the control group data; we combined all six locations for a specific dose. Differences due to location were assessed by two-way ANOVA and Bonferroni post hoc analysis with only the dose of 10 mg PM/kg body weight and without the control group data (Figure 1). The scores of the histological parameters and the labeling scores were statistically evaluated with the nonparametric Wilcoxon test. A value of $p < .05$ was considered significant. To identify associations between various effect parameters and

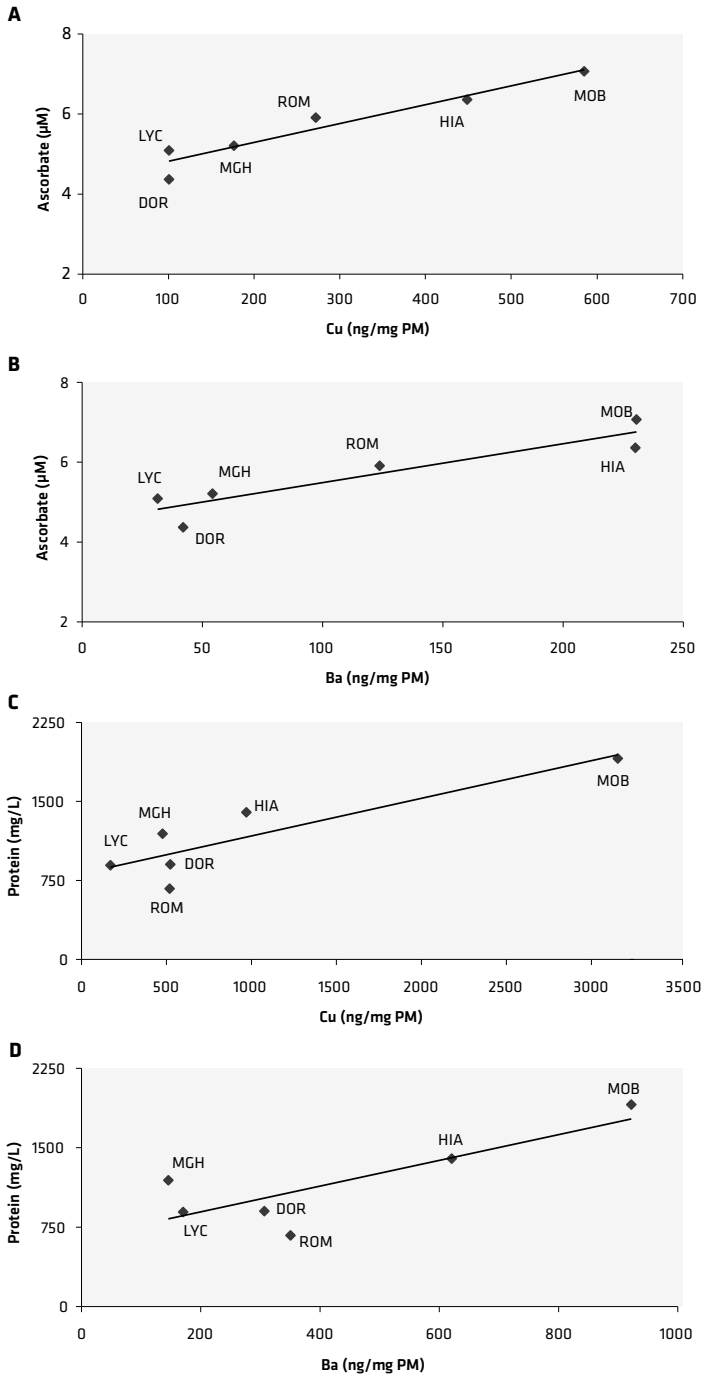


Figure 3. Correlations with distinct biological effect markers in bronchoalveolar lavage fluid: ascorbate to copper (A); ascorbate to barium (B); protein to copper (C); protein to barium (D). Ba, Barium; Cu, copper; DOR, Dordrecht; HIA, Hendrik-Ido-Ambacht; K, potassium; LYC, Lycksele; MGH, Munich Grosshadern Hospital; MOB, Munich Ost Bahnhof; PM, particulate matter; ROM, Rome.

PM composition, we calculated Pearson correlations and, for the cell differential data and histological features, the nonparametric Kendall's tau b correlation coefficients with only the dose of 10 mg PM/kg body weight without the control group data (Figures 2 and 3 and Table 4). In view of the small number of data points, the interpretation of the associations was not based only on the magnitude or statistical significance of the correlation coefficients (Table 4). Graphs were used as an aid for the interpretation in terms of effect size and number of locations that drive a correlation (Figures 2 and 3).

Results

Effects of particulate matter

Several biological parameters were affected by the PM exposure, and the majority exhibited a dose-response relationship as shown for coarse PM (Table 1) and fine PM (Table 2). Cellular damage measured by increased concentration of the cytotoxicity marker LDH in BALF was seen for all coarse PM samples at both the 3 mg/kg body weight dose and the 10 mg/kg body weight dose, while significant increases in this marker occurred only at the highest dose with the fine PM instillation. Altered epithelial permeability, expressed as the BALF protein concentration, was observed predominantly at the highest dose of the coarse PM, with the exception of the high-traffic location ROM, with evidence of attenuated responses with the fine PM samples. The BALF ascorbate concentrations were generally reduced, but only significantly so for rats exposed to the highest dose of the coarse PM fractions from the locations MOB, HIA, and LYC. The BALF antioxidant marker uric acid was only increased at one location (MOB) in the coarse fraction.

Pronounced and dose-dependent pulmonary inflammation was evoked by both PM fractions in all of the tested PM and was characterized by airway neutrophilia, increased macrophage numbers, and a mild lymphocytosis in the BALF. Again, as with the biochemical markers already described, the responses to coarse particles were generally greater than responses to the fine fraction collected at the same site (Tables 1 and 2). Pulmonary inflammation was not only observed in BALF but also in histopathological analysis of the lung (Table 3). Both coarse and fine PM caused dose-dependent alveolitis, and fine PM from LYC (10 mg/kg dose) even caused some bronchiolitis (Table 3). In all structural components of the inflammatory foci (bronchiolar

Table 1. Biological effect parameters in bronchoalveolar lavage fluid and blood of spontaneously hypertensive rats exposed to various samples of coarse particulate matter

Coarse PM		Saline			MOB		HIA		ROM		DOR		MGH		LYC	
Parameter	Units	Mean	95% CI	Dose	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
LDH	U/l	68	54-82	3	172*	150-194	141*	115-168	140*	127-153	99*	83-114	166*	134-199	107*	88-126
				10	409*	349-469	356*	312-400	261*	223-299	243*	213-273	443*	393-493	245*	207-283
Protein	mg/l	486	243-730	3	699	502-894	611	420-803	653	374-932	513	341-685	658	496-820	568	340-797
				10	1908*	1571-2246	1397*	1268-1526	672	525-819	902*	831-973	1193*	852-1535	893*	635-1151
Ascorbate	µM	8.37	6.16-10.58	3	5.43	4.21-6.65	5.58	4.16-7.00	5.23	3.89-6.57	4.91*	3.47-6.34	4.90	3.45-6.36	6.41	4.89-7.93
				10	4.81*	3.94-5.68	4.39*	3.16-5.63	5.01	3.59-6.43	5.78	3.68-7.88	5.58	4.16-7.00	4.52*	3.21-5.83
Uric acid	µM	0.70	0.29-1.10	3	1.10	0.58-1.62	0.86	0.27-1.45	1.08	0.47-1.69	0.66	0.32-0.99	1.05	0.59-1.50	0.74	0.34-1.15
				10	1.75*	1.24-2.27	1.35	0.67-2.04	0.80	0.40-1.20	1.34	0.50-2.18	1.58	1.04-2.12	1.31	0.81-1.80
MIP-2	pg/ml	499	439-559	3	663*	613-713	658	576-739	659*	598-720	627	586-667	679*	612-746	579	462-696
				10	696*	668-725	675*	618-732	662*	620-705	665	602-709	722*	572-871	621	579-663
TNF-α	pg/ml	81	75-86	3	193*	168-219	177*	155-200	188*	119-257	127	93-160	251*	216-287	126	97-155
				10	343*	295-390	288*	247-330	300*	264-336	248	218-278	407*	273-540	230	196-264
Total cells	*e^6/right lung	0.35	0.18-0.51	3	2.43*	1.92-2.94	2.52*	1.96-3.08	2.35*	1.88-2.83	1.53*	1.05-2.00	2.59*	1.84-3.35	1.77*	1.51-2.03
				10	7.77*	5.82-9.72	6.24*	5.30-7.18	4.88*	3.62-6.15	3.78*	2.94-4.62	5.38*	3.03-7.72	3.84*	2.77-4.91
Macrophages	*e^6/right lung	0.31	0.10-0.52	3	0.60*	0.37-0.84	0.75*	0.46-1.04	0.69*	0.41-0.96	0.52	0.36-0.69	0.73*	0.35-1.11	0.54	0.40-0.68
				10	1.91*	1.51-2.32	2.01*	1.62-2.40	1.13*	0.45-1.82	0.93*	0.64-1.21	1.35*	0.73-1.98	0.78*	0.55-1.00
Neutrophils	*e^6/right lung	0.04	0.01-0.06	3	1.77*	1.40-2.13	1.73*	1.15-2.32	1.57*	1.19-1.94	0.95*	0.55-1.35	1.84*	1.31-2.37	1.16*	0.90-1.41
				10	5.55*	3.95-7.16	4.16*	3.19-5.14	3.71*	2.70-4.72	2.70*	1.91-3.48	4.00*	2.26-5.73	2.96*	1.95-3.98
Lymphocytes	*e^{-2}/right lung	0.75	0.40-1.11	3	7.77*	1.53-14.00	8.19*	3.85-12.53	7.80*	1.58-14.02	2.47	0.49-4.45	5.54*	2.16-8.56	2.53	0.60-4.46
				10	21.20*	7.44-34.95	13.81*	10.06-17.56	14.83*	7.96-21.70	15.50*	7.13-23.86	11.93*	3.05-20.81	7.19*	3.18-11.19
Fibrinogen	g/l	141	126-157	3	146	141-151	153	148-157	150	145-155	144	138-149	151	142-160	150	142-158
				10	177*	165-190	183*	174-191	170*	159-181	166*	159-173	179*	164-193	159*	152-165

Values are shown as arithmetic means; *$p < 0.05$ significant compared to saline (n=8/group).
LDH, lactate dehydrogenase; MIP-2, macrophage inflammatory protein-2; TNF-α, tumor necrosis factor α.

Table 2. Biological effect parameters in bronchoalveolar lavage fluid and blood of spontaneously hypertensive rats exposed to various samples of fine particulate matter

Fine PM		Saline			MOB		HIA		ROM		DOR		MGH		LYC	
Parameter	Units	Mean	95% CI	Dose	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
LDH	U/l	70	54-87	3	95	80-111	98	79-117	101	93-110	73	57-89	84	78-91	104*	87-122
				10	144*	119-170	226*	184-267	161*	147-175	154*	103-204	190*	144-236	188*	147-228
Protein	mg/l	499	295-703	3	540	344-737	650	299-1000	550	382-718	472	277-666	411	340-482	559	397-722
				10	698	580-815	1575*	819-2331	718	632-803	794	626-961	829	553-1105	1321*	753-1888
Ascorbate	µM	7.52	6.12-8.92	3	6.26	4.19-8.34	6.55	4.58-8.52	5.23	2.93-7.54	7.90	5.00-10.81	6.62	5.18-8.06	5.67	3.98-7.35
				10	7.07	5.55-8.58	6.36	4.17-8.55	5.91	3.31-8.50	4.37	2.54-6.19	5.21	2.97-7.45	5.09	3.18-7.01
Uric acid	µM	0.76	0.41-1.11	3	0.93	0.60-1.27	0.78	0.37-1.20	1.18	0.57-1.79	1.27	0.58-1.96	0.89	0.46-1.33	0.91	0.50-1.33
				10	1.48	0.77-2.20	1.92	0.67-3.17	1.31	0.78-1.83	1.50	0.98-2.02	1.45	1.03-1.86	2.05	1.02-3.08
MIP-2	pg/ml	512	423-601	3	619	519-719	615	514-715	633	441-826	683	552-813	599	488-709	599	467-730
				10	478	394-562	457	400-514	622	454-790	684	454-914	535	428-641	631	483-779
TNF-α	pg/ml	59	13-106	3	92*	61-123	104*	71-138	107*	85-129	103*	36-171	106*	82-130	106*	57-154
				10	139*	106-172	171*	141-201	170*	138-201	159*	149-168	158*	112-205	174*	139-209
Total cells	$*e^6$/right lung	0.44	0.28-0.60	3	1.23*	0.99-1.47	1.98*	0.52-3.44	1.97*	1.34-2.60	0.97*	0.67-1.26	1.19*	0.81-1.58	2.00*	1.32-2.68
				10	3.56*	2.91-4.20	3.71*	2.22-5.19	4.65*	3.64-5.67	2.68*	2.27-3.09	4.02*	2.64-5.41	5.16*	3.67-6.65
Macrophages	$*e^6$/right lung	0.44	0.23-0.65	3	0.58	0.41-0.76	0.81	0.03-1.59	0.75	0.49-1.01	0.57	0.35-0.78	0.51	0.37-0.66	0.67	0.47-0.87
				10	0.99*	0.83-1.15	1.14*	0.35-1.94	1.60*	0.96-2.23	0.86*	0.71-1.00	1.23*	0.65-1.82	1.22*	0.36-2.09
Neutrophils	$*e^6$/right lung	0.03	0.01-0.04	3	0.61*	0.40-0.82	1.19*	0.07-2.31	0.95*	0.62-1.27	0.38*	0.23-0.52	0.52*	0.21-0.83	1.34*	0.60-2.08
				10	2.69*	1.95-3.43	2.24*	0.96-3.51	3.10*	2.38-3.82	1.70*	1.28-2.12	2.51*	1.10-3.92	3.53*	3.02-4.05
Lymphocytes	$*e^{-2}$/right lung	2.10	1.10-3.09	3	3.70*	1.56-5.84	6.91*	0-14.40	7.75*	1.13-14.37	2.00	0.73-3.28	4.93*	1.65-8.22	5.53	1.74-9.33
				10	8.81*	5.90-11.73	6.70*	3.35-10.06	12.47*	5.68-19.25	11.61*	7.09-16.13	14.80*	5.37-24.22	11.20*	3.73-18.67
Fibrinogen	g/l	152	149-155	3	154	148-159	160	145-175	165	157-174	161	151-169	159	153-164	164	156-172
				10	171*	167-175	173*		170*	165-175	162	154-170	170*	165-176	171*	162-180

Values are shown as arithmetic means; $*p < 0.05$ significant compared to saline (n=8/group).
LDH, lactate dehydrogenase; MIP-2, macrophage inflammatory protein-2; TNF-α, tumor necrosis factor α.

and alveolar) a slightly increased cell proliferation after PM exposure was measured by an increased BrdU labeling. All coarse and fine PM fractions showed a dose-related increase in the numbers and sizes of the labeled areas of inflammatory foci.

The BALF TNF-α concentrations increased significantly for both doses of the coarse samples, with the exception of those obtained from DOR and LYC, where there are moderate and low traffic densities, respectively. A similar response in the fine fraction was attenuated relative to that of the coarse fraction, but it was more consistent across the six sites examined. In contrast, MIP-2 concentrations were increased only at certain sites in response to coarse, but not fine, PM samples. Besides these biological effects in the lung, the fibrinogen responses in the blood of SH rats increased significantly when they were exposed to the highest dose of

both fractions of all PM samples, with the exception of fine PM from DOR (Tables 1 and 2).

Location-related differences

Biological effect parameters could be excluded from further analysis because no sample tested was significantly different from saline; therefore, these samples had no interpretable biological activity. However, a large variation in the control group could conceal bioactivity in these samples. Therefore, we assessed location-related differences for all toxicity endpoints with ANOVA for the dose of 10 mg/kg body weight without the control-group data. Markers indicative of cytotoxicity, inflammation, and blood viscosity revealed differences in relation to the sampling location; this was most evident for the coarse PM fraction (Figure 1). No location-related differences

	Saline			MOB		HIA		ROM		DOR		MGH		LYC	
Parameter	**Mean**	**95% CI**	**Dose**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
Coarse PM															
Alveolar inflammatory foci	0.4	0.5	3	1.3#	0.5	1.0	0.8	0.8	0.7	0.8	0.5	1.8#	0.7	1.1#	0.4
			10	1.9#*	0.4	1.3	0.5	1.9#*	0.8	1.5	0.9	2.3#	0.9	1.7*	0.5
Alveolitis	0.1	0.4	3	1.6#	0.5	1.4#	0.5	0.8#	0.5	1.0#	0.0	1.8#	0.5	1.0#	0..0
			10	3.6#*	0.5	3.1#*	0.6	2.5#*	0.5	2.6#*	0.5	2.9#*	0.4	2.1#*	0.4
Bronchiolitis	0.0	0.0	3	0.1	0.4	0.0	0.0	0.0	0.0	0.1	0.4	0.0	0.0	0.0	0.0
			10	0.3	0.5	0.3	0.7	0.3	0.5	0.4	0.5	0.6	0.7	0.8	1.2
Cell proliferation	0.1	0.4	3	1.3#	0.5	1.1#	0.4	0.9#	0.4	0.9#	0.4	1.5#	0.8	1.0#	0.0
			10	3.4#*	0.5	3.4#*	0.5	2.3#*	0.7	2.5#*	0.9	2.9#*	0.4	1.8#*	0.5
Fine PM															
Alveolar inflammatory foci	0.6	0.7	3	1.4	0.5	1.0	0.6	1.3	1.0	0.7	0.8	1.0	1.1	1.1	1.0
			10	1.8#	0.5	1.6#	0.7	2.3#	0.9	1.6#	0.7	1.6#	0.7	2.4#*	1.1
Alveolitis	0.0	0.0	3	1.1#	0.4	1.1#	0.4	0.9#	0.4	0.6#	0.5	0.9#	0.4	0.9#	0.4
			10	2.6#*	0.7	3.1#*	0.4	2.4#*	0.5	2.0#*	0.0	2.5#*	0.5	2.3#*	0.3
Bronchiolitis	0.0	0.0	3	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.5
			10	0.3	0.5	0.1	0.4	0.1	0.4	0.4	0.5	0.3	0.5	1.3#	1.2
Cell proliferation	0.4	0.5	3	1.0#	0.0	0.8	0.5	0.9	0.4	0.9	0.4	1.0#	0.0	0.9	0.4
			10	2.4#*	0.7	3.0#*	0.5	2.6#*	0.5	1.9#*	0.4	2.3#*	0.7	2.1#*	0.6

Table 3. Histological features and cell proliferation in lungs of spontaneously hypertensive rats exposed to various samples of coarse and fine particulate matter

Values are shown as arithmetic means (n = 7-8/dose); #$p < 0.05$ compared to saline; *$p < 0.05$ with respect to the corresponding 3 mg/kg of body weight group.

Table 4. Associations of biological and histopathological effect parameters with composition of coarse and fine particulate matter

	Parameters	PAHs	Zn	K	Ba	Cu
Coarse	Protein	0,35	0,56	0,21	0,82	0,88 *
	LDH	-0,09	0,60	0,07	0,38	0,52
	Ascorbate	-0,53	-0,09	-0,73	-0,41	-0,22
	MIP-2	-0,46	0,52	-0,09	0,26	0,41
	Total cells	0,07	0,33	0,47	0,47	0,47
	Macrophages	-0,20	0,60	0,47	0,47	0,47
	Neutrophils	0,07	0,33	0,47	0,47	0,47
	Lymphocytes	0,07	0,07	-0,07	0,47	0,73 *
	Fibrinogen	-0,44	0,78	0,52	0,51	0,43
	Alveolar inflammatory foci	-0,41	-0,14	-0,14	-0,28	-0,28
	Alveolitis	0,07	0,60	0,20	0,47	0,73 *
	Cell proliferation	0,00	0,69	0,28	0,41	0,69
Fine	Protein	0,00	0,92 **	0,48	0,16	0,01
	LDH	-0,35	0,86 *	0,25	0,12	-0,03
	Ascorbate	0,45	0,23	0,43	0,92 **	0,96 **
	MIP-2	-0,22	-0,52	-0,16	-0.82 *	-0.83 *
	Total cells	-0,07	0,07	0,73 *	-0,33	-0,28
	Macrophages	-0,33	-0,20	0,47	-0,07	0,00
	Neutrophils	0,20	-0,20	0,73 *	-0,07	0,00
	Lymphocytes	-0.73 *	-0,33	-0,20	-0,20	-0,28
	Fibrinogen	0,28	0,47	0,71	0,55	0,54
	Alveolar inflammatory foci	0,30	-0,30	0,75 *	-0,15	-0,08
	Alveolitis	0,07	0,20	0,07	0,60	0,69
	Cell proliferation	0,07	-0,07	0,33	0,60	0,69

**p < 0.05; **p < 0.01.*
BALF, bronchoalveolar lavage fluid; Ba, barium; Cu, copper; K, potassium; LDH, lactate dehydrogenase; MIP-2, macrophage inflammatory protein-2; PAHs, the sum of exhaust-related polycyclic aromatic hydrocarbons typical of diesel and wood burning and to a lesser extent gasoline (phenantrene, fluoranthene, and pyrene); Zn, zinc.
For the toxicity endpoint ascorbate all fine PM samples showed similar responses as saline 24 h after instillation

were found for markers related to antioxidant responses within the respiratory tract lining fluid (ascorbate or urate). The coarse PM fraction from two locations with high traffic densities, MOB and HIA, as well as PM from MGH, which represents an urban location of low traffic density, induced higher LDH responses (cytotoxicity; Figure 1A) in BALF than the PM from the three other locations (ROM, DOR, and LYC). Exposure to coarse PM from MOB caused a greater increase in BALF protein concentrations than coarse PM from any other location except HIA. Exposure to coarse PM only from HIA produced BALF protein concentrations greater than those from LYC and ROM. Coarse PM from MGH caused greater concentrations than those from ROM (Figure 1C). These results were supported by pathological changes, as exposure to coarse PM resulted in the greatest increases in alveolitis and cell proliferation in PM samples from MOB, MGH, and HIA (Table 3). Location-related differences in biological responses were found not only in the lung but also in the blood of SH rats exposed to coarse PM. Coarse PM from LYC resulted in lower fibrinogen values than PM from the locations MOB, HIA, and MGH (Figure 1E). Less differentiation among the various sites was found for the fine PM fraction, and these differences were primarily related to inflammation. Exposure to fine PM from LYC or HIA resulted in greater concentrations of BALF protein than that from MOB (Figure 1D).

Associations between chemical composition and effect parameters

All PM samples were analyzed for elemental composition, inorganic ions, and a selection of organic compounds (Bloemen et al., 2005). To determine the influence of combustion processes, the associations between biological effect parameters or key histological features and markers likely to represent traffic emissions [combustion-exhaust-related polycyclic aromatic hydrocarbons (PAHs), zinc, copper, and barium (Chella et al., 2005; Cyrys et al., 2003)] or wood burning [combustion-exhaust-related PAHs and potassium (Hedberg et al., 2002)] were explored (Figures 2 and 3 and Table 4). The PAHs phenanthrene, fluoranthene, and pyrene are typical of diesel and wood burning (Chellam et al., 2005; Hedberg et al., 2002) and, to a lesser extent, of gasoline. The sum of the amounts of these PAHs was used to characterize the PM samples. These traffic markers were correlated with the toxicity endpoints observed in the SH rats following the PM instillations. The traffic markers were carefully chosen after an extensive literature search. There

was no correlation of any of the effect markers with combustion-exhaust-related PAHs except for an increase of lymphocytes that was observed after pulmonary instillation of fine PM (r = −.73, p = .04; Table 4). In the fine fraction, a significant correlation was found between the zinc content and the BALF cytotoxicity markers protein (r = .92, p = .01) and LDH (r = .86, p = .03; Table 4). However, the association between the cytotoxicity markers and zinc is mainly driven by PM from one site of the high traffic density, HIA. The wood smoke indicator potassium correlated only in the fine fraction with total BALF cells and neutrophils, which is indicative for inflammation (both r = .73, p = .04; Figure 2, A and B, and Table 4). These results are supported by pathology, as in the fine fraction alveolar inflammatory foci were positively correlated with potassium (Figure 2C and Table 4). The fine fraction also revealed positive correlations with copper (r = .96, p = .002) and barium (r = .92, p = .01) and the antioxidant marker ascorbate (Figure 3, A and B, and Table 4), although it should be noted that none of the fine PM samples showed a different ascorbate response from that of saline and the absolute effect size is rather small. Interestingly, in the coarse fraction, we only observed positive correlations with the components barium and copper and no other components. BALF protein is positively correlated with copper (r = .88, p = .02; Figure 3C and Table 4) and barium (r = .82, p = .048; Figure 3D and Table 4) and alveolitis is only associated with copper (r = .73, p = .04; Table 4). Both correlations in the coarse fraction are driven by one single location, namely, MOB, and should therefore be interpreted with care.

Discussion

Intratracheal administration of coarse and fine PM samples from diverse locations generally induced dose-related biological and histopathological effects in the lungs and blood of SH rats. The extent of the responses varied by location, with most location-related differences in the coarse PM fraction, and the degree of cell proliferation paralleled the pathological responses. The same elemental traffic markers, copper and barium, were apparently associated with different responses dependent on the PM size range, which suggests that differing mechanisms induce the health effects. Overall, coarse PM induces a different response pattern than fine at the same dose level.

Ambient PM is regulated in the European Union and elsewhere as mass concentrations of particles with an aerodynamic diameter of 10 µm or less. Apart from mass, an understanding of the size distribution, chemical speciation, and bioavailability of PM components is clearly required if we are to fully appreciate the effects of ambient PM on human health and develop specific abatement strategies to deal with the most critical fractions, PM components, and emission sources. We examined PM collected from six European sites that differ in local traffic contributions. We selected samples on the basis of this criterion, as well as differences in toxic potency established by *in vitro* studies (unpublished results) and by chemical composition (Bloemen et al., 2005). We have shown that some biological effect markers differ significantly upon exposure to coarse and fine PM samples from various locations in SH rats when samples are instilled in equivalent doses. However, PM exposure in general did not result in substantial differences for most markers, which concurs with previous studies (Costa & Dreher, 1997; Gerlofs-Nijland et al., 2005; Steerenberg et al., 2004, 2005). The toxicity of the lowest dose for both coarse and fine PM was minimal, as indicated by the LDH and by protein concentrations in BALF. However, exposure to the low dose of all PM samples already resulted in alveolitis and cell proliferation in the lungs of SH rats. Moreover, there were no clear signs of oxidative stress at this dose level, which was reflected by the absence of changes in uric acid and ascorbate concentrations. This latter aspect might be impacted by the time course of events. Oxidative stress may have occurred very rapid after the exposure and has already been overcome at the time of necropsy. Conversely, a dose-dependent inflammatory response occurred in all animals

exposed to each of the PM samples; the response to coarse PM was somewhat stronger. This moderate pulmonary toxicity and pathological changes were accompanied by a mild increase in blood fibrinogen; coarse PM was slightly more active than fine PM in this regard.

Although we did not design this study to compare coarse and fine PM in full detail, the general outcome of the *in vivo* study is that coarse PM is often, though not always, more potent per unit mass in inducing pulmonary inflammation. Coarse PM is usually present in lesser amounts than fine PM in urban areas, and the fraction of coarse PM reaching the lower airways and alveoli is also substantially less (Cassee et al., 2002). Although the effects of the coarse fraction should not be ignored, one has to realize that the overall risk from coarse PM might actually be smaller than that of fine PM due to the differential airway deposition patterns of the two fractions. The overall risk is a function of toxicity and deposition and therefore the overall risk might actually be smaller for coarse particles. Other studies summarizing both *in vitro* and *in vivo* findings in relation to size fractions show that the biological mechanisms of coarse and fine PM differ substantially (Sandström et al., 2005; Steerenberg et al., 2006). The number of samples and thus the power of this study could be extended if coarse and fine fractions were pooled for a specific site. However, not only the difference in biological mechanisms induced by both fractions but also the fact that the matrix or availability of specific components could vary for both fractions argue against pooling coarse and fine PM fractions for one site. The bioavailability of iron appears to vary with particle size (Smith et al., 2000); for example, the relative abundance of insoluble iron oxide increases with a decrease in particle size (Hirabayashi & Matsuo, 2001). Altogether, these findings validate our approach of not combining coarse and fine samples for one location, but to analyze them separately.

Location-related differences in toxicological responses to instilled PM were qualitatively more pronounced with coarse PM. These location-dependent differences were primarily related to cell injury by fine PM samples, while exposure to coarse PM resulted in clear regional differences in both cell injury and inflammatory endpoints. The outcomes suggest that the strongest responses with coarse PM were associated with the two sites of high traffic density: MOB and HIA. In addition, coarse PM from the urban location of low traffic density at MGH also displayed considerable pro-inflammatory activity and the capacity to elicit pulmonary injury, which was supported by pathological changes. Location-related differences were found both for markers in the lung and in the blood of SH rats exposed to coarse PM. Interestingly, fine PM sampled at LYC, a rural location of low traffic density but with much PM from other combustion sources like wood burning, and that from HIA seemed to have the greatest effects of all the fine PM samples. In this study, we have linked changes in inflammatory, injury, and antioxidant response endpoints with the chemical composition of the PM samples. Since a biological effect parameter could still be driven by specific components despite the apparent lack of bioactivity, we analyzed the association of all the toxicity endpoints with some carefully selected traffic markers. Due to the many endpoints and exposure variables, we have a potential multiple-testing problem. However, we have specific hypotheses about exposures, and associations with different endpoints will be considered on the basis of consistency across the relevant endpoints. Although the study was not optimized for source apportionment, the correlations between these biological markers and some components provide useful information for understanding the link between PM sources and health effects. For example, for fine PM, copper and barium were significantly linked to BALF ascorbate concentrations. Copper and barium are released during braking and are not related to the tailpipe exhaust (Adachi & Tainosho, 2004).

Combustion-exhaust-related PAHs hardly correlated with effect markers, possibly because these PAHs represent different sources such as wood smoke, diesel, and gasoline. Another traffic marker used in this analysis is zinc, since zinc emissions could come from tire wear and corrosion of safety fencing (Berbee et al., 2004; Legret & Pagotto, 2006). Zinc is significantly related to cytotoxicity (protein and LDH) in the fine fraction, and similar associations, although insignificant, were observed for the coarse fraction. Kodavanti et al. (2002) have shown increased protein leakage after exposure toZnSO4, which is present mainly in the fine PM fraction. On the one hand, the relation between zinc and cytotoxicity in this study is less clear because the association is mainly driven by PM from the HIA site, but on the other hand, the Kodavanti et al. (2002) findings concur with our results. Traffic is a source of zinc, but wood burning is a greater source; wood burning also releases potassium (Hedberg et al., 2002). Potassium, a potential marker for wood smoke, correlated with markers of pulmonary inflammation such as the PM-induced neutrophilia and alveolar inflammatory foci. Wood smoke also exacerbates inflammatory responses *in vivo*, but without significant

increases in lung inflammatory cells (Barrett et al., 2006; Tesfaigzi et al., 2005). The reason for this discrepancy is that in our case potassium is not unique for wood smoke and contribution of other sources may explain this difference.

We have studied only six samples here, which limits our interpretations. Although the HEPMEAP project was designed to provide some comparability of sites, the *in vivo* part was restricted, for monetary and ethical reasons, to a fraction of the samples that were collected and tested in *in vitro* assays. As a result, these findings are also subject to temporal variations as well as the characteristic variations of the sites. Taking this limitation into account, the outcomes do not necessarily reflect the general toxic potency of the PM at each site in this study; they only underline the knowledge that the toxic potency of the PM is very dependent on the chemical composition. Indeed, other papers have reported the effects of the season on inflammatory mediator release and oxidative stress (Becker et al., 2005; Pozzi et al., 2005), genotoxicity *in vitro* (Gabelova et al., 2004), and adjuvant activity *in vivo* (Steerenberg et al., 2005). Unfortunately, it is difficult to trace the toxicity in the challenged rats to traffic components that comprise a small proportion of the samples tested.

In conclusion, exposure of SH rats to coarse and fine PM from several European locations that were characterized by different traffic contributions induced dose-related biological and histopathological effects in the lungs and vascular system. There were clear differences in the intensity of the effects of PM from various sites at a fixed dose level. Although there was an apparent trend toward greater toxicity in PM from sites of high traffic density, PM from sites of low traffic density also had significant effects.

The greater values in inflammatory and injury endpoints following PM instillation appeared to be associated with specific PM components derived mainly from brake wear (copper and barium), tire wear (zinc), and wood smoke (potassium). Although PM is regulated on a per microgram mass basis, these results suggest that PM composition can play a profound role in the development of adverse health effects.

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CHAPTER 3

3 PARTICLE INDUCED TOXICITY IN RELATION TO TRANSITION METAL AND POLYCYCLIC AROMATIC HYDRO-CARBON CONTENTS

Miriam E. Gerlofs-Nijland, Melina Rummelhard,
A. John F. Boere, Daan L.A.C. Leseman, Rodger Duffin,
Roel P.F. Schins, Paul J.A. Borm, Markus Sillanpää,
Raimo O. Salonen, Flemming R. Cassee

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Abstract

Exposure to ambient particulate matter (PM) is statistically significantly associated with morbidity and mortality. The objectives of this study were (a) to investigate *in vivo* pulmonary and systemic cytotoxicity and inflammatory activity in compromised animals exposed to PM and (b) to investigate the relationships of the outcomes to the chemical compositions of particular polycyclic aromatic hydrocarbons (PAH) and transition metals in the PM. The PM samples were collected in European cities representing contrasting situations. Exposure of spontaneously hypertensive rats (7 mg of PM/kg) resulted in pulmonary inflammation, cellular toxicity and the induction of blood fibrinogen. Coarse PM generally caused stronger effects per mg than fine particles. Positive correlations between lactate dehydrogenase, proteins, and some inflammation parameters and the particle metal and PAH content were found. PM rich in PAH also led to increased blood fibrinogen. Removal of particles but not the organics (i.e., PAH) of a sample led to reduced inflammation in the lungs. The present study highlights the importance of metals as well as PM bound PAH in particle biological outcomes. It supports the hypothesis that, on an equal mass basis, particle health effects differ due to differences in compositions and size.

Introduction

It has become evident in the past decades that elevated exposure to ambient particulate matter (PM) is associated with respiratory and cardiovascular morbidity and mortality (Brook, 2008; Brunekreef et al., 1995; Pope et al., 2002; Romieu et al, 1996). Both animal toxicology studies and studies with human volunteers support these findings. Inhalation studies have shown that short-term exposure to diesel engine particles or concentrated ambient particles at levels that people encounter in polluted areas results in acute lung inflammation (Ghio and Huang, 2004; Stenfors et al., 2004). Animal studies have also revealed an association between particle exposure and cardiovascular abnormalities (Cassee et al., 2005; Wichers et al., 2004).

It has become increasingly clear that toxicology studies cannot rely on PM mass alone to describe the health effects of particles. Indeed, urban air inhalable particles (PM_{10}: PM with an aerodynamic diameter <10 μm) are a complex mixture of particles of different sizes originating from a variety of sources that can differ in their composition. Numerous metals are found in ambient particles; the relative quantities vary with the size fraction of the PM. Although PAH represent a small proportion of the PM mass, their biological effects are studied particularly because of their putative health effects. Elucidating the role of the physicochemical characteristics of PM in inducing the toxic effects is essential both for ambient air regulations and for the understanding of biological mechanisms.

Therefore, the aim of this study was to investigate the *in vivo* pulmonary and cardiovascular cytotoxic and inflammatory activities of $PM_{10\text{-}2.5}$ (CPM = coarse PM) and $PM_{2.5\text{-}0.2}$ (FPM = fine PM) that differ in their metal/PAH content. Hypertensive rats were used as a model to mimic a vascular disease (Kodavanti et al., 2000) expected to be a risk factor in the human population. The choices of the PM concentration and the time to examine biological responses stem from previously acquired data (Gerlofs-Nijland et al., 2005). We hypothesized that adverse health effects observed 24 h after a single dose (7 mg PM/kg of body weight) is at least partly caused by PM components, i.e., transition metal or PAH.

Methods

Animals

Spontaneously hypertensive rats, 12 weeks old, male, and weighing 200-300 g were obtained from Charles River Laboratories (Sulzfeld, Germany). More details on housing and feeding have been described previously (Gerlofs-Nijland et al., 2007) and experiments were approved by the Animal Ethics Committee of the Dutch National Vaccine Institute.

PM sampling, preparation, and sample selection

CPM and FPM were collected on polyurethane foam (PUF) by means of a high-volume cascade impactor with a flow of 900 L/min (Pennanen et al., 2007; Sillanpää et al., 2005) in the cities Prague (PRA), Duisburg (DUI), and Barcelona (BAR) (Silanpää et al., 2005).

The CPM and FPM samples from PRA were selected to determine the relation between PM-induced genotoxicity and PM-induced pulmonary inflammation. Besides, these samples were used to examine the effect of particle size.

The PRA and DUI FPM samples were selected to determine the putative effects of transition metal content of the PM on cardiovascular end points since these revealed contrasting oxidant generation and metal content (Table 1). Metals present in the PM were inactivated with diethylene triamine pentaacetic acid (DTPA; Sigma-Aldrich, Zwijndrecht, The Netherlands), a chelator used at a final concentration of 25 µmol/ml in W1503 water (Sigma-Aldrich) and 1 M NaOH.

The PRA and BAR CPM samples exhibited marked differences in their total PAH content (1202 µg/g in PRA PM and 100 µg/g in BAR PM; Table 2), and were hence used to study the role of PM organic compounds in PM cardiovascular effects. Organic extracts from the PRA and BAR PM coarse samples were prepared by dichloromethane extraction of 10 mg dry PM.

Thereafter these organic extracts were resuspended in tricaprylin and water in a 1:9 ratio by sonication and used to expose rats.

Exposure

Five clusters of 22 rats each were exposed to a single dose of particles or solvent in a previously described procedure (Gerlofs-Nijland et al., 2005, 2007). The rat (n=10) were exposed to a volume of 2 mL PM/kg of body weight by means of intratracheal instillation, resulting in a final PM instillation concentration of 7 mg/kg of body weight. The same volume of water was used as a negative control for PM exposure. In case of organic extracts using dichloromethane (the mass of the organics associated with 7 mg PM), the material was suspended in tricaprylin dissolved in water (1:9, volume 2 mL/kg body weight). To examine the effect of metal chelation, DTPA was added to PM samples of some groups 30 min before the instillation. A DTPA solution of 25 µmol/ml was used as a negative control.

Necropsy

Necropsy was performed 24 h after exposure as described previously (Gerlofs-Nijland et al., 2007) with the minor change that the lungs were perfused with saline until all the blood was removed before collection of bronchoalveolar lavage fluid (BALF) through the right atrium into the pulmonary artery-chamber with simultaneous ventilation.

Analysis of the BronchoAlveolar Lavage Fluid

BALF samples were processed and analyzed as described previously (Gerlofs-Nijland et al., 2005, 2007) and assayed for cellular toxicity (lactate dehydrogenase (LDH), albumin, total protein, Clara cell 16 protein (CC16)), inflammation (tumor necrosis factor (TNF)-α, N-acetyl glucosaminidase (NAG), neutrophil activity marker myeloperoxidase (MPO), total cell number, and differential cell counts), and for effect on antioxidants (uric acid (UA)).

Material	City	Al	Cd	Co	Cr	Cu	Mn	Ni	Pb	V	As	Fe	Zn
FPM	Duisburg	7.856	0.018	0.007	0.055	0.246	0.403	0.053	1.008	0.082	0.045	4.320	3.601
FPM	Prague	6.047	0.015	0.004	0.006	0.188	0.066	0.029	0.550	0.042	0.092	0.992	1.521
Blank	All	3.9730	0.0001	0.0001	0.0142	0.0104	0.0039	0.0033	0.0103	0.0003	0.0004	0.1244	0.0100
CPM	Prague	13.003	0.012	0.006	0.023	0.278	0.420	0.026	0.337	0.052	0.065	9.453	1.045
CPM	Barcelona	5.282	0.002	0.006	0.032	0.488	0.293	0.035	0.215	0.112	0.005	10.787	1.165
Blank	All	5.6975	0.0001	0.0001	0.0042	0.0105	0.0033	0.0046	0.0162	0.0003	0.0007	0.0547	0.0172

Table 1. Water-soluble elemental constituents in coarse and fine particulate matter collected with a high volume cascade impactor

Abbreviations: CPM – coarse PM; FPM – fine PM; PM – particulate matter. Values are expressed as µg/mg PM.

Blood analysis

Blood was analyzed for markers of inflammation (C-reactive protein (CRP), TNF-α, inter cellular adhesion molecule (ICAM)-1) and vascular responses (fibrinogen, von Willebrand factor (vWF), angiotensin-converting enzyme (ACE)). Fibrinogen and CRP were determined in citrate plasma (U-Diagnostics, Utrecht, The Netherlands). The vWF was measured with enzyme-linked immuno sorbent assay (ELISA) (Kordia, Leiden, The Netherlands), also in citrate plasma. The ICAM-1 was measured in ethylenediaminetetraacetic acid-chelated blood (Terumo Europe, Leuven, Belgium), a blood anticoagulant, with a commercial ELISA kit (R&D Systems, Abingdon, UK). The TNF-α and the ACE were measured in the serum by using commercial ELISA kits according to the manufacturer's instructions.

Characterization of the oxidant generating activities of the samples

The suspensions as prepared for instillation were also analyzed by electron paramagnetic resonance (EPR), to determine the oxidant generating activities of the various PM samples based on the principle as described previously (Shi et al., 2003).

Statistical analysis

All biological effect parameters were log-transformed. Two-way analysis of variance (ANOVA) including Bonferroni test was used to assess differences among groups due an exposure to PM, organic extract, or DTPA. If the log transformation did not result in a normal distribution of the measured parameter, next to the two-way ANOVA the nonparametric Kruskal-Wallis ranksumtest was used to determine any differences for a specific parameter. S-Plus software (MathSoft) was used for all statistical analyses. The values are expressed as mean 95% confidence intervals (CI), and *p* values less than 0.05 were regarded as significant.

Material	City	BaP	Total
FPM	Duisburg	59.20	729.43
FPM	Prague	94.20	1558.98
Blank	All	<0.01	1.95
CPM	Prague	20.20	1202.29
CPM	Barcelona	0.34	100.46
Blank	All	<0.01	3.00

Table 2. Content of benzo(a)pyrene and total polycyclic aromatic hydrocarbons in coarse and fine particulate matter

Abbreviations: BaP – benzo(a)pyrene; CPM – coarse PM; FPM – fine PM; PM – particulate matter. Values are expressed as μg/g PM.

RESULTS

Effects of PM size

Inflammation. The instillation of CPM and FPM both collected in PRA resulted in increases of several inflammatory and toxicity markers in the BALF of the compromised rats (Table 3). Exposure to particles led to significantly increased inflammatory cells except for macrophages 24 h after the exposure. The activities of neutrophils and macrophages were assessed by measurement of the concentrations of MPO and NAG, respectively. Both MPO and NAG were enhanced 24 h after an exposure to either CPM or FPM. To complete this study of PM effects on lung inflammation, the concentration of TNF-α was measured. Both CPM and FPM induced amplified concentrations of this proinflammatory cytokine. Besides these lung inflammatory markers, we also studied PM effects on cardiovascular inflammation. However, as shown in Table 3, the PM exposure did not reveal any modification of TNF-α, CRP, or the macrophage activation marker ICAM-1 in the blood.

Toxicity. The PM exposure resulted in the augmented release of cytotoxicity and integrity cell markers such as LDH, albumin, protein, and a greater amount of UA, which is an effective antioxidant, but it did not modify the amount of CC16 in the BALF (Table 3). An increase in UA is explained as being an adaptive response to oxidative stress.

Vascular responses. Measurements of some blood parameters related to coagulation and vasodilatation showed a small increase of fibrinogen in the plasma of PM-treated animals. No significant changes were found in serum or plasma for either the vasoconstriction marker ACE or the endothelial injury marker vWF (Table 3).

Coarse and fine comparison. Statistical comparisons of FPM and CPM effects also revealed that the CPM fraction caused a greater augmentation per unit mass of inflammatory cells in the BALF as well as greater activity of MPO and NAG (Table 3). In addition, the cytotoxicity evaluated by the measure of LDH was most enhanced after exposure to CPM. The amount of fibrinogen, the only biomarker induced in the blood of PM-treated rats, was increased more by the CPM fraction than by the corresponding FPM fraction. These observations demonstrate the greater effects of CPM on several biomarkers.

Role of metals

To address the influence of metal composition on the effects of PM in compromised rats, we compared the effects of FPM samples collected in two cities that differed in metal content (Pennanen et al., 2007; Table 1). The DUI PM was richer in metals than PRA PM. The role of transition metals in inducing biological responses was further evaluated by the addition of the metal chelator DTPA to the PM samples in order to bind and inactivate transition metals prior to intratracheal instillation. Addition of DTPA resulted in partial inactivation of the oxidant generating properties of both PM samples, as shown by a reduction in the hydroxyl generating activity (Figure 1).

Inflammation. The comparison of PRA and DUI PM showed that both PM samples triggered inflammatory responses, as shown by the increased numbers of total cells, neutrophils, lymphocytes, eosinophils, and monocytes in the BALF (Table 4), as well as by increased amounts of NAG, MPO, and TNF-α. Interestingly, the comparison of both PM samples revealed a greater enhancement of most inflammation parameters with DUI PM, which is richer in metals, than with PRA PM. The exposure of rats to DTPA alone led to an inflammatory response. Nonetheless, when PM metals were chelated with DTPA, we still observed a greater inflammatory response with PM-exposed rats than with control DTPA rats (Table 4). Although it seems that binding and inactivation of the PM metals is causing a striking decrease in the multiplication of neutrophils (e.g., a 3.7-fold increase with DUI PM alone versus a 31.7-fold increase with DUI PM without DTPA). This effect is at least partly due to the shift in control value of water to DTPA. Blood inflammatory markers were less affected by DTPA than BALF inflammatory markers.

Toxicity. The concentrations of LDH, protein, and albumin that characterize cellular toxicity were induced by both PRA and DUI FPM (Table 4), though the enhancement of LDH and protein concentrations was greater with the batch of DUI particles. PRA FPM also increased the concentration of UA in the rats' BALF. Surprisingly, exposure to DTPA alone revealed some cellular toxicity, although, from *in vitro* studies, it was expected that the concentration used would not be cytotoxic. Once more, as observed with inflammation, the cytotoxicity of PM DTPA complexes was augmented compared to that found in rats exposed solely to DTPA.

Vascular responses. While both PRA and DUI PM enhanced the fibrinogen concentration, DUI FPM alone also led to a small increase of vWF and a reduction of ACE in the plasma of compromised rats. The addition of DTPA to control or PM exposed groups resulted in almost no modulation of blood biomarkers compared to samples without DTPA (Table 4).

Role of Polycyclic Aromatic Hydrocarbons

The contribution of PAH to the pulmonary and cardiovascular effects of PM was evaluated by means of two complementary methods. First, we used two batches of coarse particles (collected in PRA and BAR respectively) that were very different in PAH composition (Table 2). The PRA PM contained a markedly higher concentration of total PAH (1.2 mg/g) than the BAR PM

Table 3. Biological effect parameters in bronchoalveolar lavage fluid and blood of spontaneously hypertensive rats exposed to PRA fine and coarse particulate matter

			water		FPM		CPM	
parameters		**units**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**
	BALF							
total cells	*inflammation*	x 10^6	0.86	0.67-1.05	2.19	1.52-2.85[a]	5.18	4.46-5.91[a,b]
neutrophils	*inflammation*	x 10^6	0.084	0.034-0.133	1.34	0.83-1.84[a]	3.76	3.17-4.35[a,b]
lymphocytes	*inflammation*	x 10^3	8.00	3.71-11.65	29.0	0-61.4[a]	88.0	36.9-139.8[a,b]
eosinophils	*inflammation*	x 10^3	4.00	1.15-6.22	5.00	1.62-8.42[a]	27.0	16.3-37.2[a,b]
monocytes	*inflammation*	x 10^3	10.0	6.1-13.4	27.0	14.9-39.9[a]	77.0	49.4-103.9[a,b]
macrophages	*inflammation*	x 10^6	0.76	0.59-0.92	0.79	0.64-0.94	1.23	1.01-1.45[a,b]
NAG	*inflammation*	U/L	0.61	0.48-0.75	1.21	0.96-1.47[a]	1.63	1.38-1.87[a,b]
MPO	*inflammation*	U/L	3.11	0-9.34	9.17	3.79-14.55[a]	72.1	59.2-84.9[a,b]
TNF-α	*inflammation*	pg/mL	96	75-117	178	147-210[a]	178	147-209[a]
LDH	*toxicity*	U/L	66	52-79	90	79-101[a]	167	137-197[a,b]
protein	*toxicity*	mg/L	433	316-549	663	425-900[a]	776	508-1045[a]
albumin	*toxicity*	mg/L	246	171-321	401	234-568[a]	434	278-589[a]
uric acid	*toxicity*	µmol/L	0.054	0.013-0.095	0.30	0.10-0.50[a]	0.83	0-1.83[a]
CC16	*toxicity*	pg/mL	0.24	0.12-0.37	0.24	0.10-0.38	0.14	0.07-0.21
	Blood							
CRP	*inflammation*	µg/mL	256	200-312	218	176-259	230	187-273
TNF-α	*inflammation*	pg/mL	85	61-109	78.0	56-100	77	51-103
ICAM-1	*inflammation*	pg/mL	488	461-516	476	436-516	495	449-541
vWF	*vascular markers*	U	1.48	0.73-2.22	1.79	0.74-2.84	1.52	0.39-2.65
fibrinogen	*vascular markers*	g/L	2.14	2.03-2.26	2.27	2.14-2.40[a]	2.49	2.38-2.60[a,b]
ACE	*vascular markers*	U/L	94.8	90.3-99.3	90.7	85.5-96.0	90.1	87.0-93.3

[a] Values regarded significant compared to water; $p < 0.05$. [b] Values regarded significant compared to fine particulate matter; $p < 0.05$. Abbreviations: CI - confidence intervals; CPM – coarse particulate matter; FPM – fine particulate matter; PRA – Prague.

(0.1 mg/g). Second, soluble organic extracts from the PRA and BAR samples were used in order to distinguish between the organic compounds loosely bound to particles and the solid particles themselves. The extraction of organic compounds with dichloromethane from both PM samples led to less oxidative reactivity generated by hydroxyl radicals (Figure 2).

Inflammation. Some differences in cellular toxicity and inflammation between the CPM samples from PRA and BAR were observed. While numerous inflammation markers were stimulated in the BALF of compromised rats following exposure, the native CPM from PRA, which has the greatest PAH content, increased the total numbers of cells and neutrophils more than BAR particles (Table 5). In parallel, the amount of the pro-inflammatory cytokine TNF-α and the activity of MPO and NAG were augmented by both batches of PM, and NAG and TNF-α were more active with PRA PM than with BAR particles. These markers of inflammation were also measured in the BALF and blood of hypertensive rats exposed to PRA and BAR PM organic extracts. The exposure to either of the PM organic extracts did not lead to any modulation of most inflammatory markers when the results were compared to those for their control with tricaprylin (Table 5). Indeed, we did not observe any modification of lymphocytes, eosinophils, monocytes, macrophages, total cells, or TNF-α, and the activity of NAG remained unchanged. PRA PM organic extract slightly increased only the neutrophils and the activity of MPO. Furthermore, the comparison of the total PM suspensions and the extracted organic compounds revealed that native particles induced a greater increase than organic extracts on pulmonary biomarkers of inflammation (Table 5).

Toxicity. PRA and BAR CPM also enhanced some markers of toxicity (Table 5): there were higher LDH, protein, and albumin concentrations when rats were exposed to PRA PM than when they were exposed to BAR PM. PRA and BAR PM organic extracts neither modulated the parameters used as toxicity biomarkers in the BALF compared to their respective controls (Table 5). The exposure of hypertensive rats to whole PM collected in PRA led to greater concentrations of LDH than PRA organic extract.

Vascular responses. The CPM collected in PRA led to a larger increase of fibrinogen than the PM collected in BAR (Table 5). The PRA sample with PAH bound to PM also induced greater concentrations of fibrinogen than the organic extracts of the same sample. However, the organic extracts of CPM collected in BAR had a somewhat stronger effect on vWF than the whole BAR particles.

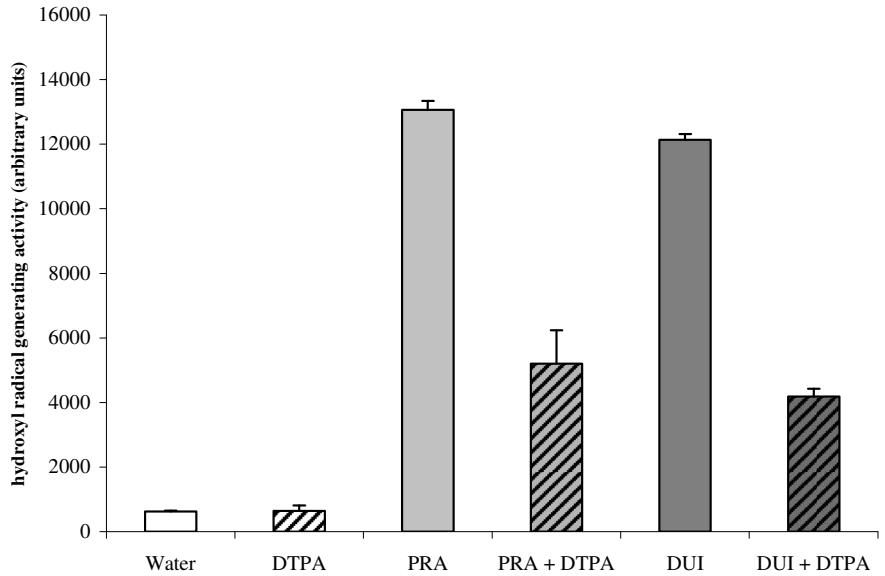

Figure 1. Action of diethylene triamine pentaacetic acid (DTPA) on hydroxyl radical activity induced by fine PM from PRA and DUI as measured by electron spin resonance: role of metals.

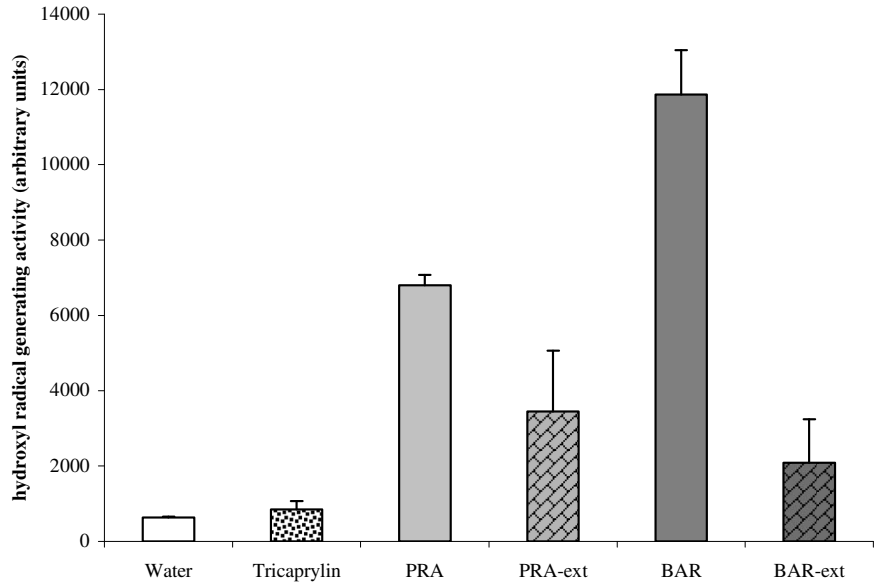

Figure 2. Action of extraction of organic compounds with dichloromethane on hydroxyl radical activity induced by coarse PM from PRA and BAR as measured by electron spin resonance: role of polycyclic aromatic hydrocarbons.

Table 4. Effect of fine particulate matter poor (PRA) or rich (DUI) in metals on different biological effect parameters and effect of metal complexation (DTPA)

			water		PRA		DUI		DTPA		PRA + DTPA		DUI + DTPA	
parameters		**units**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**
	BALF													
total cells	*inflammation*	x 10^6	0.86	0.67-1.05	2.19	1.52-2.85[a]	3.91	2.96-4.85[a,b]	1.82	1.38-2.26[a]	3.72	3.33-4.12[c,d]	5.51	4.50-6.52[c,d]
neutrophils	*inflammation*	x 10^6	0.084	0.034-0.133	1.34	0.83-1.84[a]	2.66	1.92-3.39[a,b]	0.98	0.58-1.24[a]	2.19	1.94-2.44[c,d]	3.61	2.79-4.44[c]
lymphocytes	*inflammation*	x 10^3	8.00	3.71-11.65	29.0	0-61.4[a]	28.0	5.3-50.6[a]	26.0	8.7-43.2[a]	50.0	23.0-77.1	98.0	37.1-159.3
eosinophils	*inflammation*	x 10^3	4.00	1.15-6.22	5.00	1.62-8.42[a]	7.39	1.28-13.49[a]	17.0	7.6-26.1[a]	19.0	9.2-28.7	25.0	8.23-40.8
monocytes	*inflammation*	x 10^3	10.0	6.1-13.4	27.0	14.9-39.9[a]	47.0	31.6-63.2[a,b]	28.0	20.5-35.6[a]	77.0	50.2-104.6[c,d]	84.0	54.9-113.0[c,d]
macrophages	*inflammation*	x 10^6	0.76	0.59-0.92	0.79	0.64-0.94	1.17	0.90-1.44[a,b]	0.84	0.62-1.07	1.38	1.14-1.63[c,d]	1.69	1.27-2.12[c,d]
NAG	*inflammation*	U/L	0.61	0.48-0.75	1.21	0.96-1.47[a]	1.79	1.53-2.05[a,b]	1.26	0.99-1.54[a]	1.79	1.51-2.07[c,d]	1.83	1.38-2.28[c]
MPO	*inflammation*	U/L	3.11	0-9.34	9.17	3.79-14.55[a]	35.2	25.1-45.3[a,b]	7.67	3.58-11.75[a]	8.7	4.18-13.23	26.2	19.1-33.3[c]
TNF-α	*inflammation*	pg/mL	96	75-117	178	147-210[a]	105	57-153[a,b]	87	50.3-123	99	69-130[d]	112	90-135
LDH	*toxicity*	U/L	66	52-79	90	79-101[a]	135	118-151[a,b]	181	155-207[a]	395	357-433[c,d]	418	325-511[c,d]
protein	*toxicity*	mg/L	433	316-549	663	425-900[a]	803	523-1083[a,b]	652	579-725[a]	1404	1184-1624[c,d]	1199	965-1433[c,d]
albumin	*toxicity*	mg/L	246	171-321	401	234-568[a]	467	275-658[a]	383	337-429[a]	882	726-1038[c,d]	715	585-845[c,d]
uric acid	*toxicity*	µmol/L	0.054	0.013-0.095	0.3	0.10-0.50[a]	0.39	0-0.94	0.25	0.03-0.47	1.34	0.48-2.21[d]	0.91	0.46-1.36[c]
CC16	*toxicity*	pg/mL	0.24	0.12-0.37	0.24	0.10-0.38	0.16	0.08-0.24	0.13	0.07-0.19	0.25	0.13-0.37	0.25	0.07-0.43
	Blood													
CRP	*inflammation*	µg/mL	256	200-312	218	176-259	217	172-262	216	161-272	280	209-352	268	181-356
TNF-α	*inflammation*	pg/mL	85	61-109	78.0	56-100	106	85-127	109	69-149	85	57-114	109	75-143
ICAM-1	*inflammation*	pg/mL	488	461-516	476	436-516	456	425-488	469	434-505	433	393-473	401	378-424[c,d]
vWF	*vascular markers*	U	1.48	0.73-2.22	1.79	0.74-2.84	3.13	1.22-5.03[a]	2.44	1.45-3.43	2.31	1.28-3.34	2.03	1.19-2.87
fibrinogen	*vascular markers*	g/L	2.14	2.03-2.26	2.27	2.14-2.40[a]	2.32	2.14-2.50[a]	2.26	2.10-2.41	2.46	2.32-2.61[c]	2.61	2.45-2.77[c,d]
ACE	*vascular markers*	U/L	94.8	90.3-99.3	90.7	85.5-96.0	87.7	84.0-91.3[a]	99.2	91.4-107.1	84.8	79.2-90.3[c]	86.4	80.4-92.4[c]

[a] Values regarded significant compared to water; $p < 0.05$. [b] Values regarded significant compared to fine Prague (PRA) particulate matter, $p < 0.05$. [c] Values regarded significant compared to DTPA, $p < 0.05$. [d] Values regarded significant compared to fine particulate matter without DTPA, $p < 0.05$. Abbreviations: CI – confidence intervals; DUI – Duisburg; PRA – Prague.

Table 5. Effect of coarse particulate matter rich (PRA) or poor (BAR) in polycyclic aromatic hydrocarbons and their organic extracts on different biological effect parameters

			water		PRA		BAR		tricaprylin		PRA extract		BAR extract	
parameters		**units**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**	**mean**	**95% CI**
	BALF													
total cells	*inflammation*	x10^6	0.86	0.67-1.05	5.18	4.46-5.91[a]	3.25	2.80-3.69[a,b]	1.22	0.90-1.55[a]	1.42	1.14-1.70[d]	1.06	0.91-1.21[d]
neutrophils	*inflammation*	x10^6	0.084	0.034-0.133	3.76	3.17-4.35[a]	2.00	1.59-2.41[a,b]	0.45	0.33-0.56[a]	0.77	0.58-0.96[c,d]	0.49	0.33-0.65[d]
lymphocytes	*inflammation*	x10^3	8.00	3.71-11.65	88.0	36.9-139.8[a]	63.8	44.7-82.8[a]	14.3	7.2-21.3[a]	16.4	2.4-30.4[d]	12.2	5.4-19.0[d]
eosinophils	*inflammation*	x10^3	4.00	1.15-6.22	27.0	16.3-37.2[a]	39.3	26.6-52.0[a]	13.1	6.4-19.9[a]	7.94	3.49-12.39[d]	12.3	8.5-16.2[d]
monocytes	*inflammation*	x10^3	10.0	6.1-13.4	77.0	49.4-103.9[a]	69.3	42.6-96.0[a]	20.5	9.3-31.8	18.4	9.3-27.6[d]	25.3	12.0-38.7[d]
macrophages	*inflammation*	x10^6	0.76	0.59-0.92	1.23	1.01-1.45[a]	1.07	0.97-1.18[a]	0.73	0.48-0.98	0.61	0.44-0.79[d]	0.52	0.42-0.62[d]
NAG	*inflammation*	U/L	0.61	0.48-0.75	1.63	1.38-1.87[a]	1.18	1.02-1.34[a,b]	1.33	1.06-1.61[a]	1.38	1.07-1.69	1.42	1.07-1.78
MPO	*inflammation*	U/L	3.11	0-9.34	72.1	59.2-84.9[a]	48.1	39.3-56.8[a]	2.26	0-4.67	6.35	2.11-10.6[c,d]	7.57	0-15.98[d]
TNF-α	*inflammation*	pg/mL	96	75-117	178	147-209[a]	167	107-227[a]	73	40-107	76	39-114[d]	76	48-104[d]
LDH	*toxicity*	U/L	66	52-79	167	137-197[a]	128	112-144[a,b]	108	94-122[a]	112	105-120[d]	142	70-214
protein	*toxicity*	mg/L	433	316-549	776	508-1045[a]	520	447-593[a,b]	674	346-1001[a]	615	497-734	684	276-1092
albumin	*toxicity*	mg/L	246	171-321	434	278-589[a]	286	240-333[b]	422	197-648[a]	368	280-455	408	141-674
uric acid	*toxicity*	µmol/L	0.05	0.013-0.095	0.83	0-1.83[a]	0.4	0.21-0.58[a]	0.45	0.11-0.79[a]	0.59	0.42-0.76	1.52	0-3.76
CC16	*toxicity*	pg/mL	0.24	0.12-0.37	0.14	0.07-0.21	0.16	0.04-0.27	0.11	0.05-0.17	0.1	0.05-0.16	0.12	0.08-0.16
	Blood													
CRP	*inflammation*	µg/mL	256	200-312	230	187-273	248	192-305	281	218-343	221	169-273	202	145-259
TNF-α	*inflammation*	pg/mL	85	61-109	76.9	51-103	61	34-88	81	55-107	54	26-81	67	51-83
ICAM-1	*inflammation*	pg/mL	488	461-516	495	449-541	495	454-535	483	441-524	506	462-550	466	429-502
vWF	*vascular markers*	U	1.48	0.73-2.22	1.52	0.39-2.65	1.14	0.89-1.39	2.14	0.22-4.06	1.81	0.96-2.65	1.66	1.44-1.88[d]
fibrinogen	*vascular markers*	g/L	2.14	2.03-2.26	2.49	2.38-2.60[a]	2.33	2.21-2.45[a,b]	2.2	2.04-2.35	2.32	2.17-2.47[d]	2.22	2.04-2.39
ACE	*vascular markers*	U/L	94.8	90.3-99.3	90.1	87.0-93.3	94.1	89.4-98.8	103	83-122	94.2	87.8-100.5	95.1	89.9-100.2

[a] Values regarded significant compared to water; $p < 0.05$. [b] Values regarded significant compared to coarse Prague (PRA) particulate matter, $p < 0.05$. [c] Values regarded significant compared to tricaprylin, $p < 0.05$. [d] Values regarded significant compared to coarse particulate matter without tricaprylin, $p < 0.05$. Abbreviations: BAR – Barcelona; CI – confidence intervals; DUI – Duisburg; PRA – Prague.

Discussion

This study shows an increase of several inflammatory and toxicity biomarkers in the lungs of hypertensive rats 24 h after a single dose of CPM or FPM, as well as an enhancement of the blood fibrinogen concentration. CPM possesses a stronger inflammatory potency and induces a greater concentration of LDH and fibrinogen than the FPM collected at the same site and time. PM with either high-transition metal or PAH content produces more LDH and protein release and pro-inflammatory marker release in BALF, and more coagulation than PM with low-transition metal or PAH content produces. Inactivating PM metals with a chelator or treatment of PM with an organic solvent highlights the strong contribution of metals and PAH – notably of PAH bound to particles – to the increase of pulmonary and blood biomarkers.

In recent years, it has become evident that PM mass per se, which is currently used in standard settings, may not be the causal factor that explains toxic events in populations. The roles of PM size and components need to be elucidated so that we can fully understand the effects of ambient particles on human health. The results of the present study are consistent with numerous studies that report an inflammatory reaction in the lung after an exposure to combustion particles or, more specifically, diesel or residual oil fly ash (ROFA; Kodavanti et al., 2001; Salvi et al., 1999), or to the whole inhalable fraction of ambient PM_{10} (Li et al., 1997; Schins et al., 2004). However, the biological mechanisms of FPM and CPM fractions have not yet been extensively studied *in vivo*, and data for $PM_{2.5-0.2}$ and $PM_{10-2.5}$ *in vivo* effects are still scarce (Gerlofs-Nijland et al., 2007; Happo et al., 2008; Jalava et al., 2006).

The present study also focuses on changes after exposure to PM in the blood. Fibrinogen, was induced by all PM samples. Increased plasma fibrinogen has been reported before in normal and hypertensive rats (Cassee et al., 2002; Elder et al., 2004) as well as in population or panel studies (Ghio et al., 2000; Pekkanen et al., 2000; Schwartz et al., 2001). This augmentation is corroborated by a previously observed increase of plasma viscosity during an air pollution episode (Peters et al., 1997). These effects may be predictive for cardiovascular diseases by PM and, notably, the development and progression of atherosclerosis (Mills et al., 2007). PM might promote thrombogenesis and contribute to the disruption of the atheromatous plaque by

means of its inflammatory action on endothelial cells and the generation of oxidative stress (Mills et al., 2007). However, although we describe a marked inflammatory reaction of the lungs, intratracheal instillation of CPM or FPM did not provoke any modification of the blood markers of inflammation, i.e., CRP, TNF-α, and ICAM-1, which suggests an absence of systemic inflammation. These results may appear to contradict previous data, since individual exposures of volunteers or patrol troopers to PM_{10} or $PM_{2.5}$ have been significantly related to increased concentrations of CRP and neutrophils in the blood (Riediker et al., 2004; Seaton et al., 1999). This apparent contradiction and the absence of PM modification of blood inflammatory biomarkers in our results might be explained by the altered cardiovascular inflammatory background in animals and humans affected by a cardiac disease (Haverkate et al., 1997; Kodavanti et al., 2000) or that these indicators are not affected by the exposures. Furthermore, the time required to develop systemic inflammation may be greater than the 24 h postexposure period used in the present study.

Although our study was not designed to extensively compare the effects of CPM and FPM, the use of $PM_{10-2.5}$ and $PM_{2.5-0.2}$ collected at the same sampling site in PRA enables us to highlight some differences of response between these two PM size ranges. In particular, our results show that, at the same mass, CPM has a more potent effect on inflammation than FPM as already shown in one of our previous studies (Gerlofs-Nijland et al., 2007). These findings suggest that the effects of the coarse fraction should not be neglected, even though the literature has traditionally attributed the effects of PM_{10} on pulmonary inflammation to $PM_{2.5}$. It is likely that the chemical composition, rather than the particle size, drives the toxicological effects. This implies importance of PM sources in particle-biological outcomes; the magnitude of the effects probably varies a lot from place to place and from season to season. One should, however, keep in mind that CPM concentrations in ambient air are generally lower than FPM concentrations and that the fraction of inhaled CPM deposited in the lower airways and alveolar region is substantially less than has been estimated for fine PM.

The use of PM samples of differing metal content enabled us to highlight the contribution of metals to pulmonary and cardiovascular effects. This study reveals an effect of metal rich PM on the numbers and the activity of neutrophils and monocytes/macrophages in the BALF of exposed rats that is greater than the effect of PM which has a poor metal content. In addition

to this action on inflammation, the metal rich PM sample elicits a greater pulmonary toxicity, as shown by a greater induced leakage of LDH and proteins as well as a tendency toward an increase of albumin BALF. Several studies have attributed the increase of inflammatory cells in the BALF of rodents exposed to ROFA to their metal content (Dreher et al., 1997; Hamada et al., 2002), which is compatible with our findings. The contribution of metals to ROFA-induced inflammation in epithelial cells has been confirmed by means of metal chelators, which cause a decrease of pro-inflammatory cytokines (Carter et al., 1997; Quay et al., 1998). Dye et al. (2001) studied rats exposed to particles collected in Utah Valley near a steel mill. They have found a significant increase of pulmonary injuries caused by particles collected before the steel mill closure and after it is reopening, which is in agreement with our own results.

Our results also suggest a contribution of metals to PM systemic effects: PM with high-transition metal content induced an increase of plasma vWF and a slight decrease of serum ACE that were not observed with PM with low-transition metal content. Nevertheless, the stronger induction of vWF was not associated with any increase of fibrinogen. It is tempting to believe that the increased vWF is attributable to a greater toxicity to endothelium rather than to an induced thrombotic effect of PM. This hypothesis is consistent with previous results that show increased myocardial injuries in sensitive rats treated with particles rich in metals (Kodavanti et al., 2000, 2003).

The metal complexation affected by the chelator DTPA led to results that are difficult to interpret. Indeed, DTPA partly reduced the hydroxyl-radical-generating activity of PM, and its addition to FPM collected in DUI led to an important decrease of the induction of neutrophils and their activity relative to the effect of DUI PM without DTPA. The DTPA concentration used in this study stems from *in vitro* studies in which DTPA is employed at noncytotoxic concentrations for its chelating properties (Mudway et al., 2005). Surprisingly, our results revealed an induction of pulmonary inflammation and toxicity following the addition of DTPA alone. This suggests a difference of response to this product between *in vitro* and *in vivo* studies. This chelator has been extensively used in human and animal experimental studies, especially as a permeability biomarker (Minty et al., 1981). Although the use of a metal chelator led to a marked reduction of DUI PM-induced pulmonary inflammation, some differences of pulmonary and systemic response between PM with low and high transition metal content still occurred after

the metal complexation. This could be due to the presence of components other than metals in PM that can cause pulmonary inflammation and thrombosis.

This study also demonstrates the importance of organic compounds – and probably of PAH bound to particles – in pulmonary and vascular outcomes. The results of this study underline the importance of PM sources in inducing adverse health effects. They suggest that PM composition should be considered in ambient pollution regulations, particularly with a view to the identification of components that may be used as particle indicators.

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4 PULMONARY AND CARDIOVASCULAR EFFECTS OF TRAFFIC-RELATED PARTICULATE MATTER: 4-WEEK EXPOSURE OF RATS TO ROADSIDE AND DIESEL ENGINE EXHAUST PARTICLES

Miriam E. Gerlofs-Nijland, Annike I. Totlandsdal, Evren Kilinç, A. John F. Boere, Paul H.B. Fokkens, Daan L.A.C Leseman, Constantinos Sioutas, Per E. Schwarze, Henri M. Spronk, Patrick W.F. Hadoke, Mark R. Miller, and Flemming R. Cassee

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Abstract

Traffic-related particulate matter (PM) may play an important role in the development of adverse health effects, as documented extensively in acute toxicity studies. However, rather little is known about the impacts of prolonged exposure to PM. We hypothesized that long-term exposure to PM from traffic adversely affects the pulmonary and cardiovascular system through exacerbation of an inflammatory response. To examine this hypothesis, Fisher F344 rats, with a mild pulmonary inflammation at the onset of exposure, were exposed for 4 weeks, 5 days/week for 6 h a day to: (a) diluted diesel engine exhaust (PM_{DEE}), or: (b) near roadside PM ($PM_{2.5}$). Ultrafine particulates, which are largely present in diesel soot, may enter the systemic circulation and directly or indirectly trigger cardiovascular effects. Hence, we assessed the effects of traffic-related PM on pulmonary inflammation and activity of procoagulants, vascular function in arteries, and cytokine levels in the heart 24 hours after termination of the exposures. No major adverse health effects of prolonged exposure to traffic-related PM were detected. However, some systemic effects due to PM_{DEE} exposure occurred including decreased numbers of white blood cells and reduced von Willebrand factor protein in the circulation. In addition, lung tissue factor activity is reduced in conjunction with reduced lung tissue thrombin generation. To what extent these alterations contribute to thrombotic effects and vascular diseases remains to be established. In conclusion, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms.

Introduction

Airborne ambient particulate matter (PM) is considered to play an important role in the adverse health effects associated with air pollution (Brunekreef and Holgate, 2002). Most epidemiological studies have focused on the effects of short-term exposure to air pollutants. In these short-term studies, a clear link was shown between levels of air pollutants and a tendency towards a hypercoagulable state. These associations are, for example, found with both PM_{10} and NO_2 (Baccarelli et al., 2007) or only with the traffic-related gaseous (NO_2 and CO) instead of with PM mass (Rudez et al., 2009). However, several epidemiological studies have associated long-term exposure to the fine fraction of PM ($PM_{2.5}$: PM with an aerodynamic diameter below 2.5 μm) with an increase in pulmonary and cardiovascular morbidity and mortality (Pope et al., 2004; Schikowski et al., 2005). Notably, living close to a busy road over several years has been associated with increased cardiopulmonary mortality (Gehring et al., 2006; Hoek et al., 2002). Hence, road traffic, which is a major source of $PM_{2.5}$ in urban areas, could be particularly responsible for the impact of PM exposure on human health (Beelen et al., 2009; Gauderman et al., 2005; Hoffmann et al., 2009; Schikowski et al., 2005).

These epidemiological observations are supported by controlled toxicology studies performed with animals and human volunteers exposed to PM samples from different sites. Animal exposure studies attribute a greater toxicity of PM collected at locations that contain a high proportion of traffic emissions (Gerlofs-Nijland et al., 2007; Lai et al., 2005; Seagrave et al., 2006). Interestingly, it is becoming evident that exposure to traffic-related PM has marked actions on the cardiovascular system, as well as their more well-recognized pulmonary effects (Elder et al., 2007; McCreanor et al., 2007). Short-term exposures to diesel engine exhaust, an important source of $PM_{2.5}$, cause both vascular dysfunction and impaired endogenous fibrinolysis in healthy and compromised volunteers (Mills et al., 2005, 2007). In addition, elevated thrombus formation was shown *ex vivo* after inhalation of diesel engine exhaust (Lucking et al., 2008).

In these studies, mostly fresh generated emission particles were used as a surrogate of PM, which are not necessarily representative of the PM in ambient air. Inhalation of elevated concentrations of ambient air particles collected with different size ranges at different sites by using concentrator

technology (Kim et al., 2001a, 2001b ; Sioutas et al., 1997) represents a more realistic PM exposure (Lippmann and Chen, 2009). A few hours exposure to $PM_{2.5}$ from urban traffic sites caused an increase in cardiovascular symptoms and in lung toxicity and inflammation in rodents and volunteers (Araujo et al., 2008; Cassee et al., 2005; Gong et al., 2003; Kleinman et al., 2005; Kooter et al., 2006; Lippmann et al., 2005a; Ying et al., 2009). This also suggests a major contribution of traffic-related particles to the biological effects associated with PM. Therefore, these studies, in which relatively high PM exposure levels were applied, will be useful in understanding the impact of episodic PM exposure on human health.

At present, only a few publications, all from the same well-conducted study in New York, describe the impact of prolonged exposure on normal and susceptible (i.e. mimicking a human disease) rodents at lower concentration, more environmentally relevant levels (Chen and Hwang, 2005; Chen and Nadziejko, 2005; Hwang et al., 2005; Lippmann et al., 2005b; Sun et al., 2005, 2008; Veronesi et al., 2005). The most striking results were seen on the cardiovascular system with altered vasomotor tone, induced vascular inflammation, and potentiated atherosclerosis both in Sterling Forrest (Sun et al., 2005) and more traffic-influenced Manhattan (Ying et al., 2009).

In order to investigate the contribution of traffic to the long-term effect of particles, we performed a series of experiments in which we exposed rats to filtered air, to diluted diesel engine exhaust (rich in ultrafine particles) and to $PM_{2.5}$ derived from a nearby very busy freeway. Prior to the PM exposures, a minor lung inflammation was induced by exposing the rats to ozone. We hypothesized that prolonged (i.e. 4-week) exposure to traffic-derived $PM_{2.5}$ exacerbates the existing inflammatory reaction, which could result in an induction of oxidative stress with subsequent effects on the pulmonary and cardiovascular system. In order to verify this hypothesis, a comprehensive analysis of markers for pulmonary (oxidative stress, cytotoxicity, inflammation) and cardiovascular (coagulation, fibrinolysis, endothelial damage, thrombogenicity, heart inflammation, aorta contractibility) effects due to exposure to traffic-derived PM was performed.

Methods

Animals

Male SPF F344 (DUCRL) rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in macrolon type III cages with a room temperature maintained at 22±2°C, relative humidity at 40-70%, and a 12-h light/dark cycle. Rats were allowed access to a cereal-based rodent diet (SMR-A; Hope Farms, Woerden, The Netherlands) and tap water via drinking bottles ad libitum during non-exposure periods. Exposure started after 7 days of acclimatization.

Experimental Design

A total of three experiments were conducted using different types of PM exposure (Gerlofs-Nijland et al., 2009a). At day 0, all rats were exposed (whole body) for 12 h to 0.4 ppm ozone (Marra and Rombout, 1990) to initiate a minor inflammation in the lung (Cassee et al., 2005). After the initial ozone exposure the animals were transferred to RIVM's mobile exposure laboratory (MAPCEL) and subsequently exposed for 4 weeks (5 days per week, 6 h per day) to diesel engine exhaust (PM_{DEE}) or to concentrated ambient particles with an aerodynamic diameter <2.5 µm ($PM_{2.5}$ or also known as CAPs) near a busy roadside at Utrecht, The Netherlands (Figure 1). The $PM_{2.5}$ roadside study was repeated once due to the inherent variability of the mass and composition of ambient PM.

PM_{DEE} - Exposure was performed using a 35 KVA diesel generator (Bredenoord, Apeldoorn, The Netherlands) under idling conditions. The animals (n=15/group) were exposed to 150 µg/m^3 PM_{DEE} diluted with clean conditioned air.

$PM_{2.5}$ roadside - Rats were exposed to increased levels of $PM_{2.5}$ using the Versatile Aerosol Concentration Enrichment Systems (VACES) (Kim et al., 2001a, 2001b) with a theoretical enrichment factor of 20 and at an output flow rate directed into the nose-only system of 20 LPM. The MAPCEL was placed close to (15 m), and east of a major roadside (A2; Utrecht-Amsterdam, The Netherlands), with prevailing westerly winds, used by 160,000 cars and trucks per day.

Control animals were exposed to filtered, purified air with the same temperature and relative humidity as the test atmospheres. All rats were nose-only exposed using novoplast tubes T (Münster AG, Muttenz, Switzer-

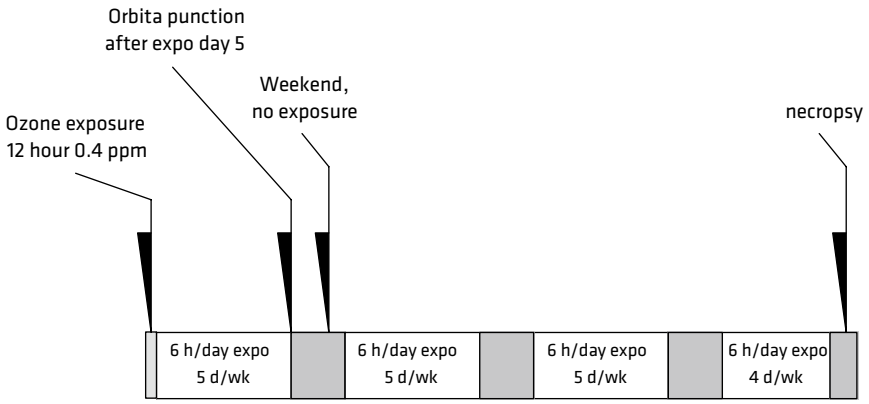


Figure 1. Experimental exposure design

land) in nose-only exposure chambers. One week before exposure, animals were trained in nose-only tubes to reduce the stress of the restraint (3 days, 1h per day). Immediately after the exposures, the animals were returned to their housing facilities.

Blood (1 ml) was obtained after the first week of exposure (directly after the fifth exposure day; Figure 1) by orbital puncture under Brevimethal anaesthesia (50 mg/kg bodyweight, intramuscularly) to measure fibrinogen, von Willebrand factor (vWF), plasminogen activator inhibitor (PAI)-1 and CC16.

Based on the initial findings, additional parameters were investigated, i.e. vascular function, measurement of cytokines in cardiac tissue, and tissue factor (TF) activity and thrombin generation in lung tissue in one of the two roadside experiments to gain more insight in a possible biological mechanism.

Necropsy was performed on the day after the last exposure day (Figure 1). Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI, Bilthoven, The Netherlands).

Characterization of the test atmospheres

A condensation particle counter (CPC model 3022A; TSI, St. Paul, MN) was used to determine the particle number concentration in the inlet of the exposure chamber. The mass concentration was measured continuously in the inlet of the exposure chamber during the exposure with a nephelometer (DATARAM 2000; MIE, Billerica, MA). In the $PM_{2.5}$ roadside experiments, the

particle number and mass concentration were measured both before the VACES inlet and after the VACES. The time-integrated PM concentrations were also measured in the inlet of the exposure chamber by means of collection on three 47-mm filters placed in parallel, two polytetrafluoroethylene (PTFE; Teflon R2PJ047; Pall Corp. Ann Arbor MI) and one Quartz filter (QMA; Whatman Int Inc, Maidstone, England). A carbon sampler tube (Anasorb CSC Lot 2000; SKC Inc., Eighty Four, PA) was placed downstream of one of the PTFE filters at the outlet to collect the volatile organic components (VOCs). One set of PTFE filters and a carbon sampler tube were used for each exposure week. Carbon monoxide (ML 9830 CO; Lear Siegler, Englewood, CO), sulfur dioxide (Model 43A; Thermo Environmental Instruments,, Franklin, MA) and nitrogen oxides (Model 42W; Thermo Environmental Instruments) were measured in the PM_{DEE} mixing chamber or at the inlet of the VACES. In the PM_{DEE} experiment, a Scanning Mobility Particle Sizer (SMPS, DMA model 3071 + CPC model 3022A; TSI) was used to measure the particle size distribution (mean diameter and geometric standard deviation) every hour in the inlet of the exposure chamber. The weekly time-integrated particle size mass distribution was measured at the inlet of the VACES with an eight-stage Micro Orifice Impactor (model No. 100; MSP Corporation, Minneapolis MN). Temperature and relative humidity were recorded once every 5 minutes in the exposure chamber and control exposure chamber and recorded every 30 minutes in the inlet of the exposure chamber. The activated carbon samplers were analyzed using GC-MS (RIVM, Bilthoven, The Netherlands) to determine the VOC concentrations.

Necropsy

The day after the final test atmosphere exposure, the rats were anesthetized with a mix of Ketamine and Rompun: 100 mg /kg of Ketamine (Aesculaap, Boxtel, The Netherlands) and 1 mg/kg Rompun (Bayer, Leverkusen, Germany). A cannula was inserted in the trachea. The abdomen was opened and a minimum of 6 ml blood was sampled through the abdominal aorta. The chest was opened and the lungs were perfused (pressure 30 cm H_2O) with saline to remove the blood from the lung using a cannula placed through the right heart chamber into the pulmonary artery. The left bronchus was clamped and the left lung was cut just behind the clamp. The left lung was weighed and fixed for 1 h under a constant pressure of 20 cm H_2O using 4% phosphate-buffered formaldehyde. The right lung was used for bron-

choalveolar lavage fluid (BALF) collection by three lavages of sterile saline (27 ml/kg body weight). The heart was dissected, split into the right and left side and frozen in liquid N_2. The descending thoracic aorta was dissected and immediately placed in Krebs buffer for organ bath measurements.

Bronchoalveolar lavage analyses

The collected BALF was centrifuged at 400g, 4°C, for 10 min. The cell-free fluid from the lavage was used for assessment of lactate dehydrogenase (marker for cytotoxicity), *N*-acetylglucosaminidase (macrophage activation), alkaline phosphatase (type II cell damage), and the levels of Clara-cell 16 protein (CC16, lung cell damages), reduced glutathione and oxidized glutathione (GSH and GSSG, respectively), albumin and total protein levels (increased permeability of the alveolar–capillary barrier), inflammatory mediators interleukin 6 (IL-6), and tumor necrosis factor (TNF)-α were determined as previously described (Cassee et al., 2005; Gerlofs-Nijland et al., 2005). Heme-oxygenase-1, a marker of oxidative stress, was determined using a commercially obtained reagent kit (Roche Nederland B.V, Mijdrecht, The Netherlands). The BALF pellet was resuspended in saline and used for total cell counts as well as preparation of cytospins for cell differential counts as previously described (Gerlofs-Nijland et al., 2005).

Hematological Analyses

Plasma levels of fibrinogen and CC16 were determined as previously described (Cassee et al., 2005; Gerlofs-Nijland et al., 2009b). vWF was measured by enzyme-linked immunosorbent assay (ELISA; American Diagnostica Inc., Stamford, US). Levels of tissue plasminogen activator, total antigen and active PAI-1 were measured in citrated plasma by ELISA (Innovative Research, Dearborn, M). Cell differentials were determined in EDTA (K3) (Terumo Europe N.V., Leuven, Belgium) anticoagulated blood in an H1-E multispecies hematology analyzer (Bayer B.V., Mijdrecht, The Netherlands). The following parameters were measured: white and red blood cell concentrations (WBC and RBC, respectively), hemoglobin, and platelet (PLT) concentrations, the mean platelet volume (MPV), and the hematocrit value. In addition, mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration, red blood cell distribution width, mean platelet component and hemoglobin distribution width were provided.

Pathology

The left lung was embedded in paraffin after fixation with formaldehyde. Tissues were cut into 5-µm slices and slides were stained with hematoxylin and eosin before light microscopic examination. Slides were screened for pathological changes as a result of the exposure. The pathological lesions and inflammation were semi-quantitatively and blindly scored as absent, minimal, slight, moderate, marked, or strong.

Vascular function

Ex vivo endothelial function and vascular responses were measured in isolated thoracic aortic rings by a modified method of Bagate et al. (2004) and Miller et al. (2009). Segments of thoracic aorta (~5 mm length) were cleaned of connective tissue and mounted in organ baths in Krebs buffer bubbled with 5% CO_2/95% O_2 at 37°C. A baseline tension of 14.7 mN was gradually applied over 10 min and vessels were allowed to equilibrate for a further 30 min.

Vessel viability was confirmed by a contractile response on addition of 80 mM KCl, repeated three times. Concentration-response curves to phenylephrine (PE; 1 nM to 10 µM) were obtained and a concentration that produced 80% maximum contraction (0.1-1 µM) was chosen for each individual rat aortic ring. Following contraction, cumulative concentration-response curves were obtained for acetylcholine (ACh; endothelium-dependent vasodilator; 1 nM to 10 µM), sodium nitroprusside (SNP; endothelium-independent nitric oxide donor; 0.1 nM to 1 µM) and isoprenaline or verapamil (endothelium- and nitric oxide-independent vasodilators; 1 nM to 10 µM). At least 30 min washout was allowed before application of subsequent drugs.

Analyses of cardiac tissue

Cytokine mRNA expression. The frozen right heart halves were homogenised in lysis-buffer and total RNA isolated using a 'Absolutely RNA™ RT-PCR Miniprep kit' (Stratagene, La Jolla, CA). mRNA in each sample was reverse-transcribed into cDNA on a PCR system 2400 (Perkin Elmer, Groningen, The Netherlands) by using a High Capacity cDNA Archive Kit from Applied Biosystems (Life Technologies Corporation, Carlsbad, CA). Quantitative real-time (QRT) PCR was performed on triplicate samples, with 18S rRNA as an internal control, using the Applied Biosystems 7500 Real-Time PCR System, with pre-designed TaqMan Gene Expression Assays (IL-6,

Rn00561420_m1; IL-1β, Rn00580432_m1; TNF-α, Rn 00562055_m1; 18S, Hs99999901_s1) and TaqMan Universal PCR Master Mix. The expression of each gene within each sample was normalised against 18S rRNA and expressed relative to a heart tissue sample from one of the control rats using the formula $2^{-(\Delta\Delta Ct)}$, in which ΔΔCt = (Ct mRNA – Ct 18S rRNA)sample – (Ct mRNA – Ct, 18S rRNA) sample control rat.

Phosphorylation of mitogen-activated protein kinases (MAPKs)
Right heart halves were homogenized in lysis-buffer (20 mM Tris-HCL pH+7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 µM leupetin, 1.5 µM aprotinin, 15 µM pepstatin A and 1% Triton-X) and examined by western analysis. Protein concentration in the samples was determined by using the BioRad DC Protein Assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherllands). Proteins (12.5-25 µg/well) from the homogenized heart tissue samples were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for the respective phosphorylated kinases (p-ERK1/2, p-JNK1/2, p-p38) prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed using the Super-Signal® West Dura chemoluminiscence system (Perbio Science Nederland B.V., Etten-Leur, The Netherlands) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with Mild Antibody Stripping Solution® from Chemicon International (Termecula, CA), and re-probed with antibodies against total mitogen-actvated protein kinase (MAPK) proteins (ERK1/2, JNK, p38). Optical quantification of the protein bands were performed by using the KODAK 1D Image Analysis Software.

TF activity and thrombin generation in lung tissue
TF activity and tissue-specific thrombin generation by means of the Calibrated Automated Thrombogram (Thrombinoscope BV, Maastricht, The Netherlands) were determined in lung tissue homogenates as described previously (Frederix et al., 2008). Briefly, thrombin generation was measured in the presence of a final concentration of 5 pM TF and 4 µM phospholipids (PL, at 20:20:60 mol% PS:PE:PC) after addition of lung homogenates in

human plasma and alternatively measurements were also implemented in the absence of both TF and PL. All TG results were normalized and expressed as percentage of normal pooled, PLT-poor plasma which was prepared from at least 80 healthy volunteers (Spronk et al., 2008).

Statistical analysis
Data are expressed as mean ± standard deviation (SD) or standard error of mean (SEM). Vascular responses are expressed as percentage of the maximal contraction to PE, where positive values represent vasodilatation and 100%

Table 1. Particle exposure characteristics of diesel engine exhaust and concentrated ambient particles near a roadside

Experiment	Week no	Mass	Number	CO	NO	NO_2	NOx	VOC	Inorganics	MMAD
		µg/m³	# 10^5/cm³	µg/m³	µg/m³	µg/m³	µg/m³	µg/m³	µg/m³	µm
	1	160	4.83	3515	2058	1128	3186	564	nd	nd
	2	162	4.44	3131	1671	937	2608	897‡	nd	0.29
PM_{DEE}	3	191	4.18	2945	1571	841	2413	5416‡	nd	0.24
	4	182	3.89	2689	1397	765	2162	4068‡	nd	0.17
	Average	174	4.34	3050	1671	918	2589	564	–	0.23
	1	484	3.72	nd	72	84	156	820	nd	1.18
$PM_{2.5}$	2	284	3.51	nd	65	76	141	70	nd	0.99
roadside #1	3	528	1.71	nd	22	42	65	45	nd	1.01
	4	643	3.53	nd	69	82	151	80	nd	0.97
	Average	485	3.12	–	56	71	127	254	–	1.04
	1	200	2.46	nd	82	48	130	6	44	1.46
$PM_{2.5}$	2	199	2.63	nd	87	71	158	6	60	1.53
roadside #2	3	224	2.32	nd	85	59	144	49	46	2.13
	4	232	1.84	nd	70	63	133	13	81	0.95
	Average	214	2.31	–	81	61	142	19	58	1.52

Abbreviations: PM – particulate matter; DEE – diesel engine exhaust; Inorganics – sum of sulfate, nitrate, chloride and sodium; VOC – volatile organic components; MMAD- Aerodynamic particle size measured by MOI; nd – not determined
‡Unreliable outcomes due to overload carbon samplers, those values are not included in the average VOC content.

vasodilatation represents a complete abolition of PE-induced tone. The outcomes of the BALF, blood analyses, TF activity and thrombin generation were compared using an unpaired Student's *t*-test. Statistical comparisons of vasodilator curves were carried out using two-way Analysis of Variance (ANOVA), or unpaired Student's *t*-test for comparisons of EC_{50} and maximum responses (estimated following linear regression of individual curves using Graphpad Prism V4.0b). $P<0.05$ was accepted as statistically significant.

Results

Ozone exposure

A separate group of 10 animals was used to confirm that ozone exposure induced a minor lung inflammation. At 24 h after the ozone exposure, there was a significant increase in lung permeability, as shown by elevated protein (487 ± 141 compared to control levels of 159 ± 49 mg/l; $P<0.001$) and albumin (248 ± 99 versus 50 ± 14 mg/l in control group; $P<0.001$) levels in BALF. Ozone exposure also increased the percentage of polymorphonuclear neutrophils (PMN) in the alveolar region by approximately 2.5% (3.05 ± 2.55% versus 0.65 ± 0.95% for control), although this increase did not reach statistical significance ($P>0.05$).

Exposures characteristics

Diesel engine exhaust. The PM_{DEE} exposures were performed at an overall average particle mass of 174 ± 15 µg/m³ (Table 1). The average particle size (geometric median diameter) was 76 nm with a geometric standard deviation of 1.95 nm as measured by SMPS, with an average particle number concentration of 434,000/cm³. During the first exposure week, the carbon sampler was used only for 1 day and the amount of VOC measured was 564 µg/m³. In addition, the concentrations of gaseous pollutants CO, NO, NO_2 and NOx were measured, with mean concentrations of 3050, 1671, 918 and 2589 µg/m³, respectively. Levels of VOC during the last three exposure weeks could not be measured due to an overload in the carbon sampler tubes.

$PM_{2.5}$ roadside #1. During the first $PM_{2.5}$ roadside study, the overall average particle mass was 485 ± 150 µg/m³ (Table 1). The average particle number concentration was 312,000/cm³ with a mean aerodynamic particle size of 1.04 µm and geometric standard deviation of 0.31 (measured by Micro Orifice Impactor (MOI) before the VACES). The mean VOC content measured was 254 µg/m³, which was mainly driven by high levels of VOC (820 µg/m³) during the first week of exposure. These appeared to be caused by high amounts of heptane, most probably due to a two-stroke engine used for lawn mowing activities nearby. The concentrations of gaseous pollutants NO, NO_2 and NOx were 56, 71 and 127 µg/m³, respectively.

$PM_{2.5}$ roadside #2. The overall average particle mass in the second $PM_{2.5}$ roadside study was 214 ± 17 µg/m³. The average particle number concentra-

Table 2. Parameters in bronchoalveolar lavage fluid after exposure to PM from diesel engine exhaust or concentrated ambient particles near a roadside

		PM_{DEE}		$PM_{2.5}$ roadside #1		$PM_{2.5}$ roadside #2	
BALF		Control	PM exposure	Control	PM exposure	Control	PM exposure
Parameter	Unit	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Macrophages	%	95.5 ± 1.5	95.0 ± 2.6	96.3 ± 1.2	96.4 ± 3.7	98.3 ± 0.8	98.6 ± 0.6
PMN	%	3.18 ± 1.31	3.33 ± 1.88	2.10 ± 1.05	2.25 ± 2.93	0.98 ± 0.40	0.71 ± 0.37
Lymphocytes	%	1.18 ± 0.62	1.45 ± 0.89	1.47 ± 0.87	1.23 ± 0.80	0.77 ± 0.47	0.63 ± 0.40
Total cells	# x 10^6	0.91 ± 0.31	1.058 ± 0.3	0.63 ± 0.18	0.57 ± 0.17	0.67 ± 0.16	0.65 ± 0.23
Macrophages	# x 10^6	0.87 ± 0.3	1.006 ± 0.28	0.61 ± 0.18	0.55 ± 0.17	0.66 ± 0.16	0.64 ± 0.23
PMN	# x 10^6	0.03 ± 0.02	0.36 ± 0.03	0.01 ± 0.01	0.01 ± 0.02	0.006 ± 0.003	0.005 ± 0
Lymphocytes	# x 10^6	0.01 ± 0	0.016 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.005 ± 0.004	0.004 ± 0
HO-1	ng/mL	0.09 ± 0.04	0.11 ± 0.04	0.31 ± 0.08	0.32 ± 0.08	0.05 ± 0.05	0.03 ± 0.04
TNF-α	ng/mL	16.3 ± 5.9	16.6 ± 10.7	29.1 ± 7.2	30.4 ± 6.9	137.0 ± 31.8	133.5 ± 32.5
IL-6	µg/mL	57.2 ± 12.3	61.5 ± 26.6	87.3 ± 19.2	90.0 ± 20.2	42.9 ± 13.3	45.5 ± 12.0
CC-16	µg/mL	5.08 ± 1.44	4.76 ± 2.05	7.02 ± 1.90	7.45 ± 2.84	8.49 ± 1.21	**9.47* ± 1.14**
ALP	U/L	36.3 ± 11.1	35.5 ± 16.9	38.4 ± 13.0	40.8 ± 12.5	44.3 ± 10.1	44.3 ± 7.4
LDH	U/L	108 ± 29	112 ± 40	180 ± 93	174 ± 57	106 ± 9	92 ± 10
Protein	mg/L	183 ± 36	177 ± 40	282 ± 156	302 ± 103	359 ± 63	363 ± 85
Albumin	mg/L	117 ± 24	115 ± 31	184 ± 108	209 ± 77	184 ± 38	185 ± 56
NAG-B	U/L	1.06 ± 0.38	1.34 ± 0.43	2.91 ± 0.64	3.38 ± 0.57		
Total glutathione	µmol/L	0.82 ± 0.44	1.27 ± 1.13	1.52 ± 0.93	1.48 ± 0.56	1.46 ± 0.90	1.70 ± 0.90
GSSG	µmol/L	0.14 ± 0.13	0.13 ± 0.18	0.45 ± 0.26	0.41 ± 0.22	0.64 ± 0.42	0.55 ± 0.21
GSH	µmol/L	0.55 ± 0.36	1.04 ± 0.96	0.74 ± 0.87	0.67 ± 0.52	0.37 ± 0.47	0.69 ± 0.67

*P<0.05 compared to experimental control

tion was 231,000/cm^3 with an associated aerodynamic mean particle size of 1.52 µm and geometric standard deviation of 0.23 (measured by MOI before the VACES). The VOC content was 19 µg/m^3 with NO, NO_2 and NOx concentrations of 81, 61 and 142 µg/m^3, respectively.

BALF analyses

Prolonged exposure to PM_{DEE} or $PM_{2.5}$ near a roadside did not induce a detectable inflammatory response in healthy rats. The number of PMN in BALF was not significantly increased after 4 weeks of exposure to $PM_{2.5}$ roadside or PM_{DEE}; nor were there any changes in the pro-inflammatory cytokines TNF-α and IL-6 (Table 2). Although some parameters (e.g. TNF-α, protein) showed strong differences with higher values after exposure to roadside $PM_{2.5}$, the only statistically significant change was an increase in BALF CC16 after exposure to $PM_{2.5}$ in the second roadside study (9.47 ± 1.14 versus 8.49 ± 1.21 in the control group; $P<0.05$; Table 2). Notably, protein and albumin levels in BALF were significantly higher in all animals that were transported to our field location near the freeway compared to those that were exposed in our laboratory at the RIVM. Apart from the fact that batch-to-batch variation among the groups of animals that we received from the breeder cannot be excluded, the only other explanation is that the transport from the field location to the lab might have resulted in increased stress and increased baseline values of the noted parameters. Since most of the other parameters that we have assessed were not to be affected in a similar manner, and we performed the (statistical) comparisons only within each of the three experiments, conclusions were not affected by this unexpected phenomenon.

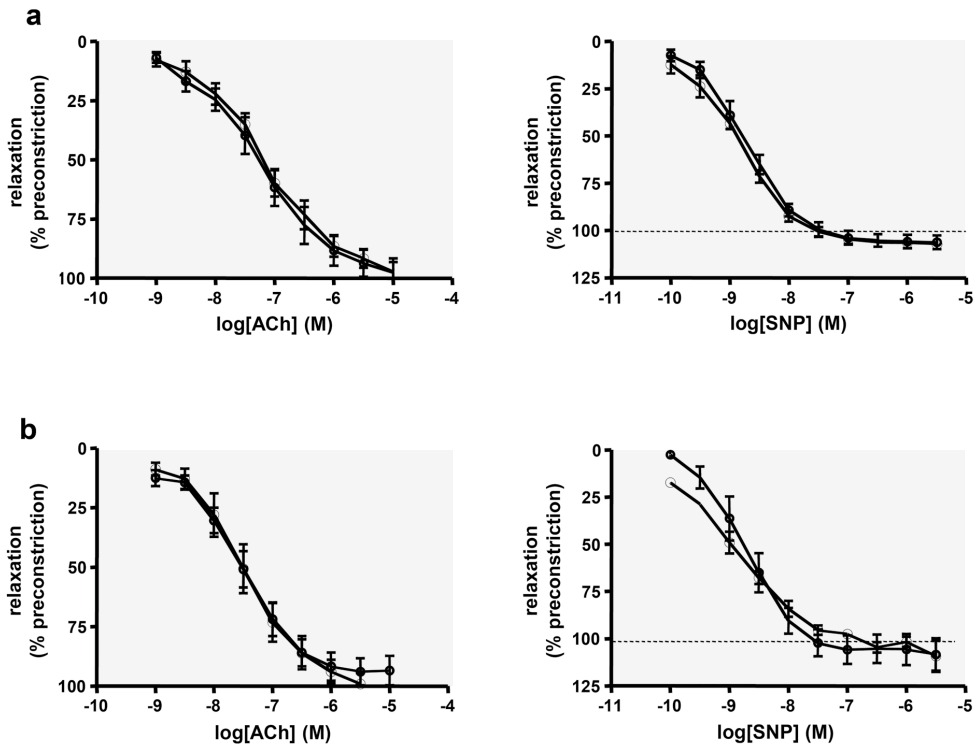
Hematological analyses

Prolonged PM_{DEE} exposure resulted in significantly reduced numbers of WBCs, lymphocytes and basophilic granulocytes (Table 3). On the other hand, neither $PM_{2.5}$ roadside exposure induced any significant changes in blood parameters, although a small decrease of lymphocyte number was observed in the second $PM_{2.5}$ roadside study. A reduction in the blood vWF levels was observed 4-weeks after exposure to PM_{DEE} (112.2 ± 34.2 mU/ml versus 132.5 ± 13.2 mU/mL in the control group; $P<0.05$; Table 3).

Table 3. Parameters in blood after exposure to diesel engine exhaust or concentrated ambient particles near a busy roadside

		PM_{DEE}		$PM_{2.5}$ roadside #1		$PM_{2.5}$ roadside #2	
Blood		**Control**	**PM exposure**	**Control**	**PM exposure**	**Control**	**PM exposure**
Parameter	**Unit**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
4 Weeks							
RBC	x 10^{12}/L	8.73 ± 0.36	8.65 ± 0.23	8.62 ± 0.23	8.59 ± 0.28	8.37 ± 0.20	8.41 ± 0.30
HGB	mmol/L	9.20 ± 0.39	9.02 ± 0.23	8.90 ± 0.31	8.93 ± 0.32	8.81 ± 0.21	8.82 ± 0.30
HCT	L/L	0.416 ± 0.016	0.412 ± 0.015	0.395 ± 0.015	0.396 ± 0.015	0.392 ± 0.010	0.393 ± 0.018
HDW	mmol/L	1.832 ± 0.079	1.855 ± 0.052	1.954 ± 0.071	1.930 ± 0.111	1.795 ± 0.101	1.806 ± 0.111
PLT	x 10^9/L	448 ± 57	431 ± 43	476 ± 89	462 ± 158	640 ± 60	671 ± 88
MPC	g/dL	22.59 ± 0.79	22.07 ± 0.93	22.87 ± 0.83	22.97 ± 0.75	22.81 ± 0.75	23.02 ± 0.61
WBC	x 10^9/L	3.69 ± 1.01	**2.91* ± 0.5**	3.34 ± 0.92	3.60 ± 1.11	4.23 ± 0.89	3.68 ± 0.82
PMN	x 10^9/L	0.77 ± 0.26	0.67 ± 0.18	0.76 ± 0.25	0.86 ± 0.28	0.68 ± 0.18	0.67 ± 0.18
Lymphocytes	x 10^9/L	2.78 ± 0.71	**2.14* ± 0.43**	2.47 ± 0.73	2.61 ± 0.85	3.36 ± 0.68	**2.85† ± 0.64**
Basophils	x 10^9/L	0.008 ± 0.005	**0.002* ± 0.003**	0.026 ± 0.013	0.028 ± 0.017	0.026 ± 0.019	0.022 ± 0.007
PMN	%	20.6 ± 02.1	23.1 ± 5.6	23.1 ± 5.2	24.2 ± 5.7	16.2 ± 2.6	18.14 ± 2.74
Lymphocytes	%	75.86 ± 2.55	73.37 ± 5.79	74.02 ± 5.11	72.44 ± 6.11	79.58 ± 3.31	77.49 ± 2.79
Basophils	%	0.196 ± 0.069	0.132 ± 0.078	0.746 ±0.263	0.778 ± 0.297	0.549 ± 0.307	0.583 ± 0.137
vWF	mU/mL	133 ± 13	**112* ± 34**	136 ± 29	124 ± 41	163 ± 22	153 ± 26
PAI-1	ng/mL	0.15 ± 0.12	0.24 ± 0.15	0.16 ± 0.1	0.22 ± 0.11	0.16 ± 0.13	0.20 ± 0.19
tPA tot	ng/mL	0.13 ± 0.04	0.12 ±0.04	0.13 ± 0.05	0.13 ± 0.04		
Fibrinogen	mg/mL	1.74 ± 0.79	1.77 ± 0.67	1.52 ± 0.49	1.46 ± 0.65	2.63 ± 0.50	2.99 ± 1.16
CC16	ng/mL	30.3 ± 21.1	25.3 ± 14.1	19.5 ± 13.2	15.6 ± 10.1	25.9 ± 2.7	27.0 ± 4.5
Day 6							
vWF	mU/mL	193 ± 83	165 ± 78	198 ± 44	222 ± 21	137 ± 20	120 ± 37
PAI-1	ng/mL			0.27 ± 0.09	0.21 ± 0.12	0.37 ± 0.17	0.26 ± 0.19
CC16	ng/mL					25.1 ± 2.8	26.4 ± 4.3
Fibrinogen	mg/mL	1.24 ± 0.48	1.27 ± 0.76	1.05 ± 0.33	0.99 ± 0.26	2.44 ± 0.45	2.69 ± 0.71

*P<0.05 compared to experimental control; †P=0.05 compared to experimental control



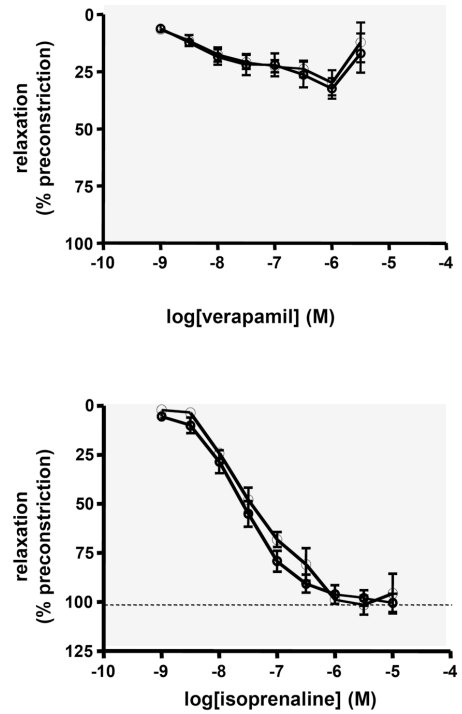


Figure 2. Effect of 4-week exposure to (a) diesel engine exhaust (PM_{DEE}) or (b) concentrated ambient particles near a busy roadside ($PM_{2.5}$ roadside) on ex vivo responses to vasodilator agents in rat aortic rings. Exposed groups (filled circles) and filtered air control (open circles). Values are shown as mean±SE (ACh, n=5-6; SNP, n=4-6; isoprenaline/verapamil, n=4-6). There were no significant differences between PM and control exposures (P>0.05 for all).

Lung pathology

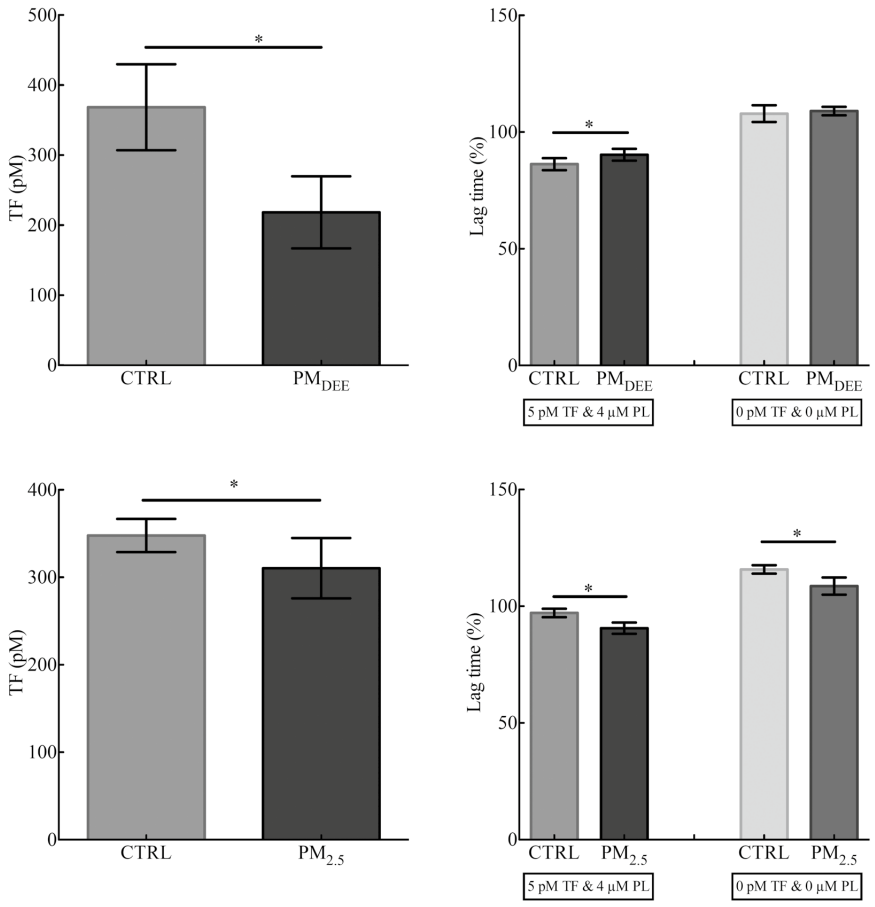
The lungs of the animals exposed to PM_{DEE} showed a number of minor changes including perivascular and peribronchial inflammatory cell infiltrates of mononuclear inflammatory cells (lymphocytes). The number of alveolar macrophages was generally low and there was no infiltration of neutrophilic or eosinophilic leukocytes. Although the incidence of a few changes was slightly increased, there were no changes that distinctly and convincingly could be related to PM_{DEE} exposure.

Roadside $PM_{2.5}$ exposures resulted in a diffuse accumulation of alveolar macrophages in the lungs of all animals, albeit in low numbers. There was no infiltration of neutrophilic or eosinophilic leukocytes. Diffuse macrophage accumulation tended to be slightly more severe in $PM_{2.5}$-exposed rats compared to rats exposed to filtered air (though not statistically significant). Alveolar macrophages of $PM_{2.5}$-exposed rats contained small dark-stained phagocytized particles, which were not observed in controls and should be therefore considered as a result of the PM exposure.

Focal subpleural accumulations of alveolar macrophages accompanied by thickened alveolar septa occurred in animals exposed to all three PM test atmospheres. However, the incidence was significantly increased in roadside $PM_{2.5}$-exposed rats ($P<0.05$, Fisher's exact test).

Because no adverse, treatment-related effects were detected, no actual data on the pathological analysis are presented here.

Tissue Factor Activity



Thrombin Generation

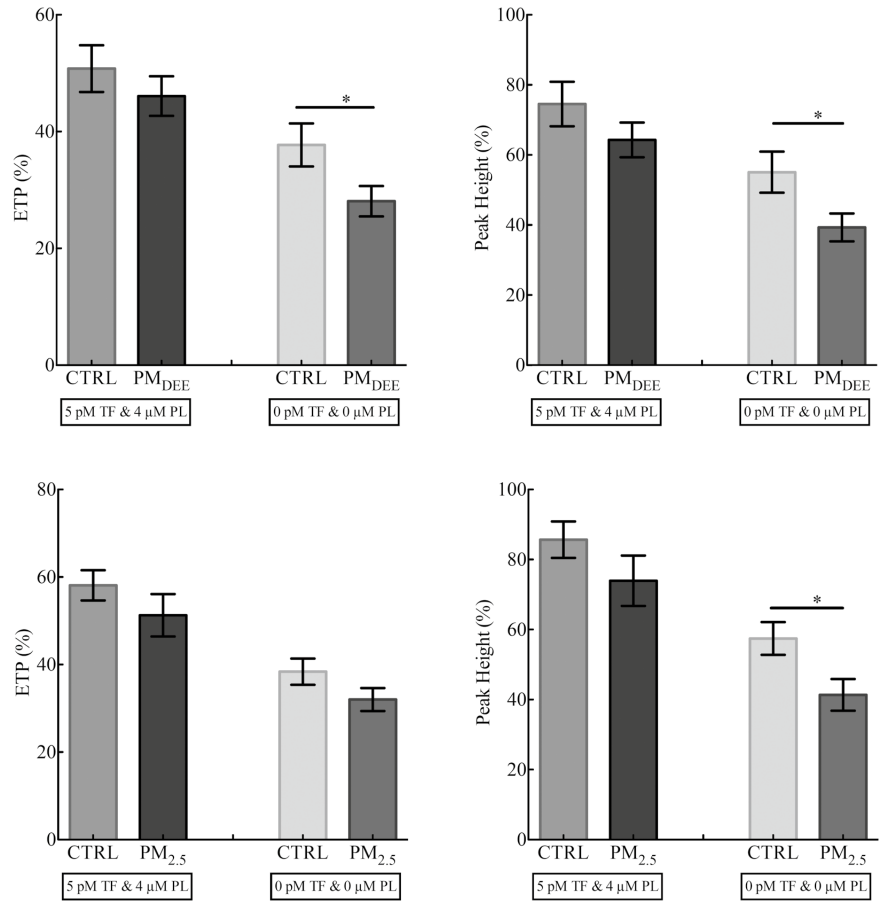


Figure 3. Tissue Factor (TF) activity in and overall procoagulant activity of lung homogenate after 4 weeks exposure to diesel engine exhaust (PM_{DEE}: upper panels) or concentrated ambient particles near a busy roadside ($PM_{2.5}$ roadside: lower panels). Lung tissue-specific thrombin generation was performed in the presence of additional 5 pM TF and 4 µM phospholipids (5 pM TF and 4 µM PL) or in the absence of both TF and phospholipids (0 pM TF and 4 µM PL). Three parameters were derived from the obtained thrombin generation curves: lag time, defined as the time reaching 1/6 of the maximum peak thrombin; ETP, the endogenous thrombin potential or the area under the curve; and peak height, the maximum thrombin generated. Tissue Factor activity is expressed as pM corrected for total protein content of 2.5 mg/mL in the lung homogenate. Thrombin generation parameters are expressed as percentage of normal human pooled platelet-poor plasma, which served as an internal control. Bars indicate mean ± SEM of n = 15 animals per groups. *P < 0.05

Vascular function

In isolated rat aortic rings, the vasodilator PE caused a concentration-dependent contraction (Figure 2). The response to PE was not different between control animals and PM_{DEE}-exposed animals or control animals and animals exposed to roadside $PM_{2.5}$ (P>0.05 for all, two-way ANOVA; n=4-6). ACh, SNP and ISP all caused concentration-dependent relaxation of PE-contracted tissue. Responses in tissue from PM_{DEE}-exposed animals or roadside $PM_{2.5}$-exposed animals were not different from their respective controls (P>0.05 for all). In light of these results, organ bath analysis was not performed in the repetition of the $PM_{2.5}$ roadside study.

Cardiac tissue

Samples of heart tissue from control and exposed rats were examined with regard to expression of IL-1β, TNF-α and IL-6 mRNA as well as to phosphorylation of MAPKs. Neither the expression of mRNA for these cytokines, nor the phosphorylation of the investigated MAPKs differed between control and PM-exposed animals (data not shown).

TF activity and thrombin generation

Lung TF activity was significantly decreased after exposure to PM_{DEE} (control 368 ± 61 pM versus PM_{DEE} 218 ± 51 pM; P=0.009; Figure 3) and slightly diminished after $PM_{2.5}$-roadside exposure (control 348 ± 19 pM versus $PM_{2.5}$ 310 ± 34 pM; P=0.047; Figure 3). Furthermore, partly coherent changes to lung TF activity were observed in thrombin generation, since the lag time is prolonged for exposure to PM_{DEE} (control 86% ± 3% versus PM_{DEE} 90% ± 3%; P=0.039; Figure 3) whereas the lag time is shortened by exposure to $PM_{2.5}$ (control 97% ± 2% versus $PM_{2.5}$-roadside 91% ± 3%; P=0.015; Figure 3) for the latter measured both with and without the addition of TF and phospholipids. Overall thrombin generation, as depicted by the ETP, was not altered upon long-term exposure to traffic-related PM: ETP control 51% ± 4% versus PM_{DEE} 46% ± 3% (p=0.231; Figure 3) and control 58% ± 3% versus $PM_{2.5}$ roadside 51% ± 5% (P=0.383; Figure 3). In addition, analysis of lung tissue thrombogenicity in the absence of additional TF and phospholipids demonstrated an overall decreased lung-induced thrombin generation for long-term exposure to PM_{DEE} (ETP: control 38% ± 4% versus PM_{DEE} 28% ± 3%; P=0.027), whereas no changes were observed after long-term exposure to $PM_{2.5}$ (38% ± 3% versus $PM_{2.5}$-roadside 32% ± 3%; P=0.197). The attenuation

of lung tissue-induced thrombin generation upon exposure to PM_{DEE} was confirmed by a decrease in peak height (control 55% ± 6% versus PM_{DEE} 39% ± 4%; P=0.018; Figure 3). Furthermore, maximum thrombin generation given by the peak height was decreased after $PM_{2.5}$ exposure (control 57% ± 5% versus $PM_{2.5}$ roadside 41% ± 5%; P=0.020; Figure 3) confirming the trend in attenuation of the ETP.

Discussion

Prolonged exposure to traffic-related PM at levels approximately 10 times higher than ambient levels, or exposure to specifically diesel engine exhaust, exerted only modest effects in relatively healthy rats. This was irrespective the fact that a mild inflammation was induced at the onset of exposure. Accumulation of particles within alveolar macrophages was observed in both $PM_{2.5}$ roadside exposures demonstrating that fine particulates are capable of reaching deep into the alveolar spaces. Biological changes were mainly of a cardiovascular nature, as shown by reduced WBC numbers, diminished levels of vWF protein and reduced lung tissue thrombogenicity or procoagulant activity.

The fact that only very mild effects were detected in this study may be related to the adequately functioning host defense system of the rats. The animals were exposed to ozone (800 µg/m^3 for 12 h) prior to prolonged exposure to traffic PM, which was intended to cause significant, yet non-severe, pulmonary inflammation to compromise the defense system at the beginning of exposure to PM. Ozone is known to provoke damage of type I epithelial cells and increased permeability of the alveolar walls (Bhalla, 1990; Dormans et al., 1990). Previous studies in our laboratory (van Bree et al., 2001, 2002) under similar conditions as in the current study (12-24 h; 800 µg/m^3 ozone) resulted in a 2-3 fold increase protein levels in BALF, as well as a moderate influx of neutrophils (10-20% of total lavage cells). However, the inflammation induced in our study was rather mild, as only a slight (2.5%) increase in inflammatory cells was observed. On the other hand, a similar rise in lung permeability was found as reported previously. The difference in response might be caused by a difference in sensitivity between Fisher-344 rats used in the present study and the Wistar rats used previously. Nevertheless, ozone exposure was found to cause a similar degree of lung permeability to that found previously. This is important as an increase in permeability of the alveolar wall may assist in the translocation of particles from the lung into the circulation; one of the key mechanisms proposed to explain the systemic actions of inhaled particles (Geiser and Kreyling, 2010). Because accumulated particles within macrophages were observed in the present study, it seems plausible that translocation to the system circulation had taken place. Many epidemiological studies have claimed that in particular people with compromised airways are more likely to develop adverse health

effects due to exposure to PM. This is in line with our observation that the rather healthy rats do not develop biological relevant adverse responses due to traffic-derived PM.

Using our diesel powered generator, a stable highly controlled test atmosphere was created that consisted of soot particles. The PM levels that were applied in this study can easily be detected in hot spots, such as road tunnels or at kerb sides of busy city streets. Ambient PM has been shown to have substantial spatial and temporal variation, both in terms of amount and physicochemical composition and that the contribution of secondary inorganic components although to play a very limited role in inducing toxicity (Schlesinger and Cassee, 2003) contributed on average ~25%. These factors might very well explain the intra- and inter-experimental variability observed for the two roadside experiments. The PM mass concentrations were higher than those applied in the PM_{DEE} exposure. On the basis of epidemiological associations that suggest a linear concentration-response relationship between PM and cardiorespiratory responses, it was assumed that PM mass concentrations would be predictive of the biological responses in the present study, however this did not appear to be the case. Because the two roadside experiments led to higher, albeit distinctly different, average PM mass concentrations, according to the general assumption, any effect seen for PM_{DEE} should also be observed in the $PM_{2.5}$ roadside experiments. However, most parameters responding in the PM_{DEE} experiment were not affected by the PM roadside exposures, which implies that other factors than PM mass (i.e., PM size and chemical composition) affect the *in vivo* responses. Indeed, previous studies by our group and others (Gerlofs-Nijland et al., 2007, 2009b; Schwarze et al., 2006;) suggested that factors such as chemical composition are driving the toxicity. Another important difference between the PM_{DEE} exposure and the exposure to roadside $PM_{2.5}$ is the higher gaseous pollutant concentrations for the exposure to diesel engine exhaust. As the PM_{DEE} gaseous components were not exceeding limit values as defined by American Conference of Governmental Industrial Hygienists (ACGIH, 1991), we can assume that these could not explain the observed vascular responses.

One more variable between the PM_{DEE} and $PM_{2.5}$ roadside exposures is the particle number concentration, with substantially higher numbers for the PM_{DEE} experiment. Diesel engine exhausts are dominated by particles of approximately <100 nm, which are also referred to as ultrafine particles. Several authors have suggested that ultrafine particles have adverse effects

on the cardiovascular system (Delfino et al., 2005; Knol et al., 2009; Schulz et al., 2005). Therefore, it may very well be that in our $PM_{2.5}$ roadside experiments, in which the numbers of ultrafine particles were lower than the PM_{DEE}, the number of ultrafines has played a more dominant role than PM mass.

Another explanation for the observed limited responses might be the development of adaptation caused by the long exposure duration. It is generally known that various biological markers have their optimal effect at different time points. Moreover, some markers like MAPKs may be activated over time in a multi-phasic way, i.e. even baseline levels vary from day to day (Chen et al., 2003; Thrane et al., 2001). Reduced vWF protein levels were already observed 6 days after exposure to PM_{DEE} and reached significance after 4 weeks. Measuring at the different time points might also implicate that changes in adaptative pathways are observed. This may explain some of the contradictory observations of other groups; increased vWF levels to traffic-related PM (O'Neill et al., 2007; Yue et al., 2007), compared to a decrease in vWF in association with air pollutants (Carlsten et al., 2008; Hildebrandt et al., 2009). Elevated plasma vWF levels may imply an increased risk for thrombosis (Franchini and Mannucci, 2008), therefore, the time course of thrombotic responses may also vary between acute, subchronic or prolonged exposure to air pollutants. Adaptation pathways to chronic exposures present in healthy animals may be impaired in disease; therefore, experiments are currently underway in our laboratory examining the actions of PM_{DEE} on the cardiovascular system in a model of atherosclerosis. The impaired lung procoagulation activity after prolonged exposure to traffic-related PM is supported by the reduced lung TF activity in conjunction with reduced lung tissue thrombin generation. In general, acute exposure to air pollution is associated to increased hypercoagulability shown by shorter prothrombin time (lag time) and elevated plasma thrombin generation (Baccarelli et al., 2007; Bonzini et al., 2010). However, these epidemiological studies provide insight in the plasma hypercoagulable state after acute exposure to air pollution, whereas impaired tissue procoagulant activity might also indicate an adaptive defense mechanism.

In our clinical studies we have shown that a 2-h exposure of healthy volunteers to Edinburgh $PM_{2.5}$ had no effect on vessel wall function as determined by forearm plethysmography (Mills et al., 2008). On the other hand, diluted diesel engine exhaust attenuated responses to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP, but not to the NO-independent vasodilator, verapamil. Previously, we have demonstrated in an animal experiment that similar responses occurred 4h after acute exposure to various PM samples by intratracheal instillation (Bagate et al., 2004). In addition, diesel engine exhaust particles directly inhibit vascular relaxation to endothelium-dependent vasodilators (Miller et al., 2009). Therefore, we assumed that prolonged exposure to PM_{DEE} and not roadside $PM_{2.5}$ was associated with cardiovascular impairment. However, no signs of impairment were observed after prolonged exposures applied in the three experiments presented in this paper.

In the present study, we noted a decrease of WBC in the PM_{DEE} as well as in the second $PM_{2.5}$ roadside experiment. Similar observations have been made in rats after acute exposure to traffic-related PM (Gerlofs-Nijland et al., 2005; Kooter et al., 2006). In human studies, Frampton and co-workers (2002) noted that NO_2 exposure resulted in reduced lymphocytes that migrate to the lung, as increased lymphocyte numbers were found in the respiratory system. Recently, changes in differential WBC was reported in patients with chronic pulmonary disease related to ambient air pollution exposure (Brüske et al., 2010). Although the biological significance and impact is still not clear, it seems that reduced circulating WBCs are related to increased exposure to air pollutants.

Freshly generated PM_{DEE} induced mild cardiovascular responses (impaired coagulation) but no respiratory effects were seen in relatively healthy rats. Also no biological relevant changes were detected after exposure to ambient roadside $PM_{2.5}$. The overall analysis of the results did not support the hypothesis that PM mass concentrations are linear related to health effects. In contrast to common belief, prolonged exposure to traffic-related PM in healthy animals may not be detrimental due to various biological adaptive response mechanisms. It could be speculated that vulnerability of humans to acute or repeated exposure to PM may be primarily dependent on the presence of co-morbidity such as coronary heart disease. We conclude that prolonged although not chronic exposures in healthy animals have very limited impact on pulmonary and cardiovascular function. Further studies are needed in animals with established disease (such as more extensive pulmonary inflammation or developed cardiovascular disease) to reveal the influence of susceptibility on air pollution induced toxicity.

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5 EFFECT OF PROLONGED EXPOSURE TO DIESEL ENGINE EXHAUST ON PRO-INFLAMMATORY MARKERS IN DIFFERENT REGIONS OF THE RAT BRAIN

Miriam E. Gerlofs-Nijland, Damien van Berlo, Flemming R. Cassee, Roel P.F. Schins, Kate Wang, Arezoo Campbell

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Abstract

The etiology and progression of neurodegenerative disorders depends on the interactions between a variety of factors including: aging, environmental exposures, and genetic susceptibility factors. Enhancement of pro-inflammatory events appears to be a common link in different neurological impairments, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. Studies have shown a link between exposure to particulate matter (PM), present in air pollution, and enhancement of central nervous system pro-inflammatory markers. In the present study, the association between exposure to air pollution (AP), derived from a specific source (diesel engine), and neuroinflammation was investigated. To elucidate whether specific regions of the brain are more susceptible to exposure to diesel-derived AP, various loci of the brain were separately analyzed. Rats were exposed for 6 hrs a day, 5 days a week, for 4 weeks to diesel engine exhaust (DEE) using a nose-only exposure chamber. The day after the final exposure, the brain was dissected into the following regions: cerebellum, frontal cortex, hippocampus, olfactory bulb and tubercles, and the striatum. Baseline levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 alpha (IL-1α) were dependent on the region analyzed and increased in the striatum after exposure to DEE. In addition, baseline level of activation of the transcription factors (NF-κB) and (AP-1) was also region dependent but the levels were not significantly altered after exposure to DEE. A similar, though not significant, trend was seen with the mRNA expression levels of TNF-α and TNF Receptor-subtype I (TNF-RI). Our results indicate that different brain regions may be uniquely responsive to changes induced by exposure to DEE. This study once more underscores the role of neuroinflammation in response to ambient air pollution, however, it is valuable to assess if and to what extent the observed changes may impact the normal function and cellular integrity of unique brain regions.

Introduction

There is an association between chronic exposure to combustion-related fine particles (present in air pollution) and an increased risk of mortality attributed to lung cancer and cardiopulmonary causes (Pope et al., 2002). One of the major contributors to particulate air pollution is diesel engine exhaust. As diesel fuel undergoes combustion in automobile engines, it produces particles of different sizes, chemical composition, and physical characteristics (Donaldson et al., 2005). Because of the substantial number of epidemiological studies showing a link between exposure to air pollution and adverse cardiovascular changes, the need for air quality controls and suggestions for future research has been addressed (Brook et al., 2004). Recent studies show that the cardiopulmonary system may not be the only vulnerable target adversely affected by air pollution. The brain may be another potential target (Kleinman et al., 2008; MohanKumar et al., 2008).

In a prospective birth cohort study, the association between exposure to black carbon, which is a surrogate for traffic-related particles, and cognition among children was assessed. The authors discovered that higher exposure to black carbon was associated with a decline in cognitive function (Franco Suglia et al., 2008). Children may not be the only sensitive subpopulation since mild cognitive impairment, associated with long-term exposure to traffic-related particulate air pollution, was also identified in elderly women (Ranft et al., 2009). These observed changes in cognitive function may be related to alteration in brain activity. Indeed, in human volunteers, brain activity assessed by quantitative electroencephalography, was altered after short-term exposure to diesel engine exhaust (Crüts et al., 2008). Although it is not known exactly how exposure to ambient air pollution or more specifically, diesel engine exhaust, may alter human brain function, studies indicate that neuroinflammation may play a role.

One of the first studies which pointed towards the potential adverse central nervous system (CNS) effects of air pollution showed that in dogs living in polluted areas of Mexico City, there is an increase in cerebral inflammation (Calderón-Garcidueñas et al., 2002). In a later study, using human post-mortem tissue, it was discovered that markers of both neuroinflammation and Alzheimer's disease (AD) pathology are increased in residents living in high (compared to low) pollution cities (Calderón-Garcidueñas et al., 2004). In a recent study, the same investigators have validated their earlier

findings by showing enhanced neuroinflammation, altered blood-brain barrier characteristics, and particulate deposition in the brain of children and young adults living in cities with high air pollution (Calderón-Garcidueñas et al., 2008). In addition, a few toxicological studies in rodents have been published that support these findings. Exposure to ambient particles, present in urban air pollution, enhances neuroinflammatory markers in the brain of mouse models (Campbell et al., 2005, 2009; Kleinman et al., 2008; Veronesi et al., 2005). In the present study, rats were exposed to diesel engine exhaust (a major contributor to ambient air pollution) to examine neuroinflammatory changes in different brain regions. We hypothesized that the accompanying neuroinflammatory response will be variable in specific brain areas.

Methods

Animals

Male Fischer F344/DUCRL rats (15-16 wks old) were obtained from Charles River (Sulzfeld, Germany). The rats were randomly allocated to either control or DEE exposure group (*n* = 15/group) and acclimatized for 7 days. Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI, Bilthoven, Netherlands).

Exposure and characterization of test atmosphere

All animals were exposed in whole body inhalation chambers to 0.4 ppm ozone for 12 hrs before initiating the diesel engine exhaust (DEE) exposures. The work described in this paper is an extension of another study aimed at evaluating the effect of prolonged exposure to traffic-related particulate matter (PM) on the cardiopulmonary system. These cardiopulmonary effects were studied in the presence of a mild inflammation status induced by ozone (Gerlofs-Nijland et al., 2009). Nose-only exposure to DEE began the next day and continued for 4 wks, 5 days/wk for 6 hrs a day. Control animals were exposed to conditioned, purified and HEPA filtered air with the same temperature and relative humidity as the test atmosphere. Diluted DEE was obtained by mixing freshly generated exhaust from an idling diesel engine (35 KVA Genset, 1500 rpm) with purified air. Particle number and mass concentration were measured continuously in the inlet of the exposure chamber using a condensation particle counter (CPC model 3022A, TSI St. Paul, Minn., USA) or a nephelometer (DATARAM 2000, MIE, Billerica, Mass., USA) respectively. Time-integrated particle concentrations were determined by gravimetric analysis. A carbon sampler tube was placed downstream of one of the PolyTetra-FluoroEthylene filters at the outlet to collect the volatile organic compounds (VOC), which were measured by means of GC-MS. The final particle mass concentration in diluted DEE was 173 $\mu g/m^3$ with a geometric median diameter of 76 nm and geometric standard deviation of 5 nm. Total VOC content was 529 $\mu g/m^3$. The concentrations of CO, NO, and NO_2, NOx measured in the mixing chamber were on average 2.6 ppm CO, 0.08 ppm NO, 1.3 ppm NO_2 and 0.5 ppm NOx. These are levels that people will experience in tunnels, workplace or at traffic hotspots.

Necropsy

Animals were sacrificed 24 hrs after the last exposure. The brain of 10 animals per group was carefully excised and the following regions were dissected on ice: olfactory bulbs and tubercles, striatum, hippocampus, cortex, and the cerebellum. A subset of these samples (*n* = 5) was used for the ELISA and gel shift mobility assays and another subset (*n* = 5) was used for the RNA isolation and quantitative PCR analysis.

Sample preparation for ELISA and gel shift mobility assays

Cytoplasmic and nuclear protein fractions were prepared using the method of Lahiri and Ge (2000). Tissue was weighed and homogenized in ice-cold buffer consisting of (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.5% NP-40). The suspension was incubated on ice for 10 min and centrifuged (4000 × g) at 4°C for 1 min. The supernatant containing the cytoplasmic constituents was collected and protease inhibitor was added. Aliquots of samples were stored at -80°C. The nuclear pellet was resuspended in a buffer composed of (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF). The samples were then centrifuged (11,000 × g) for 5 min at 4°C. The supernatant (which is the nuclear extract) was collected, protease inhibitor was added, and aliquots were prepared and stored at -80°C.

Competitive enzyme immunoassay

Levels of TNF-α and IL-1α were determined using immunoassay kits from Biosource (Camarillo CA), for the detection of the total protein in the cyto-

plasmic tissue fractions. Briefly, 50 µl of the sample was added to plates precoated with an antibody specific for either rat TNF-α or rat IL-1α. After the addition of a Biotinylated secondary antibody, the plates were washed and incubated with streptavidin-Peroxidase. After another wash, a substrate solution was added and the color generated was determined with a spectrophotometric plate reader set at 450 nm.

Electrophoretic mobility shift assay

This assay was utilized to determine the extent of NF-κB and AP-1 activation in the nuclear fractions using a protocol developed by Promega (Madison WI). The amount of protein in 2 µl of the nuclear extract was determined by the BCA protein assay kit (Pierce, Rockford, IL) and 25 µg of each sample, incubated with 32P-labeled oligonucleotides containing the NF-κB or AP-1 consensus sequence, was loaded onto a gel. A negative control containing no cell extract, as well as competitor reactions were run simultaneously with the samples. The specific competitor contained unlabelled NF-κB or AP-1 consensus nucleotide while the nonspecific competitor contained unlabelled SP-1 consensus oligonucleotide. The competitor reactions also contained 1 g of HELA cell extract (positive control). X-ray films were manually developed. The mean intensity of each band was measured and quantitated using a Kodak 1500 gel logic imaging system.

Analysis of mRNA

Total RNA was isolated from the various brain tissue regions using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA). The mRNA levels of TNF-α and TNF-RI were determined by quantitative RT-PCR. RNA was purified using the RNeasyR mini kit coupled to treatment with DNase (RNAse-free DNAse set, Qiagen). The purity and amount obtained were determined by spectrophotometry at wave lengths of 230, 260, 280, and 320 nm. From 0.5 µg RNA, cDNA was synthesized using the iScript cDNA Synthesis kit (BioRad, CA, USA) and used for qRT-PCR at a 15× dilution in RNAse-free water. PCR primers for TNF-α, TNF-RI and the housekeeping gene HPRT were designed using Primer express software (Applied Biosystems). Real time PCR was performed employing a MyIQ Single Color real time PCR detection system (BioRad) coupled to SYBRc Green Supermix (Biorad), added to the system along with 15× diluted cDNA and 0.3 µM forward and reverse primers. During the PCR reaction, a denaturation step

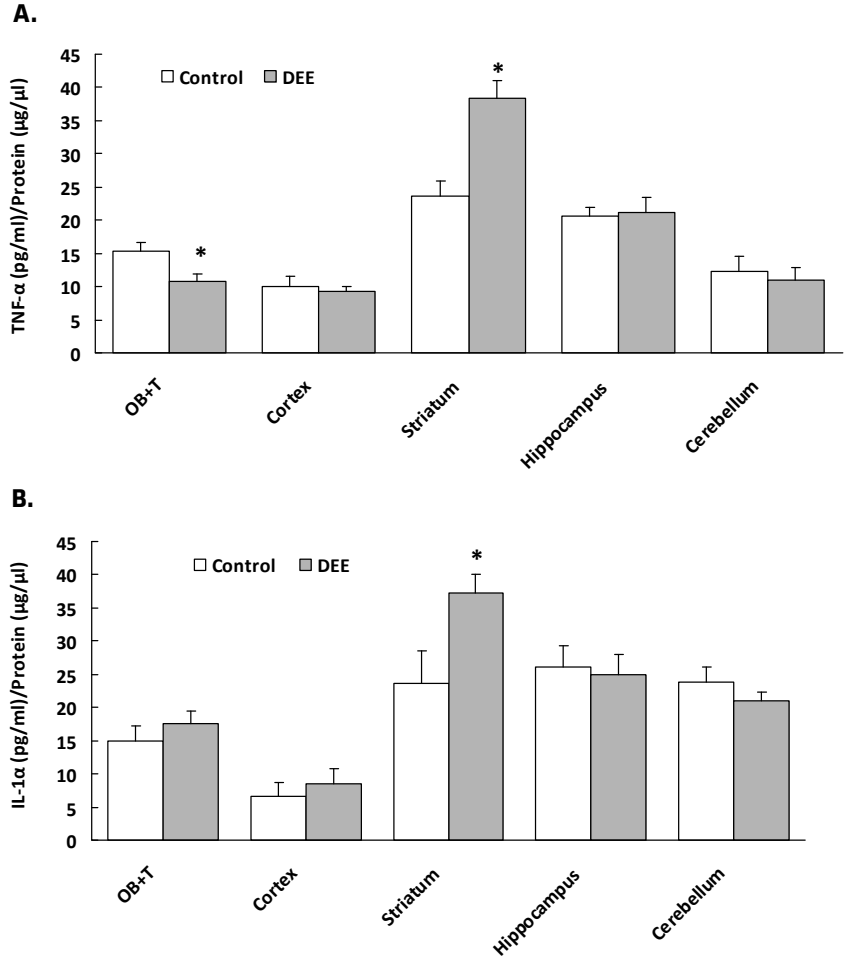

Figure 1. Pro-inflammatory cytokine levels. A. Levels of TNF-α in different regions of the rat brain after exposure to filtered air (control) or DEE (diesel-exposed). Values given as mean ± SE; n = 5; *P < 0.05 compared to control. OB+T = olfactory bulb and the tubercles.
B. Levels of IL-1α in different regions of the rat brain after exposure to purified air (control) or DEE (diesel-exposed). Values given as mean ± SE; n = 5; *P < 0.05 compared to control. OB+T = olfactory bulb and the tubercles.

at 95°C for 3 min was followed by 40 cycles at 95°C (15 seconds) and 60°C (45 seconds). To ascertain that the correct product was amplified, a melt curve (60-95°C) was produced. The efficiencies of all primer sets were tested by the generation of cDNA dilution curves. Obtained data was analyzed using MyIQ software (BioRad) and expressed as fold increase compared to the lowest expression in non-treated controls (Livak and Schmittgen, 2001).

Statistical analysis

Differences between the exposure groups were tested using analysis of variance (ANOVA). Pairwise comparisons were tested using the Tukey method.

Results

Cytokine levels

Basal levels for both TNF-α and IL-1α were detected in all brain regions and varied depending on the specific area analyzed (Figure 1). The levels of the pro-inflammatory cytokines TNF-α and IL-1α were measured in the cytoplasmic fractions derived from the following brain regions: olfactory bulb and the tubercles (OB+T), cortex, striatum, hippocampus, and cerebellum. The levels of TNF-α were slightly (but significantly) decreased in the OB+T after exposure to DEE, but there were no changes in the levels of IL-1α in this region. However, in the striatum, there was a pronounced increase in the levels of both pro-inflammatory cytokines. There were no changes in the levels of these two pro-inflammatory cytokines in the cortex, hippocampus, or cerebellum, after exposure to DEE.

Transcription factor activation

The transcription factor NF-κB is an immunologically relevant transcription factor and upon activation, promotes the transcription of a variety of immunomodulatory factors including TNF-α. The levels of activated NF-κB were assessed in the nuclear fractions derived from the olfactory bulb and the tubercles (OB+T), cortex, striatum, hippocampus, and cerebellum. DEE exposure did not alter the levels of this transcription factor (Figure 2). When the basal levels of NF-κB were evaluated, there were evident regional variations (Figure 3). The cortex had the lowest while the OB+T showed the highest constitutive activity. The regional variation in baseline activity was

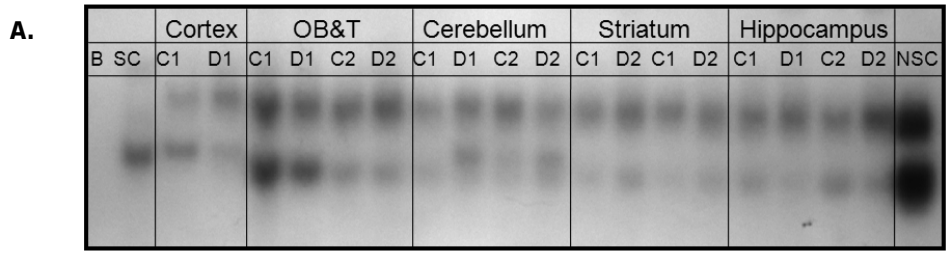

Figure 2. NF-κB activation after DEE exposure. A. A gel demonstrating NF-κB shifted bands. Samples from different brain regions were assayed on the same gel. Based on the competitor reactions, the top band is specific for NF-κB. B = blank; SC = specific competitor; NSC = non-specific competitor; C = nuclear fraction derived from the brain of animals exposed to filtered air; D = nuclear fractions derived from the brain of animals exposed to DEE; the results for two separate animals (designated as 1 or 2) are shown on this gel. B. The sum intensity of NF-κB specific shifted band (first band shown above) in different regions of the rat brain after exposure to purified air (control) or DEE (diesel). Each brain region was analyzed on different days on separate gels and therefore this figure does not allow direct comparison between various brain regions. Bars represent mean of 5 individual animals ± SE.

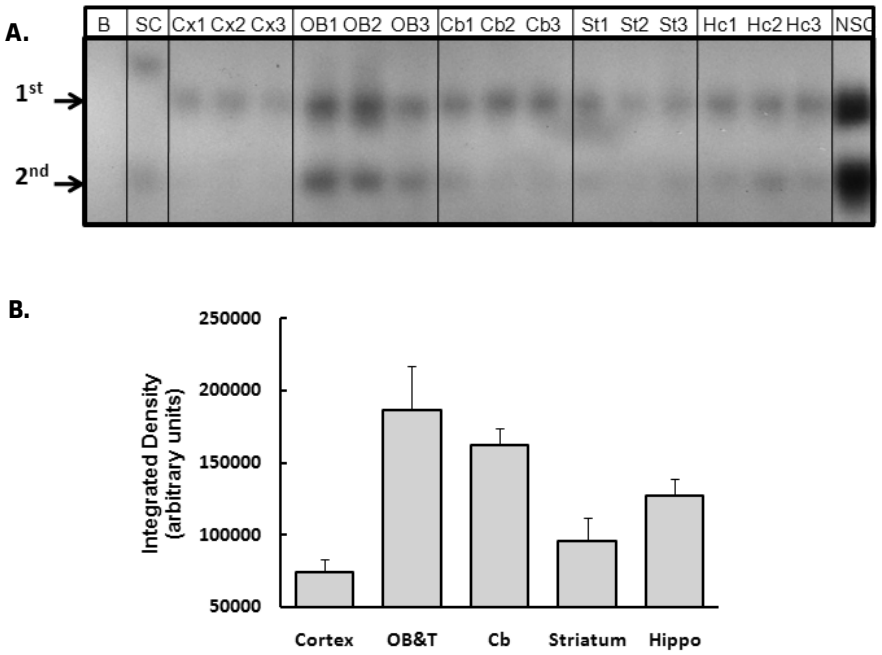

Figure 3. Basal levels of NF-κB activation. A. A typical gel showing the NF-κB specific shifted bands for three separate control samples (designated as 1, 2, or 3). B = blank; SC = specific competitor; NSC = non-specific competitor; Cx = cortex; OB = olfactory bulbs and tubercles; Cb = cerebellum; St = striatum; Hc = hippocampus. All brain regions were analyzed on the same gel and under the same conditions to allow direct comparison between regions. B. The sum intensity of the first shifted band. OB&T = olfactory bulbs and the tubercles; Cb = cerebellum; Hippo = hippocampus.

more pronounced for AP-1 (Figure 4A). Again, the OB+T showed the highest while the cerebellum had the lowest basal activity (Figure 4B). DEE exposure did not alter the levels of AP-1 (Figure 4C) in any of the regions analyzed. It should be noted that the regional differences in the 'basal' levels of transcription factor activity may have been modulated by the ozone pretreatment conducted before the initiation of the DEE exposures.

mRNA levels for TNF-α and TNF-RI

Since the TNF-α protein level was modified in specific brain regions after DEE exposures, the expression of mRNA levels for TNF-α was determined

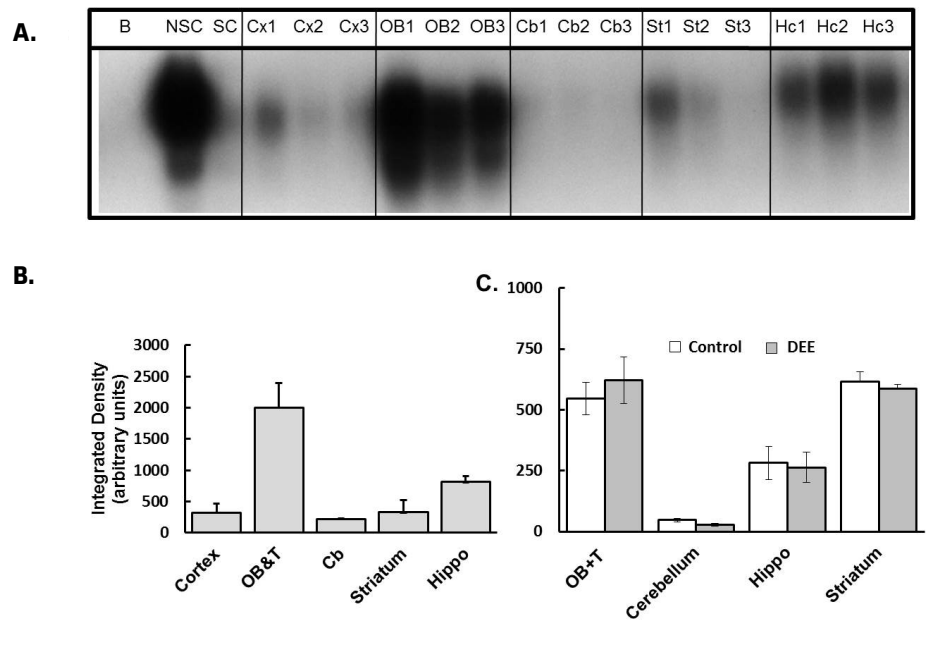

Figure 4. AP1 activation. A. A typical gel showing the AP-1 specific shifted band for three separate control samples (designated as 1, 2, or 3). B = blank; SC = specific competitor; NSC = non-specific competitor; Cx = cortex; OB = olfactory bulbs and tubercles; Cb = cerebellum; St = striatum; Hc = hippocampus. All brain regions were analyzed on the same gel and under the same conditions to allow direct comparison between regions. B. The sum intensity of the shifted band. OB+T = olfactory bulbs and the tubercles; Cb = cerebellum; Hippo = hippocampus. C. The sum intensity of AP-1 specific shifted band in different regions of the rat brain after exposure to purified air (control) or DEE (diesel). Each brain region was analyzed on different days on separate gels and therefore this figure does not allow direct comparison between various brain regions. Bars represent mean of 5 individual animals ± SE.

in various regions of rat brains. There was again a difference in basal expression and the striatum showed the lowest, while the hippocampus had the highest constitutive expression of the cytokine (Figure 5A). After exposure to diesel exhaust, there was no difference in mRNA levels for this pro-inflammatory cytokine in any of the brain regions analyzed. We also assessed the levels of TNF receptor subtype I (TNF-RI) in specific brain regions and exposure to DEE did not modify the levels of the receptor in any of the brain areas analyzed (Figure 5B).

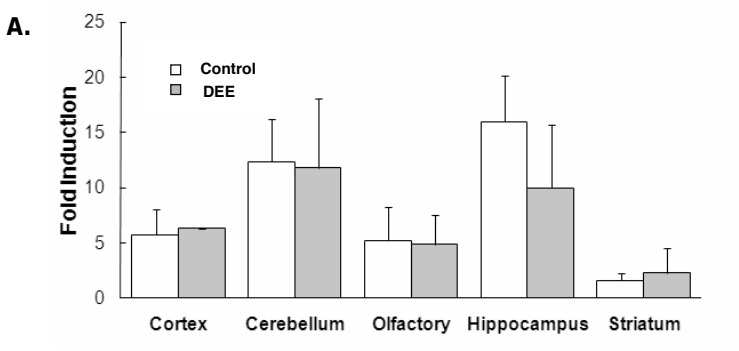

Figure 5. RNA expression.
A. RNA expression for TNF-α. Bars represent mean of 5 individual animals ± SE. There are no error bars for the cortex of DEE-exposed animals because only one sample was available.
B. RNA expression for TNF-RI. Bars represent mean of 5 individual animals ± SE. There are no error bars for the cortex of DEE-exposed animals because only one sample was available.

Table 1. Basal leveld of transcription factor activation, mRNA expression, and protein levels in different regions of the rat brain

Brain regions	Transcription factor		RNA		Protein	
	NF-κB	AP-1	TNF-α	TNFRI	TNF-α	IL-1α
OB+T	+++	+++	+	+	+/++	++
Cortex	+	+	+	+	+	+
Striatum	+/++	+	+	+	++	++/+++
Hippocampus	++	++	+/++	+	++	++/+++
Cerebellum	++/+++	+	+/++	+	+/++	++/+++

For comparison purposes, values are designated as (+ = low; ++ = medium; or +++ = high) based on arbitrary assessment between groups for each marker

Discussion

Air pollution is a complex mixture of gases and particulate matter. DEE contributes substantially to combustion-derived nanoparticles which are an important component of air pollution (Donaldson et al., 2005). In some urban areas, the air quality is so poor that the threshold considered 'safe' is consistently surpassed. In such environments, exposure to ambient air pollution and the possibility of adverse human health effects is a realistic cause for concern. While the connection between exposure to particulate matter and harmful cardiopulmonary effects has been reasonably well established (Brunekreef and Holgate, 2002; Mills et al., 2009), there is growing evidence that the CNS may be another target (MohanKumar et al., 2008). In the present study, we demonstrate that prolonged exposure to moderate levels of DEE increased TNF-α and IL-1α protein levels specifically in the striatum of rat brains. However, none of the measured transcription factors (NF-κB and AP-1) or the mRNA levels of TNF-α and TNF-RI were affected after DEE exposure.

Numerous studies show that upregulation of markers associated with neuroinflammation may be either beneficial or harmful. For instance, TNF-α and TNF-RI have been shown to be involved in both neuroprotection (Taoufik et al., 2008) as well as neurodegeneration (Tran et al., 2008). This dichotomy in TNF-α-related pathways may be attributed to the intensity (Lai and Todd, 2008) or CNS location (Sriram et al., 2006) of neuroinflammation. The striatal dopaminergic system may be more vulnerable to adverse consequences of TNF-α production. As an example, in TNF receptor deficient mice, there is less striatal dopaminergic cell death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a selective toxin for dopaminergic cells (Sriram et al., 2002).

TNF-α levels are increased in the striatum and cerebrospinal fluid of Parkinson's disease patients when compared to controls (Mogi et al., 1994). Furthermore, several studies show that certain TNF-α polymorphisms in genotype or promoter sequences increase the risk for Parkinson's disease (Nishimura et al., 2001; Wahner et al., 2007; Wu et al., 2007). Our finding that exposure to DEE increases TNF-α selectively in the striatum may suggest that such environmental exposures may further aggravate factors associated with neurodegenerative disorders such as Parkinson's disease.

In the rat brain, the levels of transcription factor activity and cytokines were different depending on the region analyzed (Table 1). It is possible that the ozone pretreatment influenced this 'baseline' transcription factor activity. However, since the ozone exposure was conducted four weeks prior to tissue harvesting, it is likely that the ozone effects may be negligible at this time point. The region-specific activation state of transcription factors may be related to the unique cellular and molecular composition and function of different brain regions. For example, the olfactory bulb is a region where progenitor cells can give rise to new neurons (Kuhn et al., 2005) and this may underlie the high basal level of AP-1 and NF-κB activity detected. Indeed, it has been shown that a subunit of AP-1 (DeltaFosB) is upregulated after insult-induced neurogenesis and induces proliferation of neuronal precursor cells after injury (Kurushima et al., 2005). NF-κB activity has been associated with neurogenesis and neuronal survival through anti-apoptotic gene expression although this transcription factor has also been implicated in neurodegeneration attributed to its pro-inflammatory characteristics. It appears that the intensity and cell type location of NF-κB activity determines its propensity to induce neuroprotection or neurotoxicity (Sarnico et al., 2009). Although the protein levels of TNF-α and IL-1α were increased in the striatum of DEE exposed rats, the levels of NF-κB activation (a factor which promotes transcription of immune-related genes) or TNF-α and TNF-RI RNA expression were unchanged. This may be due to homeostatic regulation of gene expression for the cytokines to protect against uncontrolled neuroinflammation. However, it may also be possible that the change in pro-inflammatory cytokine levels is mediated by systemic responses after exposure to DEE and the cytokines are entering the brain from peripheral sources. Exposure to particles derived from ambient air pollution increases inflammatory processes in the lungs (Dick et al., 2003; Schaumann et al., 2004) and intratracheal instillation of washed diesel exhaust particles can aggravate lung and systemic inflammation in mice (Inoue et al., 2006). Therefore, we cannot exclude that the pro-inflammatory cytokine changes in the CNS are due to systemic inflammation. However, we did not observe signs of increased inflammation in the lungs of the animals after DEE exposure (data not shown).

The CNS effects reported in this study may be direct. The route of exposure to PM is via inhalation, and thus there is a potential for compounds to rapidly enter the brain through the cribriform plate of the ethmoid bone after

deposition in the olfactory epithelium. This could then lead to direct activation of innate immune responses in the CNS. For inhaled manganese, the olfactory route has been shown to allow access of this metal into the brain (Brenneman et al., 2000; Elder et al., 2006)]. It is also possible for inhaled particles to indirectly enter the brain through the circulation. In humans, inhaled radiolabeled particles in the ultrafine size range entered the systemic circulation and were detected in extrapulmonary organs (Mills et al., 2006). The same scenario was observed in rats exposed to inhaled nano-sized silver particles (Takenaka et al., 2001). The blood-brain-barrier (BBB) is formed by tight junctions between endothelial cells comprising cerebral microvasculature. The stringent regulation imposed by the BBB prevents harmful factors present in the peripheral circulation from entering cerebral tissue. Exposure to TNF-α has been shown to increase the levels of the transporter protein P-glycoprotein which is thought to tighten the BBB by increasing the efflux of compounds out of the CNS (Bauer et al., 2007). Diesel exhaust particles dose-dependently upregulate P-glycoprotein levels and activity in isolated rat brain capillaries. This effect was dependent on TNF-α released after exposure to DEP (Hartz et al., 2008). Therefore, in our study, the increase in TNF-α detected in the striatum may be a protective effect to increase efflux of particulates from the CNS. We did not directly measure neuronal cell death and thus we cannot ascertain that the DEE-induced increase in these pro-inflammatory cytokines is harmful or protective.

The concept that gene-environment interactions play an important role in the causation and progression of chronic neurodegenerative diseases has received much attention in the past few years. Exposure to combustion-related compounds, present in air pollution, increases pro-inflammatory cytokines in the striatum of rats, and may be an environmental stress factor which contributes to neuronal cell death in this region. This may be especially important if genetic susceptibility factors are present. It has been shown that although cultured human microglial cells, derived from different individuals, show similar basal gene expression profiles (for many cytokines, chemokines, and growth factors) treatment with TNF-α leads to a completely different response depending on the individual source of the microglia (Meeuwsen et al., 2005). Similar to this finding, it may be possible that there are individual differences in CNS inflammatory responses after exposure to DEE. Further studies are warranted before it can be concluded with certainty that prolonged exposure to components of air pollution may

contribute to neuronal cell loss and whether genetic susceptibility factors may modulate this effect.

In the present study, we have shown that prolonged exposure to DEE induced a neuroinflammatory response in the rat brain in a region-specific manner. The inflammatory changes were assessed 24 hr post exposure and it is possible that this effect was transient. However, in a mouse model, neuroinflammatory markers were present two weeks after exposure to particulate matter (Campbell et al., 2005) and thus it appears that the CNS effects are long-lasting although, species differences and PM sources need to be considered. To what extent the DEE-induced enhancement of inflammatory markers may lead to neurotoxicity or contribute to the progression of neurodegenerative diseases needs to be further evaluated and is the focus of our future research.

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6 CELL TOXICITY AND OXIDATIVE POTENTIAL OF ENGINE EXHAUST PARTICLES - IMPACT OF USING PARTICULATE FILTER OR BIODIESEL FUEL BLEND

Miriam E. Gerlofs-Nijland, Annike I. Totlandsdal, Theodoros Tzamkiozis, Daan L.A.C. Leseman, Zissis Samaras, Marit Låg, Per Schwarze, Leonidas Ntziachristos, Flemming R. Cassee

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Abstract

The link between emissions of vehicular particulate matter (PM) and adverse health effects is well established. However, the influence of new emission control technologies and fuel types on both PM emission and health effects has been less well investigated. We examined the health impact of PM emissions from two vehicles equipped with or without a diesel particulate filter (DPF). Both vehicles were powered either with diesel (B0) or a 50% v/v biodiesel blend (B50). The DPF effectively decreased PM mass emissions (~85%) whereas the fuel B50 without DPF lead to less reduction (~50%). The hazard of PM per unit distance driven was decreased for the DPF-equipped vehicle as indicated by a reduced cytotoxicity, oxidative, and pro-inflammatory potential. This was not evident and even led to an increase when the hazard was expressed on a per unit of mass basis. In general, the PM oxidative potential was similar or reduced for the B50 compared to the B0 powered vehicle. However, the use of B50 resulted in increased cytotoxicity and IL-6 release in BEAS-2B cells irrespective of the expression metric. This study shows that PM mass reduction achieved by the use of B50 will not necessarily decrease the hazard of engine emissions, while the application of a DPF has a beneficial effect on both PM mass emission and PM hazard.

Introduction

Particulate matter (PM) from traffic, including vehicular exhaust emissions from diesel engines, has been linked to adverse health effects (Krzyzanowski et al., 2005). Several abatement measures, like the installation of diesel particulate filters (DPF) and the use of improved (bio)fuels, have therefore been implemented in order to reduce the PM emissions from engines and vehicles (Gill et al., 2012). New engine or exhaust aftertreatment technologies have been introduced to meet the current European PM standards. These standards are focused on reducing the mass and recently the number of emitted PM. In addition, biofuels have been introduced in an effort to reduce greenhouse gases and decrease energy dependence on fossil fuels. It is worth highlighting that the composition of exhaust emissions may differ between (blends of) biodiesel fuels depending on the biodiesel composition, the diesel/biodiesel ratio and the engine technology (Fontaras et al., 2010 ; Kousoulidou et al., 2012). In addition to reduced PM emissions, these implementations may influence the emission composition affecting the toxic potential of the engine exhaust emissions (Hesterberg et al., 2011). However, information on the influence such abatement measures may have on the toxicity and associated health effects of the PM emitted is limited (Bünger et al., 2012).

A reduction in PM emissions is estimated to have potential benefits for public health (Pope et al., 2009). DPFs that efficiently filter exhaust particles from diesel vehicles have recently been shown to prevent adverse vascular and prothrombotic effects of diesel engine exhaust in human volunteers (Lucking et al., 2011). Biodiesel not only limits the emission of solid PM (soot) (Bünger et al., 2012; Lapuerta et al., 2008; Tzamkiozis et al., 2010) but also the emissions of other regulated components (CO, CO_2, and total hydrocarbons) (Bünger et al., 2000, 2012; Swanson et al., 2007; Zou and Atkinson, 2003). Hence, the use of biodiesel as a fuel may diminish adverse health effects based solely on the foreseen reduced emissions of regulated components.

Available information on adverse health effects in relation to DPF and biodiesel usage is limited and often contradictory (Bünger et al., 2012). Effects on parameters such as cytotoxicity and the release of pro-inflammatory markers that play a role in PM-related health effects show increased, equal, or decreased toxicity with biodiesel usage (Bünger et al., 2000; Jalava et al., 2010; Kooter et al., 2011; Swanson et al., 2009). To our knowledge, only

two *in vivo* animal studies have compared the toxic potential of biodiesel and diesel (Brito et al., 2010; Tzamkiozis et al., 2010), and no human volunteer studies have been published. The *in vivo* studies demonstrated that biodiesel elicited an equal pulmonary inflammatory response compared to diesel fuel. Contradicting outcomes complicate the toxicity assessment of engine exhaust emitted using new technologies or fuels. In addition to the limited data, assessment of the toxicity of engine exhaust is hampered by confounding factors such as the engine used in exposures and other study conditions such as the animal model used and duration of exposure. We, therefore, examined the effects of a DPF and use of biodiesel and diesel (50%) blend in an *in vitro* model to assess the oxidative, cytotoxic, and pro-inflammatory potential of particles obtained under representative vehicle operation conditions.

Methods

Vehicles, fuels and lubricants

Two diesel passenger cars were selected that represent the most common engine and exhaust aftertreatment technologies currently available in the European diesel passenger car fleet. The first was a 2.2 L Euro 4 diesel vehicle (Honda Accord 2.2i-CTDi) equipped with a diesel oxidation catalyst (DOC) and a two-stage oxidation under-floor catalyst with de-NOx characteristics (Abe et al., 2004). The second vehicle was a 2.0 L Euro 4 diesel vehicle (Peugeot 407 HDi) equipped with a DOC and a non-catalytic diesel particle filter (DPF). Both vehicles featured high pressure common rail engines and exhaust gas recirculation (EGR) for NOx control.

Two different fuels were used in each vehicle, a neat fossil diesel (B0) and a biodiesel blend comprised of Rapeseed Methyl-Ester (compliant with EN14214) and neat diesel (compliant with EN590:2009) with a nominal sulfur content of 10 ppm wt. Approximately 200 ppm of a commercial antioxidant additive was used in biodiesel to ensure oxidation stability.

The lubricant used was within the specifications of the manufacturers of both vehicles. It was a SAE 0W-30 (ACEA class B3-B5) with low sulfur, phosphorus, and ash content. The lubricant was replaced in both vehicles before the start of the measurement sequence and was aged for 1000 km before testing.

Test protocol

The New European Driving Cycle (NEDC) is employed in Europe for the approval of a vehicle and is comprised of two sub-cycles, i.e. the Urban Driving Cycle (UDC) and the Extra Urban Driving Cycle (EUDC). The NEDC involves long periods of constant speeds and mild accelerations, characteristics that make it unrepresentative of actual driving conditions. In an effort to test the vehicles under more representative driving conditions, two extra cycles were employed, i.e. the Artemis Urban (A. Urban) and the Artemis Road (A. Road) cycles (Andre, 2004). By combining UDC with A. Urban and EUDC with A. Road, four new composite cycles were produced, and PM samples were collected during each composite cycle. The number of repetitions of each sub-cycle (i.e., UDC, EUDC, A. Urban and A. Road) depended on the emissions level of each vehicle and the PM mass required for toxicity tests. Table 1 shows the implemented measurement protocol.

As noted in Table 1, the number of repetitions of each sub-cycle (i.e. UDC, EUDC, A. Urban and A. Road) varied between vehicles in order to collect the required amounts of PM mass for toxicity testing. For example, the A. Urban sub-cycle that follows the UDC was repeated nine times (9x) in the case of the DPF vehicle, compared to only two times (2x) for the non-DPF vehicle. Hence, because the cold start is strictly part of the UDC sub-cycle, the relative effect of the cold start stage on the particle emission levels and composition was expected to be lower in the case of the DPF vehicle compared to the non-DPF equipped vehicle. Considering that the temperature of the exhaust system (aftertreatment devices including the engine) stabilized roughly halfway through the UDC sub-cycle, then the cold start stage of the composite cycle represents 5% and 17% of the total composite cycle distance in case of the DPF vehicle and the non-DPF case, respectively. Regarding the rural composite cycles, all sub-cylces were equally repeated for both non-DPF and DPF vehicles. As a result, the relative durations of the cold start stage in rural composite cycles are similar and these may be directly compared. This difference should be taken into account with regard to interpretation of the outcomes.

Particle emission sampling

Constant Volume sampling. The vehicle exhaust was diluted and conditioned by Constant Volume Sampling (CVS), as defined by the regulations (Directive 98/69/EC). The dilution air was filtered by a HEPA class H13 of EN 1822 before entering the dilution tunnel, with a measured efficiency greater than 99.9% in terms of particle number. In order to avoid sampling interference when shifting between vehicle configurations or fuels, it was necessary to condition the sampling system, i.e. run a series of dry tests before the actual measurements occurred. Hence, the sampling system was pre-conditioned by running three high-speed EUDCs before each actual measurement phase with a new vehicle configuration or a new fuel. After conditioning, the vehicle was left to soak for at least 8 h before conducting the tests, in order to achieve a cold start the next day.

High Volume Sampler. PM mass samples were collected on 20 cm × 25 cm PTFE-coated glass fiber filters (Pallflex, Emfab filters, TX40HI20WW) using a High Volume Sampler (HVS). The mass of PM collected was gravimetrically determined after 24 h conditioning in controlled temperature (22°C) and humidity (40%).

Particle characterization

PM samples were extracted from the CVS using an Electrical Low Pressure Impactor (ELPI) (Dekati Ltd, 2001) downstream of an ejector-type diluter operating at a nominal dilution ratio of 10:1 and ambient temperature (~20°C). The ELPI operated with oil-soaked sintered plates and a filter stage that extended the lower cutpoint to ~7 nm. In ELPI calculations, unit density was assumed for particles in order to estimate their total PM number concentrations.

Chemicals and reagents

HPLC-quality methanol was obtained from Biosolve BV (Valkenswaard, Netherlands). Chelex 100 resin was used from Bio-Rad (Veenendaal, Netherlands). LHC-9 cell culture medium was purchased from Invitrogen (Carlsbad, CA, USA), PureCol™ collagen from Inamed Biomaterials (Fremont, CA, USA), and propidium iodide (PI) and Hoechst 33342 from Sigma (St. Louis, MO, USA). All other chemicals were purchased from commercial sources at the highest purity available.

Particle extraction for toxicity assays

PM was extracted from filters according to the extraction protocol based on Salonen et al (2000). In short, filters containing the PM samples were treated with methanol in a water bath sonicator, the supernatant was dried under a nitrogen flow at 30°C, and the collected PM extract was stored at -80°C until use. PM samples used for investigating PM-induced cellular release of pro-inflammatory mediators and cytotoxicity were suspended in cell exposure medium (2 mg/ml in LHC-9 medium) and sonicated in a water-bath for 15 min prior to exposure of the cells.

Measurement of oxidative potential

The oxidative potential was measured in time at a fixed mass by indirect measurement of dithiothreitol (DTT) consumption rate and direct measurement of ascorbate (AA) consumption rate.

Dithiothreitol assay.

PM (10 µl of 1 mg/ml) was added to 0.2 ml 0.5 M potassium phosphate buffer (KH_2PO4 and K_2HPO_4 pH 7.4) and water to a final volume of 900 µl. The reaction was run in duplicate at different time points (0, 10, 20, and 30 min). Addition of freshly made DL-Dithiothreitol (100 µl 1 mM DTT) and incubation at 37°C started the reaction. The reaction was stopped at different time points by addition of 1 ml freshly prepared cold 10% Trichloroacetic acid ACS reagent (TCA). Subsequently, 0.5 ml of the reaction mixture was added to 1 ml 0.4 M Tris HCl (pH 8.9 in 20 mM EDTA) and 25 µl 10 mM 5,5'-dithiobis-(2-) nitrobenzoic acid (DNTB) in methanol. The DTT consumption was visualized by measuring the coloring reaction of unused DTT with DNTB on a spectrophotometer (SpectraMax190, Molecular Devices, Sunnyvale, USA) at 412 nm. The results were plotted graphically and the DTT consumption rate was calculated.

Ascorbic acid assay. All buffers and solvents were treated for 1 h with Chelex 100 resin to remove traces of metals that are known to contribute significantly to the redox activity in this assay. PM (160 µl of 12.5 µg/ml water) was pre-incubated with 20 µl water or 20 µl of 200 µM diethylene triamine pentaacetic acid (DTPA) for 10 min at 37°C in duplicate in a UV translucent 96 wells enzyme-linked immunoabsorbent assay (ELISA) plate (UV-star, VWR, Breda, Netherlands). DTPA is a metal chelator and was added to determine the ascorbic acid depletion caused by non-metal components. After incuba-

tion 20 µl 2 mM L-ascorbic acid was added to start the reaction. The ascorbic acid (AA) consumption was followed by measuring the absorbance at 265 nm every 2 min for 2 h using the Spectra Max 190™ Spectrophotometer plate reader. The maximal AA depletion rate was calculated.

Cell culture

BEAS-2B cells, a SV40-transformed human bronchial epithelial cell line, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in LHC-9 medium in collagen (PureCol™)-coated flasks (NUNC A/S, Roskilde, Denmark) in a humidified atmosphere at 37°C with 5% CO_2, with refreshment of medium every other day. One day prior to exposure, BEAS-2B cells (passages 14-21) were plated into collagen (PureCol™)-coated 35 mm 6-well culture dishes (Corning, MA, USA) at a density of 460,000 cells/well.

Cell exposure

Cells were incubated for 24 h with various concentrations of each particle sample (0-200 µg/ml). Total exposure volume was 1.5 ml in 6-well 35 mm cell culture dishes. An applied particle concentration of 100 µg/ml corresponded to ~16 µg/cm^2, if all the suspended particles were deposited on the cells on the surface of the culture dishes.

Particle-induced cell damage

Following exposure, floating and attached cells were stained with PI (10 µg/ml) and Hoechst 33342 (5 µg/ml) for 30 min to determine particle-induced plasma membrane damage. Cell morphology was evaluated using a Nikon Eclipse E 400 fluorescence microscope. Cells with clearly condensed and/or fragmented nuclei were counted as apoptotic, PI-stained cells as necrotic, and non-apoptotic cells excluding PI as viable cells. The percentage of apoptotic and necrotic cells were determined as a fraction of the total number of counted cells. In order to express cytotoxicity on a per distance driven basis, the PM emission rates (mg/km) needed to be taken into account. This was achieved by using the PM concentrations at which 20% cytotoxicity occurred as representative values in order to compare the potency of all samples. Second, a value of 1 was assigned as a reference to the B0 case (separately for urban and rural driving conditions) and a relative distance where 20% cytotoxicity occurred was calculated for all other configurations (Table 2).

Particle-induced cytokine release

After exposure, cell culture supernatants were collected and centrifuged twice for removal of dead cells (300g) and particles (8000g), and stored at -70°C for quantification of particle-induced cytokine release. Concentrations of IL-6 and IL-8 in cell culture supernatants were determined by ELISA (R&D Systems, Minneapolis MN, USA), according to the manufacturer's manual. The increase in color intensity was measured and quantified using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) with software (Magellan V 1.10). Cytokine concentrations are expressed in ng/ml. When expressing the PM-induced IL-6 release on a per distance driven basis, the same procedure as with cytotoxicity was used combined with the maximum levels of IL-6 release. As PM collected from the urban cycle of the B0 non-DPF configuration did not reach the maximum IL-6 level at the highest concentration tested, this concentration was taken as the peak concentration (Table 2).

Statistical analysis

Differences in the oxidative potential for specific parameters (fuel, driving cycle, or filter usage) could not be analyzed on log-transformed basis due to the low number of observations and the fact that the observations in the separate groups may have different shapes. Therefore, no statistical tests were performed for the oxidative potential endpoint and only a trend was given. PM-induced effects on cytotoxicity and cellular release of IL-6 and IL-8 were analyzed statistically by application of a one-way analysis of variance (ANOVA) with a Dunnett multiple comparison test on log-transformed data using GraphPad Prism software (version 4.03, Inc., San Diego, CA). For all endpoints, $p < 0.05$ was considered to reflect statistically significant differences.

Results

Characterization of PM emissions

PM emission factors. The Euro 4 PM limit (25 mg/km) was exceeded under representative urban driving conditions with a non-DPF vehicle fuelled with B0 almost by a factor of two (Table 1). PM emission rates were strongly decreased (~95%) using a DPF and dropped even below the Euro 5 limit (4.5 mg/km). Use of B50 reduced the PM mass and emission rate on average by 50% under all conditions tested (Table 1).

Particle number emissions

The total particle number emitted by the DPF-equipped vehicle was more than three orders of magnitude lower than in the non-DPF case, at levels substantially below the recently enforced particle number emission limit for Euro 5 diesel cars ($6.0x10^{11}$ km^{-1}; Table 1). Use of B50 with the DPF led to an increase in the total particle number compared to the B0 with DPF vehicle, but this was rather insignificant as emissions levels were very low. Use of B50 decreased the total particle number for the non-DPF vehicle, and was consistent with a decrease in PM mass (Table 1).

Table 1. Samples, measurement protocol, mass and emission rates of particulate matter and particle numbers

Sample code	DPF	Fuel	Composite driving cycles	Mass (mg)	Emission rate (mg/km)	Total PN (#/km)
B0 urban	no	diesel	1x UDC + 2x Artemis urban	24.3	46.1	9.0×10^{13}
B0 rural	no	diesel	1x EUDC + 1x Artemis road	28.18	29.72	6.2×10^{13}
B50 urban	no	biodiesel blend	1x UDC + 2x Artemis urban	13.52	25.76	5.0×10^{13}
B50 rural	no	biodiesel blend	1x EUDC + 1x Artemis road	12.68	13.37	4.9×10^{13}
B0 urban DPF	yes	diesel	1x UDC + 9x Artemis urban	3.82	1.47	3.8×10^{10}
B0 rural DPF	yes	diesel	4x EUDC + 4x Artemis road	3.58	0.67	2.6×10^{10}
B50 urban DPF	yes	biodiesel blend	1x UDC + 9x Artemis urban	1.58	0.61	4.7×10^{10}
B50 rural DPF	yes	biodiesel blend	4x EUDC + 4x Artemis road	2.25	0.42	3.5×10^{10}

Note: Numbers in column composite driving cycles represent number of repetitions of each sub-cycle. Abbreviations: B0 – diesel; B50 – 50% v/v biodiesel blend; DPF – diesel particulate filter; EUDC –Extra Urban Driving Cycle; PN – particle numbers; UDC – Urban Driving Cycle

Oxidative potential

PM collected from the DPF vehicle had less or equal oxidative potential per mass compared to PM emitted by the non-DPF vehicle during urban driving conditions (Figure 1A), while the opposite (higher or equal) was observed in the case of rural driving conditions (Figure 1B). In general, decreased oxidative potential per mass was observed with the use of B50 as a fuel for urban driving (Figure 1 A). PM collected during rural driving conditions showed no general consensus with the use of B50 and the oxidative potential per mass decreased or increased depending on the assay used, i.e. DTT or AA as well

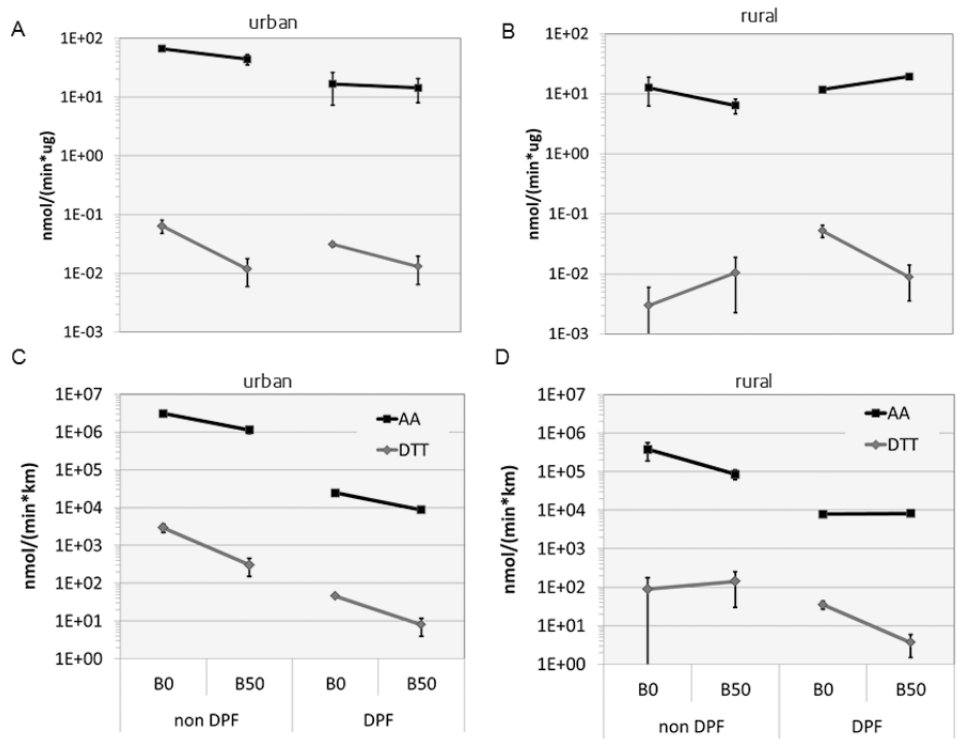

Figure 1. Oxidative potential of particulate matter (PM) samples measured at a fixed PM mass (A, B) or expressed per kilometer (C, D) collected during urban (A, C) or rural (B, D) driving conditions. The oxidative potential is measured as consumed dithiothreitol (DTT) or ascorbic acid (AA) and given as mean ± SD. Vehicles were equipped with or without a diesel particulate filter (DPF) and fueled with diesel (B0) or a 50% v/v biodiesel blend (B50).

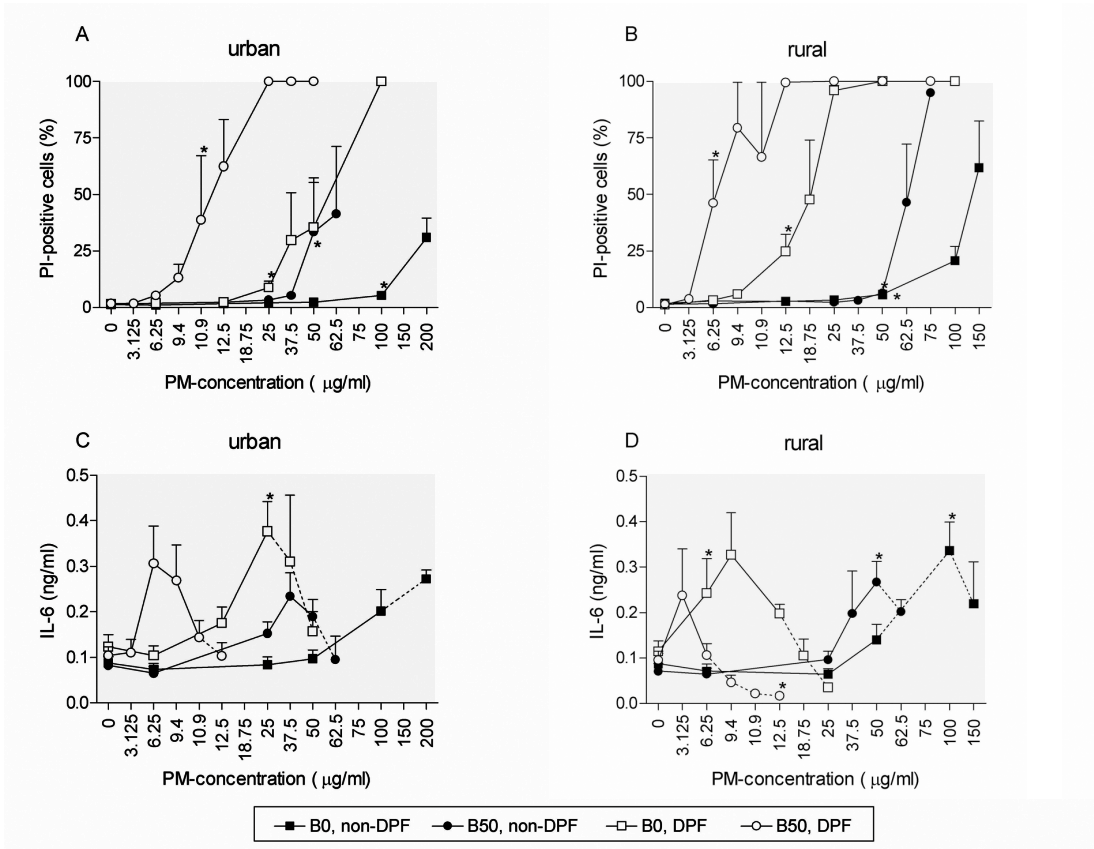

Figure 2. Particle induced cytotoxicty (A, B) and release of IL-6 (C, D) in human bronchial epithelial (BEAS-2B) cells measured after exposure to increasing particle concentrations (0-200 µg/ml) for 24 h under urban (A, C) or rural (B, D) driving conditions. Dashed line indicates PM concentration at which significant cytotoxicity occurs. Bars represent mean ± SEM of separate experiments (n≥3). * p<0.05: exposed versus unexposed cells measured by ANOVA with a Dunnett multiple comparison test on log-transformed data.

as whether a DPF was used or not (Figure 1B). In a majority of situations PM collected during urban driving conditions demonstrated a higher oxidative potential compared to PM from rural driving. Diesel particles emitted from a non-DPF vehicle simulating urban driving induced the highest oxidative potential.

Expressing the oxidative potential on a per distance driven basis (Figure 1 C, D) takes into account the PM emission rates (mg/km). Expressing the data per distance driven showed a clear decrease in the oxidative potential of PM when the DPF is introduced. This was the case for PM collected both during urban (Figure 1C) and rural (Figure 1D) driving conditions and was due to a much lower PM emission rate compared to the non-DPF equipped vehicle. In general, B50 fuel usage reduced the oxidative potential of PM emitted on a per distance driven basis under all conditions tested (Figure 1 C, D).

Cytotoxicity

On a per mass basis, PM generated from a vehicle equipped with a DPF induced cytotoxicity at lower concentrations than PM generated from a vehicle not equipped with a DPF (Figure 2 A, B). Furthermore, increased toxicity was mostly seen in cells exposed to PM samples collected from a vehicle fuelled with B50 compared to B0 (Figure 2 A, B). In general, PM emitted during driving cycles simulating rural driving conditions (Figure 2B) appeared to be more cytotoxic than particles emitted during cycles simulating urban driving conditions (Figure 2A). On a per mass basis, biodiesel particles emitted from a vehicle equipped with DPF during rural driving conditions were most cytotoxic, with a significant increase in toxicity at a particle concentration of 6.25 µg/ml. The microscopic analysis of the particle-exposed cells after PI and Hoechst staining revealed that the cytotoxicity was primarily characterized by a concentration-dependent increase in necrotic cells. In general, the percentage of apoptotic cells remained below 1% for all particle samples tested (data not shown).

When expressed on a per distance driven basis, the PM emissions from the DPF-equipped vehicle were less cytotoxic than the PM emissions from the non-DPF equipped vehicle. Furthermore, the emissions from the vehicles fuelled with B50 were in most cases more cytotoxic than emissions from vehicles fuelled with fossil diesel (Table 2).

Release of pro-inflammatory markers

A PM concentration-dependent increase in the release of IL-6 was detected for all PM samples (Figure 2 C, D solid line), which decreased at higher concentrations when cytotoxicity become more evident (Figure 2 C, D dashed line). No significant differences in the release of IL-8 were detected (data not shown). Both IL-6 and IL-8 appeared to be induced to a similar extent by the different PM samples at the mRNA level (data not shown).

In general, the PM-induced release of IL-6 followed the same trend as the PM-induced cytotoxicity. PM emitted from the DPF vehicle or a vehicle fuelled with B50 appeared to be more potent on a per mass basis than without DPF or using diesel fuel (Figure 2 C, D). Furthermore, PM emitted during cycles simulating rural driving conditions appeared to be more potent in inducing release of IL-6 than PM emitted during cycles simulating urban driving conditions (Figure 2 C, D).

The levels of PM-induced IL-6 release as per distance driven basis revealed that the application of a DPF had a beneficial effect (Table 2). However, the use of B50resulted in a much greater potential to induce release of IL-6 compared to vehicles fuelled with fossil diesel, irrespective of DPF application (Table 2).

Table 2. Cytotoxicity and inflammatory potential of engine exhaust particulate matter

Sample code	20% Cytotoxicity PM (µg/ml)	Il-6 peak PM (µg/ml)	Correction factor	20% Cytotoxicity Equiv. distance	IL-6 peak Equiv. distance
B0 urban	157	> 200	–	1.0	1.0
B50 urban	44	37.5	1.8	0.5	0.3
B0 urban DPF	34	25	31.4	6.7	3.9
B50 urban DPF	10	6.3	75.6	4.7	2.4
B0 rural	98	150	–	1.0	1.0
B50 rural	54	50	2.2	1.2	0.7
B0 rural DPF	12	9.4	44.4	5.3	2.8
B50 rural DPF	4	3.1	70.8	3.1	1.5

Note: Correction factor – emission rate B0 divided by emission rate PM sample for urban or rural driving conditions separately. For example, correction factor 1.8 for B50 urban is emission rate of B0 urban (Table 1; 46.10) divided by the emission rate of B50 urban (Table 1; 25.76). Equivalence distance is calculated by PM concentration of sample where 20% cytotoxicity or peak IL-6 release occurred divided by the PM concentration of B0 for urban or rural driving conditions separately multiplied by the correction factor. For example, 0.5 is the 20% cytotoxicity equivalence distance for B50 urban, which is calculated by the PM concentration where 20% cytotoxicity occurred for B50 urban (Table 2; 44 µg/ml) divided by the PM concentration for B0 urban (Table 2; 157 µg/ml) multiplied by the correction factor for B50 urban (Table 2; 1.8). Abbreviations: B0 – diesel; B50 -50% v/v biodiesel blend; DPF – diesel particulate filter; IL- interleukin; PM –particulate matter.

Discussion

In the present study, we have shown that the application of a non-catalytic coated DPF resulted in a reduction of PM mass (85%) and particle numbers (33%), which lowered the hazardous effects of the engine emission. This beneficial effect, compared to PM from a vehicle without a DPF, was mainly evident when the emission rates of the vehicles were taken into account and therefore most probably due to reduced PM mass. B50 fuel usage also resulted in a reduction (50%) of the PM mass emission, and the oxidative potential that particles elicit was equal or lower compared to PM collected from the B0-fuelled vehicle under most conditions tested. However, exposure of human airway epithelial (BEAS-2B) cells to PM from B50 vehicles caused more cytotoxicity and a greater release of pro-inflammatory markers than exposure of cells to B0, irrespective of the dose metric and irrespective of whether a DF was used or not. B50 fuel usage might therefore be equal or more harmful compared to fossil diesel, despite having lower PM emissions.

In this study a non-catalytic coated DPF technology was used with the catalyst added as particles to the fuel. Non-catalytic DPFs have been shown to filter particles without producing oxidative derivates (e.g. nitrogendioxide) to the same extent as catalytic DPFs (Richards et al., 2006). DPF-technology significantly reduces the emission of total polycyclic aromatic hydrocarbons (PAHs) (Carrara and Niessner, 2011; Portet-Koltalo et al, 2011). However, in the presence of NOx more carcinogenic nitro-PAHs may be formed (Bünger et al., 2006; Heeb et al., 2008). As non-catalytic traps produce lower levels of oxidative derivates than catalytic filters, the production of harmful substances might also be affected (Mayer et al., 2003). The results of this study suggest the non-catalytic coated DPF had a beneficial effect on PM-related health effects including the reduction in oxidative potential. The benefit of a DPF compared to non-DPF equipped vehicle might not simply be explained by PM mass reduction but may also result from the filtration and reduced production of harmful substances.

Another factor that might contribute to the reduced toxicity of PM samples collected from the DPF vehicle under urban driving conditions compared to the non-DPF vehicle could be the lower percentage of cold starts. In our study, the contribution of the cold start was less for the DPF versus the non-DPF vehicle (5% and 17%, respectively) in urban driving conditions. This difference was caused by the contribution of the Artemis urban cycle within the urban composite driving cycles, namely, two versus nine Artemis urban cycles for the non-DPF versus the DPF-vehicles, respectively (Table 1). The cold start contribution for rural driving conditions in this study was 25% for both the DPF and non-DPF vehicles. Given that more harmful components are produced during cold starts, when aftertreatment systems are not fully operational, it is expected that a lower cold start percentage might result in lower toxicity. The higher cold start percentage of rural driving conditions therefore might explain the elevated toxicity of PM collected under rural compared to urban driving conditions.

Oxidative stress and the formation of reactive oxygen species (ROS) is assumed to play a central role in the biological effects of diesel engine exhaust exposure (Ghio et al., 2012). The oxidative potential of PM can be measured by different chemical or biochemical assays and be used as a marker for toxicity. Increased PM oxidative potential values reveal a higher potential of PM to induce adverse effects *in vivo*. This study showed, as demonstrated previously (Cheung et al., 2009), a reduced oxidative potential per kilometer of PM collected under transient conditions when a DPF was used. This was most likely due to substantially lower PM and oxidative compound emissions. PM emitted from vehicles with soot removing control devices contain a higher amount of semi-volatiles per unit mass compared to non-controlled vehicles (Biswas et al., 2009). The semi-volatile fraction contributes to greater than 70% of the oxidative activity of vehicles with soot exhaust treatments (Biswas et al., 2009). This may explain why the benefit of a DPF in terms of reduced oxidative potential is only evident when expressed on a kilometer basis. The importance of the expression metric was also evident for other health effect parameters measured. Inflammation, involving expression and release of several pro-inflammatory mediators, is considered to play a key role in the adverse health effects associated with exposure to vehicular emissions (Ghio et al., 2012). Interleukin (IL)-6 and IL-8 are pro-inflammatory cytokines expressed by the airway epithelium in response to stimuli like diesel engine exhaust (Park & Park, 2009; Tal et al. 2010). The present study showed an increased pro-inflammatory response and cytotoxicity after exposure to PM derived from a DPF vehicle when expressed on a per unit mass basis, while a decrease was observed when expressed on a per distance driven basis. Tzamkiozis et al.(2010) also found more pronounced inflammation in mice after exposure to different PM mass concentrations from the DPF equipped vehicle.

In general, the oxidative potential of PM from a biodiesel-fuelled vehicle was similar or diminished in comparison to fossil diesel. Diminished oxidative potential for biodiesel PM collected under transient conditions was shown before when expressed on a per km or kWh basis (Cheung et al., 2012; Kooter et al., 2011), while a slight increase was observed when expressed per unit mass basis (Cheung et al., 2009). The present study also indicated that PM from biodiesel exhaust obtained under specific conditions (rural driving conditions) could have a higher oxidative potential when expressed on a per unit mass, but this parameter was reduced when expressed on per distance driven basis. While B50 fuel usage resulted in a reduction (50%) of PM mass emission, exposure of human airway epithelial cells to PM from B50 vehicles caused more cytotoxicity and increased release of pro-inflammatory markers compared to PM from diesel-fuelled vehicles, irrespective of the dose metric. A rise in the inflammatory response caused by biodiesel exposure was reported previously by Swanson et al.(2009). In general, the results for the health-related parameters measured in the present study suggest that B50 fuel usage could be equal or more harmful compared to fossil diesel despite the lower PM mass emission.

Our study as well as other experiments (Bünger et al., 2000; Cheung et al., 2009; Jalava et al., 2010; Kooter et al., 2011; Swanson et al., 2009) revealed that the metric used to express the outcomes is critical to interpret the health effects. Therefore, we have applied a risk estimation approach by reconstructing impact based on emissions per kilometer driven. It is gradually being recognized that total mass alone might not be the best metric to express health outcomes. Use of a DPF will result in increased hazard on a per mass basis and suggests that a DPF vehicle is more harmful then a non-DPF equipped car. However, the inclusion of the emission rate corrects for the reduction in PM emission. By expressing the emissions on per unit of distance driven basis, one is including a parameter (distance) that is related to exposure relevant for risk assessment purposes. In addition to the metric used, the outcomes of any study could differ due to different study design. Several factors e.g., engine technology and operation, fuel type, and the application of catalysts and particle trap properties and implementation, could affect the composition and thus the toxicity of the engine exhaust mixture, which makes it difficult to compare experiments. Caution is necessary when comparing the observed health effects from different studies and determining relevance to human health.

The present study indicated that although the use of new vehicle emission control technologies and fuel type reduced the mass of emitted PM, in some cases, the generation of PM with increased hazard appeared. Therefore, beneficial effects of these interventions cannot be assumed based on mass reduction. The toxic potential can differ depending on the method or expression metric used. The results of this study are useful in understanding how various vehicle and fuel technology may influence health relevant responses and provide information necessary for developing sampling and analysis protocols for future studies. Therefore, a harmonized test protocol should be considered to test various conditions and technologies on a range of relevant health endpoints in combination with a solid exposure assessment strategy. Only such a consistent approach will allow conclusions on the impact on human health risks. We therefore suggest that the biological effects of vehicle PM interventions should be thoroughly investigated before being implemented on a large scale.

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CHAPTER 7

GENERAL DISCUSSION 7

Highlights thesis research

- Road traffic is a major source of hazardous air pollutants (Chapter 2);
- Both engine exhaust and non-exhaust PM from wear emissions contribute to traffic-related health effects (Chapter 2);
- Coarse particles exert significant adverse toxicity (Chapter 2 and 3);
- Transition metals and polyaromatic hydrocarbons (PAHs) are likely to be crucial factors affecting pulmonary toxicity (Chapter 3);
- Particle size and composition seem to represent more important determinants of the health risks imposed by air pollution than PM mass (Chapter 2 and 3);
- Prolonged exposure to diesel engine exhaust did not appear to be related to adverse cardiopulmonary effects in healthy animals (Chapter 4);
- Prolonged exposure to diesel engine exhaust resulted in the induction of pro-inflammatory markers in specific brain regions (Chapter 5);
- The application of a particulate filter on combustion engines has a beneficial effect both on PM emission and the hazard of PM (Chapter 6);
- Significant reduction in PM emission by the use of 50% biodiesel blend as a fuel is not necessarily linked to reduced toxicity (Chapter 6).

The objective of the research described in this thesis was to examine the adverse health effects of air pollution and assess in experimental studies the toxicological contribution of components and sources of PM emissions. The focus has been on traffic-generated air pollution as many people, especially in urban areas, are exposed to this part of the complex air pollution mixture. Hence, it would be highly beneficial for abatement strategies to know more precise what is causing the adverse effects of traffic-related air pollution. The impact of traffic on human health has recently been underlined by the adaptation of the classification of diesel engine exhaust from “probably carcinogenic to humans” to “carcinogenic to humans” following evaluation of the scientific evidence by the International Agency for Research on Cancer (IARC). However, it is worth highlighting that the ground to adapt the classification is heavily based on studies from underground miners that may have less relevance to the general population (Attfield et al., 2012; Silverman et al., 2012). Thereby, diesel engine exhaust has various make ups and cannot be considered invariably over time due to changed technologies and fuels. Yet, it is pivotal to elucidate what actions could be taken by manufacturers, engineers, policymakers and other stakeholders to reduce the most harmful components in the air that we all breathe. The research presented in this thesis was performed to strengthen the link between scientific evidence and interventional measures taken in order to limit the adverse health effects of traffic-related air pollution.

Traffic-related air pollutants and health effects

Sources

"Road traffic is a major source of hazardous air pollutants"

The study described in Chapter 2 of this thesis was carried out when the health effects of traffic-generated emissions, specifically, were poorly understood. In an *in vivo* animal study using spontaneously hypertensive rats, we compared the pulmonary and systemic responses caused by PM exposure from different sites with contrasting traffic profiles. It was observed that there was a trend towards a greater toxicity of PM from sites of high traffic density based on changes in biological markers indicative for cytotoxicity, inflammation and blood viscosity. The outcomes suggested that road traffic is an important source of harmful air-pollution-related health effects. Yet, low traffic density sites, but with substantial levels of PM from other combustion processes like wood burning, also had significant effects (Chapter 2).

Cell based *in vitro* experiments, in which cells of a mouse monocyte/macrophage cell line were exposed for 5 hours to PM from the same traffic-related sites as used in our own Chapter 2 study, did not confirm our *in vivo* findings. Contrary, the *in vitro* study illustrated that PM from high road traffic environments did not show an increased capacity to trigger inflammation compared to sites with low traffic contributions (Guastadisegni et al., 2010). Apart from the apparent differences between these experiments (e.g. the lack of different cell types *in vitro*, time after exposure to assess inflammation, and cytokine measure of PM inflammatory potential), these contrasting observations are not necessarily related to traffic density and the concentrations of PM in terms of mass. Instead, other characteristics such as particle size and composition might be important. Indeed, a review of toxicological studies of traffic-related PM revealed that traffic density does not always explain local differences in PM toxicity (de Kok et al., 2006). Instead, the authors suggest particle size (e.g. ultrafine particles) and composition (e.g. metals and polycyclic aromatic hydrocarbons (PAHs) content) are likely to be the most crucial factors affecting pulmonary toxicity. In case of the *in vitro* study, the bacterial endotoxin content explained the observed pro-inflammatory response.

Nowadays, a large body of evidence demonstrates that traffic-related air pollution is of importance and has a diverse array of health impacts including the onset and exacerbation of asthma, allergies, respiratory symptoms, impaired lung function and cardiovascular diseases (Anderson et al., 2011; Brunekreef et al., 2009; Gehring et al., 2010; HEI, 2010; Kagawa, 2002; McConnell et al., 2010; WHO, 2006). However, the evidence regarding exposure to traffic-related air pollution and most of these health effects is not always sufficient to infer a simple causal relationship (HEI, 2010; Laumbach and Kipen, 2012). Nevertheless, the associations has been strengthen as most studies have been conducted nearby busy roads and just recently, it is shown that road traffic participation did result in adverse respiratory effects (Strak et al., 2010; Zuurbier et al., 2010, 2011). Over the past few decades, considerable efforts have been made to regulate traffic-generated emissions, through reduction in vehicle exhaust in combination with attempts to minimize traffic-related exposure. To support the changing policies in this area it is important to scrutinize the health effects of traffic-related air pollution in order to reduce the health risks of the vast number of people exposed to these pollutants, especially in urban areas.

Non-exhaust emissions

"Both engine exhaust and non-exhaust PM from wear emissions contribute to traffic-related health effects"

Correlations between PM composition and health effects showed that not only vehicular exhaust is of significance. Non-exhaust emissions need to be considered. For example, work from our own group (presented in Chapter 2), highlighted brake and tyre wear as potential sources of harmful PM as indicated by an association of the toxicological responses (inflammation and lung injury) and specific PM components derived from brake (copper (Cu) and barium (Ba)) or tyre (zinc (Zn)) wear. Our study was one of the original studies addressing non-exhaust sources of traffic-derived PM. Similar studies suggested the importance of non-exhaust emissions revealing the role of resuspended road dust (correlations with calcium (Ca), iron (Fe), aluminium (Al), and manganese (Mn)) as well as wear emission in exerting urban air PM-related health effects (Happo et al., 2008, 2010; Table 1).

Over the past decade, the health risks (considering both toxicity and exposure) of PM have increasingly focused on road traffic emissions. This is partly because the hazardous nature of combustion-related PM is, in

comparison to other sources, more established (WHO, 2007). Traffic-related abatement strategies are aimed at reduction of PM from combustion engines and generally overlook PM mass from non-exhaust emissions (Cheung et al., 2012). Research in the Los Angeles Basin showed that the levels of Cu in the air, an element from non-exhaust abrasion origin, have been reduced to a lesser extent in recent years than chemicals from combustion origin (Cheung et al., 2012), suggesting the effectiveness of the taken combustion-related abatement policies. Consequently, the relative contribution of abrasion dust to PM mass might increase and this may imply an even more significant role of non-exhaust emission within the total of air-pollution-associated health effects. The relevance of this development for air quality policy and the 'state-of-the-art' scientific knowledge was discussed in an international workshop in 2011 entitled "Policy relevance of wear emissions from road transport, now and in the future" (Denier van der Gon et al., 2012). The workshop highlighted the increasing recognition and importance of non-exhaust PM in air-pollution-related health effects and a useful tool for abatement strategies.

The fact that non-exhaust emissions contribute significantly to PM_{10} mass and could, therefore, be of relevance as an abatement measure to reach the air quality limits (Charron and Harrison, 2005) is one of the main findings of the international wear workshop. Furthermore, the wear particles from non-exhaust emissions are generally larger and contain more transition metals compared to vehicular exhaust emissions and, therefore, are likely to exert a different toxicity (Denier van der Gon et al., 2012). A relative comparison of the harmfulness of both emissions is of the utmost importance and, although still missing, might in the future help develop policy abatement strategies.

Particle size

To fully explore particle toxicity, it is essential to consider the size range of PM. The respirable proportion of PM is classified by size fraction based on aerodynamic diameter. The particles are identified as either $PM_{2.5\text{-}10}$ or "coarse particles" (particles with an aerodynamic diameter between 2.5 and 10 µm), $PM_{0.1\text{-}2.5}$ or "fine particles" (aerodynamic diameter between 0.1 and 2.5 µm), and $PM_{0.1}$ or "ultrafine particles"/"nanoparticles" (diameter smaller than 0.1 µm). Concentrations of coarse PM are regulated using PM_{10} standards, while the fine fraction and ultrafine particles are regulated within

both the $PM_{2.5}$ and PM_{10} limits. Particle size determines the region of the respiratory tract and lung where a particle will deposit (Figure 1) as particles with the similar aerodynamic diameters will tend to have the same settling velocity (de Boer et al., 2002). When inhaled, high proportion of coarse and ultrafine particles will deposit in the airways, whereas a significant part (± 70-80%) of the fine particles are exhaled. However, the particle fraction forming most of the mass in ambient air is within the fine particle range. Therefore, no linearity exists between ambient PM mass concentrations and deposition in the lungs (Cassee et al., 2002). Instead, smaller particles may have a high likelihood to reach the peripheral regions of the bronchioles, and interfere with gas exchange inside the lungs and even reach the systemic system (Kreyling et al., 2006, 2010).

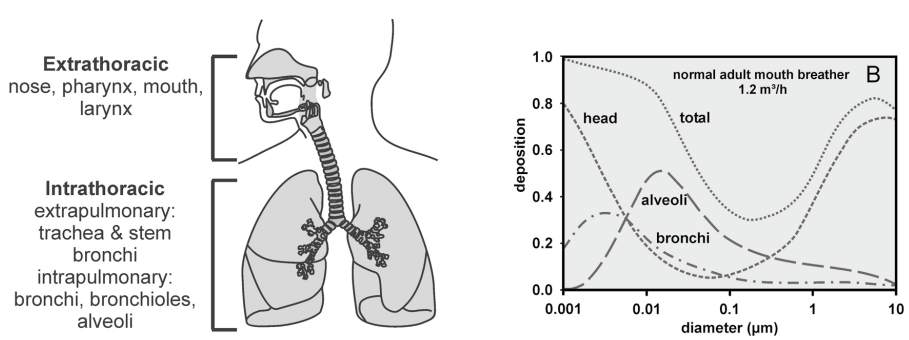

Figure 1. Particle deposition as a function of particle size (Geiser and Kreyling, Particle Fibre Toxicol, 2010, 7:2)

"Coarse particles exert significant adverse toxicity"

In our studies (Chapter 2 and 3), in which we compared the *in vivo* toxicity of ambient PM from different locations, we observed that both coarse and fine particle fractions exerted a similar toxicological potential on a per mass basis (albeit, with a trend towards a higher toxicity for coarse PM). Coarse PM exerting higher toxicity than fine PM was especially observed for inflammatory responses and less evident for cytotoxicity (Chapter 2 and 3). An increased inflammatory potential of coarse compared to fine PM seemed to be a consistent finding in both *in vitro* (Guastadisegni et al., 2010; Hetland et

al., 2005; Jalava et al., 2006,2007, 2008) and *in vivo* (Cho et al., 2009; Happo et al., 2007, 2010) studies despite the source of PM. This might be caused by bacterial endotoxin generally observed in higher concentrations in ambient coarse than fine PM (Guastadisegni et al., 2010; Osornio-Vargas et al. 2003). Although, other components including non-exhaust-related elements were also associated to the inflammatory response of coarse PM (Happo et al., 2007, 2008), and a correlation with endotoxin is not always observed (Happo et al., 2010; Table1). While coarse PM may have an inherently greater toxicity per unit of mass, the overall risk is a function of toxicity and deposition (the fraction of inhaled PM that is deposited in e.g. the lungs). Therefore, the low deposition of coarse PM in the alveolar region means that the pulmonary actions of coarse PM could well be smaller than for fine PM. Nevertheless, toxicological research including our own (Table 1) as well as epidemiological evidence (Brunekreef and Forsberg, 2005) has shown that the effects of

Table 1. Toxicological effects of ambient PM samples from different PM size fractions collected with high volume cascade impactor

Study	Animal model	Exposure	Material	Health measurements	Outcomes	Correlation components	Comments
Cho et al., 2009	CD-1 mice	25 or 100 µg/mouse; oropharyngeal instillation; necropsy 4 and 18 hr after exposure	ambient near and far road CPM, FPM and UF PM samples	inflammation cytotoxicity ischemic perfusion injury	↑CPM highest dose no significant effects ↑ UF PM>FPM>CPM	CPM: Ca^{2+}, K^{+}, NO_3^{-} UF PM: SO_4^{2-}, NH^{4+}	
Gerlofs-Nijland et al., 2007 (Chapter 2)	spontaneously hypertensive rats	3 and 10 mg/kg; intratracheal instillation; necropsy 24 hr after exposure	ambient CPM and FPM; contrasting traffic contribution	inflammation (e.g. TNF-α, PMN) cytotoxicity (LDH) vascular markers	dose-response↑ CPM; 10 mg/kg↑ FPM fibrinogen↑ 10 mg/kg CPM/FPM	CPM: Cu, Ba; FPM: Zn	correlation inflammation CPM driven by one single location
Gerlofs-Nijland et al., 2009 (Chapter 3)	spontaneously hypertensive rats	7 mg/kg; intratracheal instillation; necropsy 24 hr after exposure	ambient urban air CPM and FPM from different European cities	inflammation (e.g. TNF-α, PMN) cytotoxicity (e.g. LDH, protein) inflammation blood vascular markers	↑ CPM>FPM ↑ CPM=FPM except LDH↑ CPM>FPM no changes fibrinogen↑ CPM>FPM	metals, PAHs metals, PAHs metals, PAHs	effect of PAH bound to particles; metals rich in soil and wear elements
Tong et al., 2010	CD-1 mice	100 µg/mouse; oropharyngeal instillation; necropsy 24 hr after exposure	ambient CPM, FPM and UF PM pollution particles	inflammation coronary flow rate recovery cardiac function ischemic perfusion injury	↑ CPM most significant ↓ only UF PM ↓ only UF PM ↑ only UF PM		
Happo et al., 2008	C57BL/6J mice	10 mg/kg; intratracheal instillation; necropsy 4 and 12 hr after exposure	ambient urban air CPM, FPM, UF PM from six European cities	inflammation (e.g. IL-6, TNF-α) cytotoxicity (protein)	↑ responses both CPM and FPM ↑ 12 hr highest response	CPM:NO_3^{-}, K^{+}, Mg^{2+}, Cu; FPM: Ca^{2+}, Al, Fe, V, dicarboxylic acids CPM: Ni, Fe; FPM: Ca^{2+}, Ni	FPM negative correlation PAHs CPM nearly correlation with endotoxin
Happo et al., 2010	C57BL/6J mice	10 mg/kg; intratracheal instillation; necropsy 4 and 12 hr after exposure	urban seasonal air particulates CPM, FPM and two UF PM fractions collected in Helsinki	inflammation (e.g. IL-6, TNF-α)	4 hr highest response CPM>FPM>UF	CPM: Al, Co, Cu, Mn, Fe, Ca^{2+}; FPM: Al, Co, Cr, Cu, Mn, Pb, Fe, Zn, Ca^{2+}, NO_3^{-}; CPM,FPM: not with endotoxin	

coarse PM are significant and should therefore not be ignored. Recently, this was again confirmed by a European multicenter panel study (Karakatsani et al., 2012), in which associations with coarse particles were observed with respiratory symptoms in susceptible individuals.

Particles from both fractions will be derived from different processes. Fine and ultrafine PM are mostly from combustion processes, while non-exhaust emissions (e.g. vehicle-induced suspension and abrasion from tyres and friction of brakes) contribute significantly to coarse PM fractions (Charron and Harrison, 2005). Although difficult to disentangle, our own studies seem to support this rough PM fraction classification with the exception that tyre wear contribute heavily to the fine PM fraction indicated by high Zn concentrations (Chapter 2). The different size fractions of PM not only originate from different processes but also invoke various biological mechanisms that can lead to adverse health effects. Ultrafine particles are so small that there is the possibility that these particles may cross the alveolar wall ('translocation') and reach the systemic circulation where they may directly induce cardiovascular effects (Amatullah et al., 2012). Coarse and fine particles, on the other hand, are unlikely to translocate and thus are more likely to be related to changes in lung function and respiratory diseases (Amathullah et al., 2012). Indeed, exposure to coarse particles increase the risk of asthma, allergy morbidity, cause pulmonary inflammation and exacerbation of chronic pulmonary disease (Zanobetti and Schwartz, 2009). Contrary, fine and ultrafine particle exposure increase cardiovascular morbidity and mortality and triggers acute cardiovascular events (Langrish et al., 2008; Zanobetti and Schwartz, 2009). In the research described in this thesis, we did not evaluate ultrafine particles that are known to be elevated at roadside microenvironments (McCreanor et al. 2007) and, therefore, are likely to be highly significant in terms of potential traffic-related health impacts (Knol et al. 2009). Furthermore, our studies did not explore the biological mechanisms of PM adverse effects and the examined cardiovascular effects were limited, though overall coarse PM did induce a different response pattern than fine PM at the same dose level (Chapter 2 and 3) supporting various mechanisms. In addition, similar research as the ones presented in this thesis of Tong et al. (2010) do support that coarse PM results in significant pulmonary toxicity while ultrafine PM exerts cardiovascular effects (Cho et al., 2009; Tong et al., 2010; Table 1).

Particle composition

Besides particle size, our investigations (Chapter 2 and 3) identified that the chemical composition of PM is an important factor in the induction of toxicity, a finding also confirmed by others (Bell, 2012; Valavanidis et al., 2008). Since we know that not all PM components are equally toxic, identifying the most harmful compounds, and the associated sources of these emissions, would be useful for policymakers to develop effective abatement strategies for air pollution. However, it is extremely challenging to quantify the health effects of specific components and sources since PM consists of thousands of known, and unknown, chemicals. Furthermore, the toxicity of each constituent may be potentiated when combined with other constituents.

"Transition metals and polyaromatic hydrocarbons (PAHs) are likely to be crucial factors affecting pulmonary toxicity"

Considerable insight has been gathered on the role of metals and organic compounds on the adverse health effects of PM (Kelly and Fussell, 2012; Stanek et al., 2011; Strak et al., 2012). However, only a few years ago it became apparent that PM mass, per se, might not be the most important property to explain adverse health effects of PM. Our study described in Chapter 2 revealed that pulmonary toxicity of PM might be linked to specific components (metals) and sources (wood burning). The study presented in Chapter 3 was, therefore, conducted to further elucidate the role of PM composition on its toxicity. The toxic effects on the lung and blood were examined as a function of the chemical composition using PM samples with contrasting levels of metals or PAHs (Chapter 3). Our study showed clear variations in pulmonary responses among PM samples on an equal mass basis, with greater toxicity for PM with higher metal and PAHs content.

Since the metal content of our PM samples were mainly different for soil (chromium (Cr), Mn, Fe, Al) and tyre wear (Zn) related elements, the outcomes suggest that non-exhaust PM is especially important for pulmonary toxicity. In addition, carbon-containing components seemed to be of significance although we could not specify the particular PAH type. Our finding with regard to the significance of metals of non-exhaust origin in pulmonary toxicity is supported by similar studies (e.g. Happo et al., 2010), but no correlation or even a negative correlation is observed with PAHs and pulmonary inflammation (Happo et al., 2007, 2008; Seagrave et al., 2006). According to Happo et al. (2008), the negative correlation might be the result from an

immunosuppressive effect of PAHs. In addition, it is known that PAHs exert mutagenic effects and chemicals most closely related to pulmonary toxicity differ from those involved in mutagenicity (Kelly and Fussell, 2012). Therefore, it might well be that other PM components abundantly present in PAHs-rich PM samples were responsible for the observed pulmonary effects in our study.

The assignment of specific metals or other PM components would be useful for source apportionment and consequently would be of great interest for abatement policy. One of the difficulties in performing source apportionment is to find source specific tracers that are sufficient to identify the original source. The transition metals Fe, vanadium (V), Nickel (Ni), Zn, Cr, and Cu are present within traffic emissions and are likely to exert some biological effects, however, they are also found in emissions from other combustion sources such as industrial activities. In the HEPMEAP project, hopanes and steranes were used as traffic-specific markers to assess the contribution of road traffic to the different PM sites used in the study described in Chapter 2 (Bloemen et al., 2005), as these components originate from lubricant oil (Schauer et al., 1996), especially that used for diesel engines. However, the study revealed that the concentrations of hopanes and steranes did not represent road traffic sufficiently well to be in accordance with the actual site-specific traffic contributions. Another issue is that expressing composition as a mass by mass concentration might overlook the complexity of the real world situation due to over- or under-estimation of a specific source depending on the weather conditions (Bloemen et al., 2005). Despite these difficulties source apportionment studies have still been able to indicate significant health risks for exposure to motor vehicle emission (Vinzents et al., 2005; Maciejczyk and Chen, 2005) and raised attention to traffic as an important source of harmful PM that could be targeted to reduce PM-related health effects.

"Particle size and composition seem to represent more important determinants of the health risks imposed by air pollution than PM mass"

We noted several times that the effects of PM exposure differed on an equal mass basis, which seemed to be related to differences in size and composition (Chapter 2 and 3). As such, our studies contributed to the evidence that there might be more important determinants of PM toxicity than mass. Recently, a review on the evidence for a role of composition in the adverse

health effects of PM, encompassing epidemiological, toxicological and human controlled exposure studies, supported that PM mass concentration is not the sole driving force of health effects and that PM composition is of significance (Rohr and Wyzga, 2012). Legislation is currently based on the total mass and size ranges of PM, but a cost effective PM reduction policy should ideally consider which constituents/sources of PM have to be reduced in order to maximize the health benefits of that specific regulatory strategy. However, linking specific components with adverse health effects should be interpreted with care. As highlighted by the recent study of Rohr and Wyzga (2012) any given component could be actually only an indicator for another (more significant, although less abundant) component that could well be the true toxic agent. Theoretically, even if all hazardous components were known, it would still remain difficult to conclusively prove any association with health effects due to the high co-existence of components and the difficulty in disentangling causality (Stanek et al., 2011).

Long-term exposure

Short-term exposure, as assessed with intratracheal instillation studies presented in Chapter 2 and 3, make it difficult to relate a "no effect" to real life exposure, which in contrast to short-term exposure is both prolonged and repetitive (Ghio et al., 2012). An assessment by Künzli et al (2000) revealed that in Europe exposure to outdoor and traffic-related air pollution has greater adverse effects on public health in the long-term, versus the short-term. This is shown by an increased relative risk for the same health condition (WHO, 2006) but not solely as public health impact also depends on the exposure distribution (Künzli et al., 2000). The sizeable health effects of traffic-derived PM is in part due to high traffic densities in areas with larger populations. As rather little is known about the impact of long-term exposure to traffic-generated PM from a toxicological point of view, we have examined the pulmonary and cardiovascular effects of daily exposures (5 days/week) for a prolonged period (4 weeks) in rats (Chapter 4).

"Prolonged exposure to diesel engine exhaust did not appear to be related to adverse cardiopulmonary effects in healthy animals"

Surprisingly, our study revealed no major adverse health effects of prolonged exposure to traffic-related PM (concentrated ambient particles or diesel engine exhaust), assessing changes in a large panel of haematological

and biochemical assays. This could be attributed to adaptive response mechanisms present in healthy animals. The absence of a response after prolonged exposure in normal compared to compromised animals is seen more often (Lippmann et al., 2005 a,b). In addition, the animal species used in our study might have influenced the response as McDonald et al. (2012) showed that rats are more sensitive than mice to prolonged exposure. Since epidemiological studies strongly associate prolonged exposure to traffic-related emissions with a greater health risk (Künzli et al., 2000; WHO, 2006), similar studies are needed in specific susceptible subpopulations or animal models of disease. Although, susceptibility is not always a prerequisite for an adverse response as only healthy individuals and not asthmatics demonstrate an inflammatory response after short-term exposure to diesel engine exhaust (Stenfors et al., 2004). The pro-inflammatory response after short-term exposure to diesel engine exhaust in healthy individuals is only observed at higher concentrations with a threshold dose approximating 300μg/m^3 and is depending on the time effects are assessed (Ghio et al., 2012). The limited response in our prolonged exposure study may be related to the used moderate exposure dose to mimic real-world exposures rather than using higher doses mostly employed in acute toxicological studies. The use of lower doses in contrary to a design in which clear toxicological effects are assured by high dose exposures imply a risk to fully assess the health effects (Cassee et al., 2012) and might well explain our findings. Finally, differences in the type and composition of the particulate or co-pollutant in ambient PM exposures and even controlled exposures like diesel engine exhaust, as we observed for pulmonary responses after short-term exposure to traffic-related sites (Chapter 2) and prolonged exposure to roadside PM (Chapter 4), may explain these discrepancies.

Brain as a target organ

Oberdörster and Utell (2002) suggested the potential translocation of inhaled particles from the respiratory tract into the systemic circulation and the brain. It is opposed that inhaled manganese particles can access the brain by the olfactory route (Brenneman et al., 2000) and ultrafine particles can enter the brain after exposure by inhalation (Takenaka et al., 2001; Oberdorster et al., 2004). These studies show that a fraction of the inhaled ultrafine particles that are deposited on the olfactory epithelium in the nose by diffusion can subsequently be transported along the olfactory nerves to

the olfactory bulb. Very small particles can transfer to other organs including blood, liver and spleen as indicated by bio-distribution studies (Kreyling et al., 2009; Phalen et al., 2010). Moreover, it has been shown that nanoparticles can cross the blood-brain-barrier and physically enter the central nervous system (CNS) in animals (Lockman et al., 2004). Yet, sufficient evidence that particulate matter is directly toxic to the CNS is lacking.

"Prolonged exposure to diesel engine exhaust resulted in the induction of pro-inflammatory markers in specific brain regions"

In our prolonged *in vivo* inhalation study with diesel engine exhaust, we also examined the impact on the brain (Chapter 5). We showed that prolonged exposure to diesel engine exhaust increased the protein levels of the pro-inflammatory markers tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1 alpha (IL-1α) in the striatum of normal healthy rats. Our study is one of the studies demonstrating that inhaled particles may be involved in adverse CNS effects. PM-related CNS outcomes include neuropathological effects (Calderon-Garciduenas et al., 2002), alterations in neuroinflammatory markers (Chapter 5; Calderon-Garciduenas et al., 2008; Campbell et al., 2005, 2009), central neurodegeneration (Lucchini et al., 2012; Veronesi et al., 2005), as well as cognitive and behavioral changes in children (Fonken et al., 2011). Recently, it was suggested that neuroinflammation induced by diesel engine exhaust might be the result of a systemic response rather than a direct effect of particles entering the brain (Levesque et al., 2011).

The mechanism(s) underlying PM-CNS toxicity are still unclear but neurotoxicity may be related to oxidative stress and innate immune activation. Oxidative stress might be a factor that links PM exposure and neurotoxic effects (Peters et al., 2006). Research suggests that microglial activation may be a key component in the initiation of neuroinflammation in response to air pollution and as such involved in neuronal damage (Block et al., 2012; Lucchini et al., 2012). Microglia are the resident innate immune cells in the brain and are predominant regulators of neuroinflammation (Block et al., 2012). Several studies have indeed shown microglia activation, neuroinflammation and cellular damage (Block et al., 2004; Calderon-Garciduenas et al., 2008; Levesque et al., 2011; Morgan et al., 2011; Sama et al., 2007). High microglia density caused a pronounced pro-inflammatory response *in vitro* (Pintado et al., 2011).

To what extent this region specific response observed in our study (Chapter 5) is related to microglia density and neurodegenerative diseases remains to be elucidated. Levesque et al. (2011) showed activation of microglia especially in the midbrain region that contains the substantia nigra, which is one of the regions with the highest microglia density (Kim et al., 2000; Pintado et al., 2011). It could be that the vulnerability of specific brain regions to exert an inflammatory response depends on a combination of factors such as cell characteristics (e.g. microglia density) as well as type and route of exposure (Block et al., 2012). Exposure to air pollution including traffic-related PM is increasingly suggested to be a risk factor associated with CNS-related disorders. To what extent PM contributes to neurological impairment and the role of specific components in air pollution is unknown. Children may be at particular risk from air pollution exposure since childhood and adolescence are crucial periods of brain development associated with dynamic behavioral, cognitive and emotional changes (Lucchini et al., 2012). Although at present still speculative, it might very well be that ultrafine particle exposure can worsen neurodegenerative disease such as Alzheimer and Parkinson (Moulton and Yang, 2012).

Changing emissions from combustion engines

The introduction of new fuel additives, biofuels or engine emission control technologies (particle traps and catalysts) can be supported as part of an abatement air pollution policy strategy to reduce traffic-related emissions. However, while these technologies are designed to reduce the levels of harmful pollutants, there is the potential that they could inadvertently increase levels of other harmful co-pollutants. Furthermore, as physio-chemical composition plays an important role in air-pollution-toxicology, it is feasible that the PM emissions from new fuels or that altered by exhaust technologies have more pronounced, or different, adverse health-related effects that negate any benefits from the overall decreased levels of PM. However, to evaluate the impact for the full source to health risk chain and avoid unintended consequences, sufficient data should be available of all levels of the life cycle chain. A recent review of biofuels concluded that there is not sufficient information to determine their impact on human health (Ridley et al., 2012). In addition, the marked differences in the study design of the few health-related studies examining the impact of new fuels and/or emission control technologies make it difficult to compare potential adverse

health effects. In particular, there is an urgent need for harmonized testing of engine emission toxicity (Bünger et al., 2012). In order to build upon to the limited data, we assessed the impact of a particulate exhaust filter and the use of a biodiesel fuel blend on the cellular toxicity and oxidative potential of engine exhaust particles (Chapter 6).

"Significant reduction in PM emission by the use of 50% biodiesel blend as a fuel is not necessarily linked to reduced toxicity"

To evaluate the toxicological effects of biodiesel we collect particles utilizing a Euro 4 vehicle fuelled with diesel or a 50% biodiesel blend (Chapter 6). Next to a significant reduction (~50%) in PM mass emission using biodiesel compared to diesel fuel, we observed similar or decreased oxidative potential for PM collected from vehicles fuelled with the biodiesel blend. However, exposure to 50% biodiesel increased cytotoxicity and the release of the pro-inflammatory marker IL-6 *in vitro* compared to diesel even when health parameters were expressed on a per distance driven basis, which is more relevant for risk assessment as expression per unit of mass. These results suggests that a reduction in PM emission as caused by the use of a 50% biodiesel blend is not necessarily linked to reduced health effects.

For a long time it has been taken for granted that emission reduction of the regulated component PM would result in reduction of the health risk. However, the identification and evaluation of potentially hazardous and unregulated components in vehicle emissions is growing and the outcomes so far already suggest a need to be cautious predicting the health risk of vehicle emission on solely PM emission. Some of these studies revealed that the nature of the harmful characteristics of a fuel might change adversely with the addition of biodiesel (Fontaras et al., 2010; Karavalakis et al, 2009, 2010; Zhu et al., 2010). Biodiesel combustion led to an increase of the soluble organic fraction of the total emitted particles with increasing blend concentration (Karavalakis et al., 2009; Zhu et al., 2010). In addition, elevated emissions of PAHs, especially oxygenated PAHs, are observed with a 10% biodiesel blend, while the highly mutagenic nitro-PAH, 1-nitro-pyrene, is reduced (Karavalakis et al., 2010). These changes in composition of engine exhaust depend not just on the fuel but the blend percentage and the feed-stock origin are important as well.

"The application of a particulate filter on combustion engines has a beneficial effect both on PM emission and the hazard of PM"

In the study described in Chapter 6, we also examined the toxicological implications of a diesel particulate filter by expressing the hazard of PM per unit of mass and per distance driven, thereby forming a surrogate of the exposure metric. A beneficial effect of the diesel particulate filter was mainly observed per unit distance driven (Chapter 6). The beneficial effect might be due to the incredible reduction in PM mass brought about by this type of technology, but also might partly be explained by a change in the toxicity of specific components. An absence of vascular impairment was observed in humans exposed to filtered diesel engine exhaust, compared to non-filtered exhaust, the latter still containing the combustion particles (Mills and Miller et al., 2011; Lucking et al, 2011). The importance of combustion-derived particles, as opposed to pure carbon particles, in establishing adverse cardiovascular effects was recently been shown by Mills and Miller, et al. (2011) emphasising the importance of specific chemicals absorbed onto the particle surface. Most new technologies that have been applied since 2007 have not only influenced the amount of PM mass emitted, but also significantly changed the composition of PM (Hesterberg et al., 2011). There is sufficient evidence now to consider that studies exploring the health effects of pre-2007 diesel engines have less relevance towards the assessment of the potential health risks of modern diesel engines containing new technologies (Hesterberg et al., 2011). This also questions the classification of diesel engine emissions, in general, as carcinogenic (IARC, 2012), since there is still a clear lack of toxicological studies that can be used for the risk assessment of old versus new engine emissions. It is worth considering though that the average age of engines is still about 5-10 years old in the developed world and far older in the developing world. Nevertheless, it still remains a distinct possibility that the modern "cleaner" engines can emit high numbers of ultrafine particles (that are not picked up by many sampling techniques) and as such impose a risk for public health (Oberdorster and Utell, 2002).

Strengths and limitations of experimental studies

There are a number of important issues to consider when translating the outcomes of animal studies to real-life scenarios in man and their exposure-related health effects. First, the collection of PM material for *in vivo* and *in vitro* experimental studies is a frequently used approach. Most devices used for PM collection require an extraction step to separate PM from the collection matrix (e.g. Teflon filter). Extraction of PM is often difficult and/or inefficient, and changes in PM composition and size are likely, and consequently a different biological response may be observed (Jalava et al., 2005). To overcome this disadvantage, determination of the physicochemical properties of the extracted material should be performed and, although expensive and time consuming, such an approach allows direct associations to be made between PM-induced health effects and specific components, chemical groups or sources, which is not straightforward in real life exposures.

The exposure dose is another aspect to consider. Most experimental studies are performed with high doses and dose rate that far exceeds real life exposure concentrations. This dose discrepancy makes direct translation of the findings to real life scenarios difficult, as different biological pathways may be involved in response to high dose exposure. These high doses are, however, used to determine if adverse effects can occur (plausibility), as low dose exposure may induce effects that are below the threshold of detection. As such, assessment of the hazard is an essential first step and subsequent dose-response relationships could be performed to support the translation to population-based health risk. Therefore, experimental toxicological studies with a range of doses (including high dose) accompanied by good physiochemical characterization of the PM from various sources/locations is extremely informative, providing additional plausibility to support the associations found in epidemiological studies. Furthermore, toxicological studies provide a way to identify and evaluate the biological mechanisms involved, including effects on different target organs, and the effects of short- versus long-term exposure (Solomon et al., 2012).

Of particular importance is the method of exposure used in experimental studies. The human population is exposed to air pollutants in the air pollution mixture, largely, by inhalation. Intratracheal instillation is a common method of administering PM directly into the lungs via the trachea, and one

we routinely use in our own laboratory (e.g. Chapter 2 and 3), mostly used to screen PM samples and compare the relative toxicity. However, it needs to be acknowledged that it is not a physiological route of exposure, and has limitations in that it delivers specific pollutants only, instead of exposure to the complete complex air pollution mixture (Driscoll et al., 2000). It could be expected that the observed health effects occurring with intratracheal instillation differ from research studies using inhalation exposure especially due to differences in the deposition, clearance and retention of particles (Driscoll et al., 2000). For example, particle size will have an impact on the biological effects of each method of administration. With inhalation exposure, the nose will act as a barrier filtering out the larger particles (especially in rodent models), which on the other hand could still deposit in the lung using the instillation exposure method. Additionally, with inhalation exposure ultrafine particles may reach the brain through the olfactory bulb and exert an effect directly in the brain, with the possibility of subsequent effects on peripheral organs. However, overall qualitatively similar results are observed using both exposure methods (Driscoll et al., 2000; Henderson et al., 1995). This is also illustrated by a study of Miyabara et al. (1998) in which diesel engine exhaust enhanced airway inflammation in mice using inhalation as well as intratracheal instillation exposure. Intratracheal instillation is a powerful screening tool but for risk assessment purposes inhalation exposure is the preferred route (Driscoll et al., 2000). However, rodents are obligatory nose breathers while in man a large proportion of breathing is done via the mouth and it is unlikely that nasal filtration is as efficient in man. Therefore, it is questionable using rodents if inhalation is indeed the best route of exposure. Overall, the disadvantages of intratracheal instillation need to be balanced by the relative technical ease of this technique and that the use of PM suspensions for instillation allow for more exact quantification of effects in relation to PM composition.

The pros and cons of toxicological methods, in regards to how reliable various approaches are for studying and quantifying the links between air pollutants and adverse health effects, are discussed by Solomon et al. (2012). In particular, they highlight how useful rodent models of disease can be for determining factors (e.g. diabetes) that influence the impact of air pollution to gain insight in susceptibility. The strengths and limitations of the techniques used for this thesis are just briefly discussed. It is beyond the scope of this thesis to discuss the many issues surrounding the interpretation of the findings of *in vitro*/*in vivo* approaches but overall, toxicological studies are powerful tools for studying the plausibility observed in epidemiological research (Solomon et al., 2012).

Public health impact and policy abatement strategies

The scientific evidence presented in this thesis provides a structure to develop current health-based air pollution policy. Important key questions for guidance of EU policy were recently formulated in the framework of a joint WHO/EC project entitled: "Evidence on health aspects of air pollution to review EU policies – REVIHAAP". The chapters in this thesis directly address the REVIHAAP health question on the role of different size fractions, chemical constituents or sources. Despite a large amount of data there is still a need to generate further data to fully answer this key question and develop efficient PM abatement and air pollution management strategies (Kelly and Fussell, 2012).

The research presented in this thesis is centered around the role of particle size and composition on the health effects of traffic-related air pollution. From an air-pollution-policy perspective, health effects linked to a specific source is of most interest as abatement measurements aimed to reduce the pollutants from a specific source may be more effective rather than overall reduction in pollutants. An important driver to better tackle air pollution is the sheer size of large populations exposed in everyday life, by that explaining the interest of policymakers for the source traffic. We clearly highlight in this thesis that non-exhaust abrasion emissions from brakes and tyres are of significance and this might provide a new tool for policymakers to diminish traffic-related health effects even further. Although not discussed in detail in this thesis, it is worth noting that people are not only exposed to motor vehicle exhaust, but also to engine exhaust from other modes of transport like trains and ships, generator engines apart from PM exposure from non-exhaust wear emissions, as well as more specific occupational PM.

While we emphasise the need to identify specific harmful constituents of current pollutants, the other side of this argument also needs to be considered. Air pollution and its health risk are controlled by policies based on limit

values for emissions of single pollutants only. This has its limitations, as it is the mixture and the interaction of pollutants that is likely accountable for its effect. PM mass is just a measure of a complex pollution mixture with multiple sources and can be easily used for policy purposes and regulations. PM affects many people and it is, therefore, important to minimize human exposure. Several policy measures can be taken on different levels to reduce personal exposure including personal measures, e.g. wearing a facemask (Langrish et al., 2009) or source specific measures. An example of the latter is the ever more stringent emission standards (Euro classes) for both diesel and gasoline engines, cleaner (bio)fuels and vehicle inspection programs (HEI, 2010). This type of regulation has resulted in a relatively effective control of engine exhaust emissions.

With the tighter vehicle emission standards, both new emission control technology and (bio)fuels were introduced that have decreased the emission of PM (and other co-pollutants), and consequently lead to a reduced occupational and environmental exposure. However, these standards only apply to newly introduced engines and it will take time to replace the old equipment. Unfortunately, an increased number of vehicles and the distances driven, has lessened the effect of emission policy control (Laumbach and Kipen, 2012). As such it remains to be seen how changes in engine emission composition, as a result of the utilization of those new technologies and (bio)fuels, will affect human health in the near future and long-term. Our own research indeed suggests that reduction in exhaust emissions not necessarily results in diminished adverse health effects.

Paracelus was the first to mention that the dose makes the poison. The risk for the population is determined by both the (personal) exposure and the hazard (risk = exposure x hazard). It follows that high exposure concentrations to low hazards could imply a similar risk as low exposure to severe hazards, and cumulative exposures may result in a different response due to mechanisms to adapt to these exogenous factors. The focus of mitigation strategies has been mostly on the sources with the largest mass emission rates to the outdoor atmosphere, instead perhaps on which sources lead to the greatest exposure to (and greater effect on) humans (Nazaroff, 2008), while exposure is essential for the adverse effects to occur. Furthermore, the effective dose depends not only on the exposure concentration and exposure time, but also physiological factors like particle size and deposition play a role. Steep gradients of concentrations of traffic-related air pollution

(especially fine PM) exists near roadways, with reduction of traffic-related air pollution concentration to background levels within several hundred meters away from roadways (Gilbert et al., 2003; Zhu et al., 2004). Nevertheless, this perhaps overlooks the heightened exposure individuals have living/working in the close vicinity of major roads in urban areas.

Haber's rule (Miller et al., 2000) states that, for a given poisonous gas or aerosol the product of concentration and exposure duration is constant within certain limits. This suggests that a peak exposure followed by a period of low exposures may result in a similar dose (and effect) as a steady state exposure at moderate concentrations. Thus, the rule states that for a given effect doubling the concentration should be combined with half the exposure time to result in the same response.

Besides concentration and time, the time-activity pattern is another important determinant of exposure. The mode of transport (biking versus car versus bus) have been shown to result in important differences in exposure concentration and inhaled dose, with in general the highest doses for cyclists and the lowest for electric bus riders (Zuurbier et al., 2009). Additionally, engine emissions will age with distance and time away from the emitted source undergoing complex chemical and physical processes like oxidation that alter exposure and toxic properties in ways that are not fully understood.

A report by the U.S. National Research Council (2004) provides recommendations to improve air quality management. Policy strategies could be improved by moving from the current pollutant-by-pollutant approach to a more integrated multi-pollutant approach, which also considers human exposure. A multi-pollutant approach could reduce the risk further, as this strategy includes unmeasured/infrequently measured air pollutants that exist which might exert significant health effects. In the long run policies measures addressing multiple pollutants may actually be less costly than separate measures for single pollutants. Quantifying the relative risk across pollutants and the integrated risk of pollutants for specific urban or regional situations may dramatically improve risk-based policy.

Conclusions

The research presented in this thesis contributed to our understanding of the toxicological impact of traffic-related air pollution, and provides insight into alternatives or modifications for health-based policy control of air pollution. This research confirms the importance of road traffic exhaust as a source of harmful PM, but also highlights the significance of non-exhaust emissions.

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SUMMARY

Numerous studies have been published on the health effects associated with exposure to air pollution. Air pollution is acknowledged as a public health risk and air quality regulations are set for specific air pollutants to protect human health. A major pollutant, well known for its adverse health impact, is particulate matter (PM) of which road traffic is a major source. Therefore, the health effects of traffic-related air pollution have been under considerable scrutiny and the research described in this thesis contributes, at least in part to unravelling of the puzzle.

This thesis is based on the research of two European Commission funded projects and two projects funded by the Ministry of Infrastructure and the Environment and conducted at the National Institute for Public Health and the Environment. The overall objective of the conducted research is to examine the adverse health effects of inhalable PM and assess in experimental studies the toxicity of components and sources of PM emissions. The research presented in this thesis was also performed to strengthen the link between scientific evidence and interventional measures taken in order to limit the adverse effects of air pollution generated by road traffic.

The research described in Chapter 2 has been conducted at a time that the health effects of traffic-derived PM were less evident. As such, the research described in Chapter 2 has contributed to our knowledge on the role of road traffic emissions exerting biological responses and toxicity of PM. In the *in vivo* study, we observed a trend of enhanced capacity to trigger an inflammatory response of PM from high compared to low traffic environments (Chapter 2). At that time, ***the importance of road traffic as a source of harmful air pollutants*** supported the efforts of policymakers to reduce traffic emissions and exposure to traffic-derived air pollutants.

In addition, the results of our study suggested that other sources like wood combustion and wear emissions (e.g. from brake and tyre wear) could be of relevance based on the associations with specific elements like potassium, copper, barium and zinc (Chapter 2). ***Both engine exhaust and non-exhaust PM from wear emissions are of significance in terms of toxicity*** and the contribution of non-exhaust emission to traffic-related health effects is now beginning to gain recognition by policymakers. However, to what extent various sources of air pollution contribute to the health risks is still surrounded by uncertainty.

PM mass seems not the best metric to predict air-pollution-related health effects and research from our own group (Chapter 2 and 3) has emphasized the ***importance of particle size and composition as determinants of the health risks imposed by air pollution***. The *in vivo* studies examining the short-term detrimental effects of PM from different locations (Chapter 2 and 3), showed that ***coarse particles (2.5-10 µm) exert significant adverse toxicity***, which emerged the importance of coarse particles, in addition to fine (<2.5 µm) PM. This implies that coarse particles should not be ignored by air pollution regulation. In addition, the specific role of metals and polyaromatic hydrocarbon content (PAHs) has been examined in more depth *in vivo* using PM samples with significant contrast in those PM components (Chapter 3). ***Transition metals and PAHs are likely to be crucial factors affecting pulmonary toxicity*** as we observed increased adverse pulmonary responses in relation to these PM components and particle composition may be considered useful to establish a strategy for reduction of harmful PM levels.

As rather little is known about the impact of long-term exposure to road traffic PM from a toxicological point of view, we have examined the pulmonary and cardiovascular effects of prolonged exposure to roadside and diesel engine exhaust particles *in vivo* (Chapter 4). Contrary to what was expected ***no adverse cardiopulmonary effects occurred in healthy animals after prolonged exposure to PM generated from road traffic***. This might be explained by adaptive response mechanisms present in healthy animals or a lack in toxic potency of the pollution studied. Still, ***prolonged exposure to diesel engine exhaust resulted in the induction of pro-inflammatory markers in specific brain regions*** (Chapter 5), suggesting the susceptibility of the central nervous system to air pollutants and the impact of air pollution that may have been underestimated in the past.

The last research chapter of this thesis (Chapter 6) considers interventions that aim to decrease emissions from road vehicles e.g. introduction of a particle trap and/or a biodiesel blend as a fuel. Policies are already in place promoting the use of such approaches based on their expected PM mass emission reduction and expected benefits for human health. Our study revealed that ***significant reduction in PM emission by the use of 50% biodiesel blend as a fuel is not necessarily accompanied by reduced toxicity of the emitted PM*** as shown by increased cytotoxicity and the release of the pro-inflammatory marker IL-6 *in vitro* compared to diesel engine exhaust PM.

Yet, we observed that ***the application of a particulate filter on combustion engines has a beneficial effect on both PM mass emission and the hazard of PM***. To elucidate systematically the impact of road traffic-derived emissions, and technologies or fuels used to tackle them, an international harmonised methodology is needed.

Finally, the outcomes and implications of the conducted research described in this thesis are considered in Chapter 7. This research confirms the importance of road traffic exhaust as a source of harmful PM, and especially the significance of non-exhaust emissions. Despite regulation on air pollution, considering the sheer number of people exposed to road traffic-generated emissions globally, road traffic-specific health-based policy measures will remain significant for the near future, and the research in this thesis highlights the importance of integrating scientific research findings with that of regulatory policy.

SAMENVATTING

Er zijn veel publicaties verschenen over de gezondheidseffecten van luchtverontreiniging. Omdat blootstelling aan luchtverontreiniging wordt gezien als een risico voor de volksgezondheid zijn voor bepaalde luchtverontreinigende stoffen grenswaarden voor luchtkwaliteit opgesteld. Fijn stof is een belangrijk onderdeel van luchtverontreiniging en het is algemeen bekend dat blootstelling aan fijn stof de gezondheid nadelig beïnvloed. Aangezien het wegverkeer een belangrijke bron van fijn stof is, zijn de gezondheidseffecten van verkeersgerelateerde luchtverontreiniging uitvoerig onderzocht. Dit proefschrift draagt bij aan het ontrafelen van deze complexe materie.

Het onderzoek is uitgevoerd bij het Rijksinstituut voor Volksgezondheid en Milieu vanuit twee Europese projecten en twee projecten van het Ministerie van Infrastructuur en Milieu. Het doel van deze projecten is het beoordelen van de nadelige gezondheidseffecten door blootstelling aan luchtverontreiniging en de bijdrage van componenten en emissiebronnen van fijn stof. Het in dit proefschrift beschreven onderzoek is ook uitgevoerd om interventie maatregelen, die genomen kunnen worden om de nadelige effecten door blootstelling aan verkeersgerelateerde luchtverontreiniging te verminderen, te ondersteunen met wetenschappelijk bewijs.

Het onderzoek beschreven in Hoofdstuk 2 is uitgevoerd in een tijd dat het verband tussen nadelige gezondheidseffecten en fijn stof afkomstig van het wegverkeer nog niet zo vanzelfsprekend was. Dit onderzoek heeft daarmee bijgedragen aan onze algemene kennis over de rol die emissies van het wegverkeer spelen bij de schadelijkheid van luchtverontreiniging. We vonden dat dieren die werden blootgesteld aan fijn stof afkomstig van een locatie met veel wegverkeer een verhoogde mate van ontsteking in de longen hadden in vergelijk met fijn stof afkomstig van een plaats met weinig wegverkeer. ***Het belang van wegverkeer als een bron van schadelijke luchtverontreinigende stoffen*** ondersteunde indertijd de inspanningen van beleidsmakers om verkeersemissies en blootstelling aan verkeersgerelateerde luchtverontreiniging terug te dringen.

Onze studie liet ook zien dat andere bronnen van fijn stof zoals houtverbranding en slijtage-emissies van remmen en banden bijdragen aan de mate van toxiciteit van fijn stof gebaseerd op bron-specifieke componenten van fijn stof afkomstig van deze bronnen zoals kalium, koper, barium en zink (Hoofdstuk 2). ***Fijn stof afkomstig van zowel uitlaat- als slijtage-emissies is belangrijk***. De bijdrage van het niet uit de uitlaat afkomstige fijn stof aan de

gezondheidseffecten van verkeersgerelateerd fijn stof wordt tegenwoordig meer en meer erkend door beleidsmakers. Echter, in hoeverre verschillende bronnen van luchtverontreiniging bijdragen aan de gezondheidsrisico's is nog steeds omgeven door onzekerheden.

De massa van fijn stof lijkt niet de beste maat om gezondheidseffecten door luchtverontreiniging te voorspellen. Ons onderzoek (Hoofdstuk 2 en 3) heeft laten zien dat de ***deeltjesgrootte en chemische samenstelling van fijn stof belangrijke factoren zijn bij de beoordeling van de gezondheidsrisico's***. De studies die we hebben uitgevoerd om de acute schadelijke effecten van fijn stof afkomstig van verschillende locaties te onderzoeken, laten zien dat ***blootstelling aan de grove (2,5-10 µm) fractie van fijn stof kan leiden tot nadelige effecten***. Uit deze bevinding blijkt het belang van grof stof naast de fijnere (< 2,5 µm) fractie. In de regelgeving moet de grove fijnstoffractie dan ook niet genegeerd worden. We hebben tevens de specifieke rol van metalen en polyaromatische koolwaterstoffen (PAKs) onderzocht door dieren bloot te stellen aan fijn stof afkomstig van locaties met een behoorlijk contrast in deze componenten (Hoofdstuk 3). De nadelige effecten in de long namen toe in relatie tot deze fijnstofcomponenten. ***Overgangsmetalen en PAKs lijken een belangrijke rol te spelen bij longtoxiciteit***. Kennis over de samenstelling van fijn stof kan nuttig zijn om een strategie te ontwikkelen om de concentraties van het meest schadelijke fijn stof te verminderen.

We hebben in een dierstudie de effecten op de longen, het hart en de bloedvaten onderzocht van langdurige blootstelling aan deeltjes afkomstig van het wegverkeer of dieselmotoruitstoot (Hoofdstuk 4). Aanleiding voor dit onderzoek was de weinig beschikbare informatie over de impact van langdurige blootstelling aan fijn stof. In tegenstelling tot onze verwachting werden er ***geen nadelige effecten op het hart en in de longen waargenomen in gezonde dieren na langdurige blootstelling aan fijn stof afkomstig van het wegverkeer***. Dit kan verklaard worden door het adaptatie mechanisme aanwezig in gezonde dieren of door de onverwacht geringe toxische potentie van het onderzochte mengsel. Toch zagen we een ***toename in stoffen die leiden tot ontstekingsreacties in specifieke delen van de hersenen na langdurige blootstelling aan dieselmotoruitstoot*** (Hoofdstuk 5). Dit suggereert dat het centrale zenuwstelsel gevoelig is voor luchtverontreinigende stoffen en de impact van luchtverontreiniging kan in het verleden onderschat zijn.

Het laatste onderzoekhoofdstuk van dit proefschrift (Hoofdstuk 6) beschouwt interventies waarmee de emissies van voertuigen afnemen zoals

de introductie van een roetfilter en/of het gebruik van biodiesel als brandstof. Uit onze studie blijkt echter dat een ***significante afname van fijnstofemissie bij het gebruik van 50% biodiesel als brandstof niet noodzakelijk gepaard gaat met een afname in toxiciteit***. In een *in vitro* studie ondervonden de cellen meer schade en vonden we een toename in de afgifte van de ontstekingsmarker IL-6 na blootstelling aan 50% biodiesel in vergelijk met fijn stof afkomstig bij het gebruik van diesel als brandstof. Anderzijds heeft het ***gebruik van een roetfilter een positief effect op zowel de fijnstofemissies als de schadelijkheid van het fijn stof***. Om systematisch de impact van de uitstoot van het wegverkeer en de introductie van technologieën of brandstoffen op de gezondheid te bepalen is een internationaal geharmoniseerd protocol een vereiste.

Tot slot zijn in Hoofdstuk 7 de uitkomsten en de implicaties van het uitgevoerde onderzoek beschreven. Het onderzoek bevestigt het belang van wegverkeer als een bron van schadelijk fijn stof en vooral het belang van niet-uitlaatemissies. Ondanks de pogingen om luchtverontreiniging te reguleren, blijft het in de nabije toekomst belangrijk om verkeers-specifieke gezondheids-gerelateerde beleidsmaatregelen te nemen gezien de aantallen mensen die wereldwijd aan verkeersemissies worden blootgesteld. Het onderzoek in dit proefschrift benadrukt het belang van het integreren van de bevindingen van wetenschappelijk onderzoek en beleidsvoorschriften.

AFFILIATIONS OF CO-AUTHORS

Damien van Berlo
IUF-Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

Bert Brunekreef
Division of Environmental Epidemiology (EEPI), Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, the Netherlands
and
Julius Centre for Health Sciences and Primary Care, University Medical Centre Utrecht, Utrecht, the Netherlands

A. John F. Boere
Department for Environmental Health (DMG), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Paul J.A. Borm
Centre of Expertise in Life Sciences, Zuyd University, Heerlen, the Netherlands

Henk J.T. Bloemen
Centre for Environmental Quality (MIL), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Leendert van Bree
Netherlands Environmental Assessment Agency (PBL), The Hague, the Netherlands

Arezoo Campbell
Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, California, United States of America

Flemming R. Cassee
Department for Environmental Health (DMG), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
and
Division of Toxicology (TOX), Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, the Netherlands

Ken Donaldson
ELEGI/Colt Laboratory, Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

Jan A.M.A. Dormans
National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Rodger Duffin
ELEGI/Colt Laboratory, Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

Paul H.B. Fokkens
Department for Environmental Health (DMG), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Cecilia Guastadisegni
Department of Environment and Primary Prevention, Instituto Superiore di Sanita, Rome, Italy

Patrick W.F. Hadoke
Centre of Cardiovascular Science, University of Edinburgh, Edinburgh, Scotland, United Kingdom

Nicole A.H. Janssen
Department for Environmental Health (DMG), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Al A. Jimenez
ELEGI/Colt Laboratory, Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

Frank J. Kelly
Environmental Research Group, MRC-HPA Centre for Environmental Health, King's College London, London, United Kingdom

Evren Kilinç
Laboratory for Clinical Thrombosis and Haemostasis, Department of Internal Medicine, Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, Maastricht, the Netherlands

Marit Låg
Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway

Daan L.A.C. Leseman
Department for Environmental Health (DMG), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Mark M. Miller
Centre of Cardiovascular Science, University of Edinburgh, Edinburgh, Scotland, United Kingdom

Ian S. Mudway
Environmental Research Group, MRC-HPA Centre for Environmental Health, King's College London, London, United Kingdom

Leonidas Ntziachristos
Laboratory of Applied Thermodynamics, Mechanical Engineering Department, Aristotle University, Thessaloniki, Greece

Melina Rummelhard
National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

Raimo O.Salonen
National Institute for Health and Welfare, Department of Environmental Health, Kuopio, Finland

Zissis Samaras
Laboratory of Applied Thermodynamics, Mechanical Engineering Department, Aristotle University, Thessaloniki, Greece

Thomas Sandström
Department of Respiratory Medicine and Allergy, University of Umeå, Umeå, Sweden

Roel P.F. Schins
IUF-Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany

Per E. Schwarze
Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway

Markus Sillanpää
Finnish Environment Institute, Laboratory, Helsinki, Finland

Constantinos Sioutas
Department of Civil Engineering & Environmental Science, University of Southern California, Los Angeles, California, United States of America

Henri M. Spronk
Laboratory for Clinical Thrombosis and Haemostasis, Department of Internal Medicine, Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, Maastricht, the Netherlands

Annike I. Totlandsdal
Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, Oslo, Norway

Theodoros Tzamkiozis
Laboratory of Applied Thermodynamics, Mechanical Engineering Department, Aristotle University, Thessaloniki, Greece

Kate Wang
Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, California, United States of America

ABOUT THE AUTHOR

Curriculum vitae

Miriam Elisabeth Nijland was born in Monnickendam, the Netherlands on January 26th 1970. After graduating her secondary school at the Bonhoeffer College in Castricum, she started the MSc programme Molecular Sciences at the Wageningen University. The Master study included two work placements on molecular biology and toxicology. In 1997 Miriam graduated and started a PhD at the Radboud University Nijmegen on "The role of podocytes in the induction of acute albuminuria by injection of monoclonal anti-aminopeptidase A antibodies". Since 2002, she is working at the National Institute for Public Health and the Environment (RIVM) in Bilthoven examining the adverse effects of air pollution including the emission of combustion products like biofuels with a special interest for particulate matter (PM). Her work started with the coordination of *in vivo* toxicity studies examining the hazard of various ambient PM samples conducted in the framework of two European projects (HEPMEAP and PAMCHAR) and continued within two programmes for the Dutch Ministry of Infrastructure and the Environment. Miriam is an inhalation toxicologist by profession and will continue her work on adverse health effects of ambient particulate air pollution at the RIVM Department for Environmental Health (DMG).

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