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# DOSSIER ON CERIUM OXIDE - ANNEX 5

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# The biological effects of subacute inhalation of diesel exhaust following addition of cerium oxide nanoparticles in atherosclerosis-prone mice☆

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## Abstract

### Background

Cerium oxide ($CeO_2$) nanoparticles improve the burning efficiency of fuel, however, little is known about health impacts of altered emissions from the vehicles.

### Methods

Atherosclerosis-prone apolipoprotein E knockout ($ApoE^{-/-}$) mice were exposed by inhalation to diluted exhaust (1.7 mg/m$^3$, 20, 60 or 180 min, 5 day/week, for 4 weeks), from an engine using standard diesel fuel (DE) or the same diesel fuel containing 9 ppm cerium oxide nanoparticles (DCeE). Changes in hematological indices, clinical chemistry, atherosclerotic burden, tissue levels of inflammatory cytokines and pathology of the major organs were assessed.

### Results

Addition of $CeO_2$ to fuel resulted in a reduction of the number (30%) and surface area (10%) of the particles in the exhaust, whereas the gaseous co-pollutants were increased (6–8%). There was, however, a trend towards an increased size and complexity of the atherosclerotic plaques following DE exposure, which was not evident in the DCeE group. There were no clear signs of altered hematological or pathological changes induced by either treatment. However, levels of proinflammatory cytokines were modulated in a brain region and liver following DCeE exposure.

### Conclusions

These results imply that addition of $CeO_2$ nanoparticles to fuel decreases the number of particles in exhaust and may reduce atherosclerotic burden associated with exposure to standard diesel fuel. From the extensive assessment of biological parameters performed, the only concerning effect of cerium addition was a slightly raised level of cytokines in a region of the central nervous system. Overall, the use of cerium as a fuel additive may be a potentially useful way to limit the health effects of vehicle exhaust. However, further testing is required to ensure that such an approach is not associated with a chronic inflammatory response which may eventually cause long-term health effects.

**Keywords:** Cerium oxide, Diesel exhaust, Atherosclerosis, Nanomaterial, Neuroinflammation, Inhalation

## Highlights

► $CeO_2$ nanoparticles added to diesel decreases the number of particles in exhaust. ► Atherosclerosis is associated with exposure to standard diesel fuel in ApoE mice. ► $CeO_2$ nanoparticles in diesel induce cytokines in the brain of mice.

## 1. Introduction

High levels of particulate matter (PM) in air pollution are associated with an increase in the morbidity and mortality caused by cardiorespiratory diseases (Dockery et al., 1993; Mills et al., 2007; Pope et al., 2004). Overall, PM levels in the air have decreased over the last 60 years, however, within this time there has been a marked increase in combustion-derived particles from road vehicles. Diesel engine exhaust is especially rich in nano-sized particles that, while contributing little to particle mass, can penetrate deep into the lungs and have a large surface area to volume ratio on which to carry toxic chemicals into the body.

Nanoparticulate cerium oxide or ceria ($CeO_2$) can be used as an additive to improve the burning efficiency of fuels, reducing fuel consumption, greenhouse gases and particle numbers in vehicle exhaust (Logothetidis et al., 2003; Selvan et al., 2009). $CeO_2$ acts as a catalytic converter in diesel fuel (Wakefield et al., 2008) and is already in commercial use by some coach companies in the United Kingdom (Park et al., 2008). While the use of $CeO_2$ contributes to a reduction in PM, it is necessary to demonstrate that it does not alter the intrinsic toxicity of particles or co-pollutants emitted in the exhaust (Cassee et al., 2010). Direct evidence via a controlled inhalation study is lacking (Cassee et al., 2010) and the potentially harmful effects of emissions may not be suitably predicted by studies using cells or healthy animals. The association between exposure to urban PM and cardiovascular disease is well established and pose a serious problem (Araujo and Nel, 2009; Brook, 2008; Mills et al., 2007; Hassing et al., 2009). Therefore, technologies that alter PM and co-pollutants require testing in models of cardiovascular disease.

Here we use an atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mouse model to study the effect of a sub-acute (4 week) diesel exhaust exposure on the development of atherosclerosis, proinflammatory cytokine levels in two regions of the central nervous system (CNS), systemic inflammation, endothelial function and overall pathology. We hypothesize that the addition of $CeO_2$ to diesel fuel will lead to a decrease in particle number in diesel engine exhaust, that may be associated with fewer or lesser pathological changes compared to exposure to exhaust from regular diesel fuel.

## 2. Materials and methods

The design of this study was based on the Organisation of Economic Co-operation and Development (OECD) Test Guideline nr 412 (adopted 7 Sept 2009), and within the scope of the Sponsorship Programme for the Testing of Manufactured Nanomaterials (OECD, 2007).

### 2.1. Animals & experimental design

ApoE$^{-/-}$ mice (B6.129P2-ApoE$^{tm1Unc}$ N11; 5 males and 5 females per group; Taconic, Denmark) were fed a "Western" diet high in fat (21% fat; AB diets, The Netherlands) ad libitum for 7 weeks from an age of 11 weeks until the end of the study. After three weeks of feeding (age 14 weeks), mice were exposed (nose only) to filtered air or diluted exhaust originating from an engine fueled with standard diesel with (DCeE) or without (DE) cerium oxide, 5 day/week for 28 day. Preliminary experiments showed that pre-exposure, mice will have developed only modest atherosclerotic lesions in the aortic arch and vessels branching from this point. To assess the dose-dependence of the exposures, three different lengths of exposure (20, 60 or 180 min) of a single concentration of exhaust (see below) were used to provide three "dose" levels within each exposure type as described in the OECD TG412 guidelines. Animals were sacrificed 3 day after the last exposure, with the exception of "recovery groups" which were sacrificed 14 day later (17 day after last exposure) to explore the reversibility or possible delayed effects of exposure (high dose groups only). Where possible, experimental variation was limited by randomization of treatment and analysis (e.g., allocation of animals to treatment groups, alternating the time of day for the exposure to DE or DCeE and introducing a random assignment into the sectioning and blood-taking schedule).

### 2.2. Generation and characterization of the test atmosphere

An Ingersoll Rand (4IRD5AE) engine (run at 1500 rpm with a generator load of 35 kW) fueled with low sulfur (10 ppm) diesel (EN590, purchased at a regular gas station in the Netherlands; CAS number 68334-30-5) with or without 9 ppm nanosized cerium oxide was used to generate exhaust for exposures. A cerium oxide suspension "Envirox", (2.5% Ce in non-flammable aliphatic hydrocarbon solvent (Exxsol D-80), Oxonica, Haddenham Bucks, UK),) was mixed into the regular fuel in barrels of 100 L that were used in the engine. The exhaust was captured and diluted with humidified compressed air to obtain a particle concentration of 1.7 mg/m$^3$ for the DE exposure. This level was based on our previous studies in mice that did not cause severe pathological effects (Hiramatsu et al., 2005). Identical engine parameters were used for the DCeE exposure. The dilution of the engine exhaust was adjusted to get equal mass concentrations for both fuel types at a given exposure day (i.e., with and without cerium oxide). Although cerium oxide did slightly affect the mass emissions of the engine, the major influence came from the ambient temperature.

Continuous mass recordings were made using a TEOM 1400a (Rupprecht & Patashnik, Albany, NY, USA) and time-integrated mass concentrations were determined gravimetrically using particles collected on 47 mm Teflon filters. Particle number concentrations were measured with a condensation nucleus counter (CPC 3022A, TSI Inc, St Paul, MN, USA; logfile 10 s interval) and the surface area was measured using a Nanoparticle Surface Area Monitor (NSAM model 3550, TSI Inc, St Paul, MN, USA). Carbon monoxide and nitrogen oxides were detected with on-line analyzers (Envitec model 300E, Teledyne, City of Industry CA, USA and model 42 W, Thermo Electron Corporation, Hopkinton, MA, USA, respectively). Aerodynamic particle size distributions were recorded using a scanning mobility analyzer (SMPS model 3080 with CPC 3785, TSI Inc, St Paul, MN, USA). The sizes of the cerium particles in "Envirox" were determined by dynamic light scattering. Cerium content was measured by high resolution inductively coupled plasma mass spectrometry (HR-ICPMS, Thermo Fisher, Germany), following digestion in nitric acid and hydrogen peroxide at 100 °C for 1 h. Based on possible interferences, Ce was measured as $^{140}$Ce in medium resolution mode. An external calibration with internal standard correction was applied. The procedure is comparable to other applied analysis within this field (e.g., Ulrich and Wichser, 2003). Transmission electron microscopy (TEM; TECNAI F30ST electron microscope, operated at 300 kV and with a high angle annular dark field detector) and local energy dispersive X-ray (EDX) analysis were also

used to assess particle size and to detect cerium oxide in soot.

### 2.3. Pathology

One half of the lung was preserved on ice to determine cerium content of the lung. The remaining lung was infused with fixative (4% solution of formaldehyde in neutral aqueous phosphate-buffered) under water pressure for 1 h to insure fixation and expanded lung structure. Bronchoalveolar lavage was not performed in order to maintain the fine structure of the lungs for histological analysis. All other tissues were preserved in a neutral aqueous phosphate-buffered 4% formaldehyde. Fine dissection was used to examine pathological changes in the adrenals, brain, heart, kidneys, liver, lung (left lobe at three levels, including main bronchi), nasopharyngeal tissues (at least 4 levels, one level including the nasopharyngeal duct and the nasal associated lymphoid tissue (Woutersen et al., 1994)), spleen, testes and thymus.

### 2.4. Hematology and clinical chemistry

Hematology and cell differentials were conducted in EDTA-anticoagulated blood taken one day after the last exposure via an orbita puncture as described in Gerlofs-Nijland et al. (2010b) with volume adjustments for mice. The following parameters were measured: white and red blood cell concentrations, hemoglobin concentration, platelet concentrations, the mean platelet volume, hematocrit value, mean corpuscular volume, mean cell hemoglobin, mean cell hemoglobin concentration, red blood cell distribution width, mean platelet component and hemoglobin distribution width.

Immediately after sacrifice, plasma was obtained from heparinised blood samples to measure albumin (assesses nutritional status), alkaline phosphatase activity (ALP; increase is associated with hepatobiliary and bone disorders), alanine aminotransferase activity (ALT; liver disease), calcium (parathyroid diseases, a variety of bone diseases, chronic renal failure), glucose (diabetes mellitus, adrenal gland aberrations), inorganic phosphate (PHOS; renal disease), urea (renal disease), aspartate aminotransferase activity (AST; liver, heart, muscle, brain, and kidney tissue damage), gamma glutamyl transferase activity (GGT; liver disease), total protein (TP; dehydration or cancer of blood cells), total bilirubin (TBIL; hemolysis or liver disease), creatinine (kidney function), phospholipids (PLIP; lipid maintenance) and triglycerides (TRIG; lipid maintenance).

### 2.5. Assessment of atherosclerosis

The brachiocephalic artery was fixed in formalin, embedded in wax and histologically sectioned every 100 μm (3 sections/slide, 4 replicates per 100 μm) from the first complete ring until the last, with on average 6 serial sections (range: 2–12 sections) per vessel. Slides were stained with United States Trichrome (UST), a structural stain that highlights the composition of atherosclerotic plaques clearly (Hadoke et al., 1995). Cross-sectional area of the plaque was measured and standardized as a percentage of the area of the vascular media (It was not possible to perfuse fix vessels, therefore, plaques were not expressed as a percentage of the lumen). The mean plaque size of all sections was calculated to provide a single score of atherosclerotic burden throughout the brachiocephalic artery of each animal. Maximum plaque size for a single section of the brachiocephalic was also recorded and used as an additional method to compare plaque size between groups.

The presence of buried fibrous caps is taken as a general marker of plaque complexity either from the ongoing development of a single plaque or the merging of two separate sites of plaque growth (Rosenfeld et al., 2000). However, it has also been suggested that buried fibrous caps represent the growth of a new plaque over a site of a previous plaque rupture (Jackson et al., 2007; Johnson and Jackson, 2001). These possibilities were considered by counting the number of potentially distinct plaques within an artery (either existing separately or adjoining with a clear fibrous divide indicating the merging of two separate plaques)

and the number of buried fibrous caps within each section, whereby a buried fibrous cap was defined as "a length of fibrous/cellular matter that completely bisects a lipid-rich regions of two overlying plaque sections".

A single section from each artery was chosen for analysis of plaque composition, specifically lipid cavities (enclosed regions of plaque, unstained with UST; Johnson et al., 2005) and macrophage content (MAC-2 immunohistochemistry). For macrophage (MAC-2) staining, a rat anti-mouse antibody was used for the primary antibody (1/12000; CL8942AP, VH Bio, Gateshead, UK) with rat IgG (1/12000; I-400, Vector Labs, Peterborough, UK) as a negative control, followed with a goat anti-rat IgG biotinylated secondary antibody (BA-9400, Vector Labs). Slides were then incubated with extravidin-peroxidase solution (1/200), followed by avidin biotin complex (ABC; PK-4001, Vector Laboratories, Peterborough, UK) before detection of MAC-2 with 0.05% 3,3′-diaminobenzidine (DAB; SK-4100; Vector Labs, Peterborough, UK). Nuclei were counterstained with Harris' hematoxylin. Sections were photographed and images were imported into Adobe Photoshop v-11.0, and a color range was selected from three randomly chosen positively stained sections, which was then used to identify positively stained plaque components from all subsequent slides.

### 2.6. Cytokine assessment in the brain

Half of the brain was placed in 4% paraformaldehyde for immunohistochemical analysis. The remaining half was immediately frozen and further subdivided into the cerebellum & brain stem (referred to as "cereb") and other regions (referred to as "brain"). Cytoplasmic fractions were prepared (Lahiri and Ge, 2000) and analyzed for levels of tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) using immunoassay kits (Invitrogen, Carlsbad, California).

### 2.7. Data analysis and statistics

Arithmetic means±standard error of the mean are reported in the text and on figures, whereas ±standard deviation is given in the tables. Body and organ weight, clinical chemistry and hematology data were analyzed using two-way analysis of variance (ANOVA). If variances were not homogeneous or data not normally distributed, the data were stepwise log or rank transformed prior to the ANOVA. Histopathological data was analyzed using Fisher's exact probability test. Cytokines in the CNS and atherosclerotic plaque data were analyzed by one-way ANOVA or unpaired *t*-test as appropriate. $P<0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Exposure characterization

The overall exposure characteristics of both exhaust types are shown in Table 1. The concentrations indicated in Table 1 are those attained after dilution, i.e., the levels that were used to expose the animals. There was no difference in the mass of particulate matter between the two exhaust types, however, there was a 30% reduction in average particle number in exhaust from cerium oxide enriched diesel ($P=0.011$) that equated to an ~10% reduction in particle deposition on the tracheobroncheolar surface of the lung ($P=0.04$). An opposite trend was noted for the gases, which exhibited a slight increase of 6, 9 and 8% for CO, NO and $NO_x$, respectively. The data obtained from this study do not allow clear conclusions to be drawn on the fuel efficiency for both fuel types. Atmospheric conditions during the 4th week (outdoor tempartures fell to ~0 °C where the engine was situated) resulted in different engine operating conditions and different exhaust composition (24% larger median particle size, 33% lower particle numbers, 23% reduced particle surface area and 35–55% lower $CO/NO_x$ concentration), however, these changes were observed in both the DE and DCeE groups (data not shown).

Prior to adding to the fuel, the most abundant particle size in the ENVIROX cerium oxide additive was 15.6±0.2 nm. A few large particles with sizes ~200 nm were seen infrequently. The particle size (mass mean diameter) of diesel exhaust particles were similar for DE and DCeE groups (Table 1). TEM analysis of loaded filters from DCeE exposures identified numerous types of particles consisting of a wide range of elements (K, Na, Cl, Mg, Si, Al, O, $FeO_x$, $TiO_x$, $AlO_x$, Ni, Cr, Fe, Ca, S, Ba, Sn). Small nanoparticles were observed within the clusters of soot particles that yielded EDX spectra with C and Ce peaks (Fig. 1). Filters from DCeE exposures contained 5.5±4.0 μg Ce/mg loading of soot in comparison to 0.2±0.1 μg Ce/mg of soot from DE exposures. Unexposed collection filters contained insignificant levels of Ce (<10 ng abs; $n$=3).

### 3.2. Assessment of biological effects: Sex differences, recovery groups and dose effects

With the exception of body and organ weight, biological responses, were similar in male and female mice. Therefore, for most parameters, the data is pooled to attain an $n$=10. Biological responses to the two exposure types were also similar in mice sacrificied 17 day after the last exposure (data not shown), suggesting that there were no delayed response to the exposure within this time frame. In general there was no clear dose-response in mice receiving either 20, 60 or 180 min of exposure. Therefore, unless otherwise stated, only the responses to 180 min exposure are reported in key experiments.

### 3.3. Body and organ weight and cerium content

The average organ (thymus, heart, left lung, brain, liver, kidney, adrenal gland, spleen, testis) and body weights of male or female mice was not affected in a dose-dependent manner by either exposure (data not shown). The cerium and cerium oxide levels in lung tissue were 1.06±0.07 for males and 1.30±0.09 μg/g for females in DCeE exposed mice, whereas the levels in the DE group were below the detection limit (<0.02 μg/g tissue).

### 3.4. Pathological observations

No treatment-related macroscopic abnormalities were observed at necropsy (Table 2). Microscopic examination of the lungs revealed greater number of macrophages laden with dark-brown pigment in the alveolar and bronchiolar lumina (and occasionally in the interstitium) of DE-exposed mice. There was a similar frequency of these observations in animals exposed to the highest concentration of DCeE. Cells with a golden-brown pigment were very occasionally observed in the spleen and the brain, especially the meninges (Table 2), although, incidences were similar in mice receiving filtered air, suggesting this is an endogenous pigment e.g., melanin. There was no evidence of restructuring of the pulmonary tissue (e.g., fibrotic thickening of the airways and cuffing of alveolar blood vessels) in response to either exposure.

### 3.5. Hematology & clinical chemistry

A 4-week exposure to DE had little effect on blood cell differentials, hemoglobin concentration, hematocrit, platelets and mean corpuscular volume (Table 3) or associated hematological indices (see Methods, data not shown). There were no statistically significant differences between the DE and DCeE groups for any of the hematological indices studied.

There were small, but significant, differences in several of the clinical chemistry parameters between the exposure types (Table 4). Albumin, ALP & TP levels were slightly greater in the DE group than the DCeE group at all doses, whereas ALT levels were lower in the DE group than the DCeE group. Blood calcium levels also showed significant differences between the DE and DCeE groups, although a dose-response relationship was not evident.

### 3.6. Atherosclerosis

Overall, there was a trend that "standard" diesel exhaust increased the atherosclerotic burden, with an ~35% increase in plaque size compared to control (Fig. 2(a)). This effect was not seen in the DCeE group. However, these differences did not achieve statistical significance ($P$=0.12 for Control vs DE; $P$=0.10 for Control vs DCeE). No dose related effect was seen and similar findings were observed if the data on the maximum plaque size (rather than burden throughout the artery) was used (data not shown).

Histological analysis of plaques showed lesions rich in foam cells, cholesterol crystals and regions that were dense in fibro-elastic matrix and smooth muscle cells. All sections were assessed for the number of individual plaques within a single section (Fig. 2(b)(i)) and, where this was observed, as two overlying plaques separated by a buried fibrous layer (Fig. 2(b)(ii)). The number of plaques in a single section varied from one to five, with a mean of 1.8±0.1 adjoining plaques and 0.6±0.1 buried fibrous layers in air-treated ApoE$^{-/-}$ mice. There was a significantly greater number of adjoining plaques (2.3±0.4 plaques, $P$=0.049) and buried fibrous layers (1.2±0.4 buried fibrous layers, $P$=0.019) in mice exposed to high DE, but not in DCeE-treated mice (Fig. 2(c)). Exposure to DE or DCeE (high dose only) had no significant effect on either lipid or macrophage/foam cell content of plaques (Fig. 3).

### 3.7. Proinflammatory cytokines in the CNS, spleen and liver

When compared to exposure to DE, there was an increase in the levels of the proinflammatory cytokine, TNF-α in the cerebellum fraction of the brain after exposure to DCeE ($P$=0.05; Fig. 4). Levels of IL-1β were also increased after exposure to DCeE, although, this did not reach statistical significance ($P$=0.10). There was no change in the levels of these proinflammatory cytokines in the spleen, however, in liver tissue a decrease in the levels of both proinflammatory cytokines was observed after DCeE exposure compared to DE exposure ($P$=0.04; Fig. 4).

## 4. Discussion

### 4.1. Exposure characteristics

The use of $CeO_2$ as a diesel fuel additive changed the physicochemical composition of the exhaust, reducing the total number of particles and, to a lesser extent, the total particle surface area predicted to be deposited in the tracheobronchial region. The decrease in particle numbers was accompanied by an increase in $NO_x$ and CO levels, however, these alterations were small and non-significant. In the present study we maintained a fixed mass concentration for the DE exposure. Switching to cerium-spiked fuel may have a more dramatic reduction in particle numbers if the running conditions of the engine are kept constant rather than the mass of PM in the exhaust. Similarly, it is unlikely that levels of gaseous co-pollutants will increase when simply switching to cerium enriched fuel. Improved combustion may result in higher $CO_2$ levels, however, this was not measured in the present study and may require attention from an environmental perspective. Sajith et al. (2010) reported that the addition of 80 ppm $CeO_2$ (~9 fold higher than the concentration of cerium used in the present study) resulted in a doubling of the CO production, and marked reductions in the levels of $NO_x$. In the present study we used an engine under a fixed load, whereas it is notable that Sajith et al. initially used an idling engine and when higher engine loads were used the changes in gases became less evident. Thus, different engines, engine load and fuel composition will influence the exhaust characteristics, and therefore, the potential biological consequences caused by exposure to these emissions.

Interestingly, the particle size distribution in the diesel engine exhaust was unchanged by addition of cerium to the fuel, yet despite particle mass remaining constant, the overall surface area of the particulate was reduced. These characteristics suggest that the net effect of using $CeO_2$ is the formation of more condensed particle agglomerates in the exhaust. Cerium itself could be detected in the exhaust as 1–3 nm

(geometric diameter) spherical $CeO_2$ nanoparticles captured within or on the surface of agglomerates of diesel exhaust soot. Therefore, the toxicity of $CeO_2$ will depend on the complex formed in exhaust (e.g., the availability of the cerium on the outer surface of the diesel exhaust agglomerates) and this may differ from $CeO_2$ aerosolized from a dry powder, which has formed the basis of risk-assessment studies in the past (Park et al., 2008; Cassee et al., 2005), or applied via intratracheal instillation into the lung (Ma et al., 2011).

### 4.2. General toxicology

Exposure to nano-sized $CeO_2$ at the current levels in ambient air have been suggested to be unlikely to lead to pulmonary oxidative stress and inflammation (Park et al., 2008; Ma et al., 2011). These events are considered potential precursors for respiratory and cardiac health problems. However, these predictions have been made from the results of in vitro assays and cell culture experiments. Our study is conducted in an in vivo disease model using a biologically relevant route of exposure with realistic exposure atmosphere (albeit higher than can be expected to occur in urban environments) to accurately assess the toxicity of airborne cerium and the effect of cerium on the properties of engine exhausts. The overall evaluation of the effects of a 4-week diluted engine exhaust exposure reassuringly revealed very little adverse biological changes for either DE or DCeE, and consequently, no apparent dose-response relationships. While there were significant differences between the DE and DCeE groups for a select number of parameters, the changes observed were small and did not appear to be related to the dose of exhaust. Additionally, these changes might be transient, since 17 day after the final exposure, the effects of the highest dose of exhaust were no longer detected. Furthermore, the clinical consequences of these modest effects on individual parameters may be limited, since the changes are mostly within the range of reference values (Fox et al., 2007) and in some cases in the opposite direction than that associated with various diseases.

The modest effects of both exhaust types is surprising since previous studies in our own lab (Van Berlo et al., 2010; Gerlofs-Nijland et al., 2010a), as well as by others (Kobayashi, 2000; Sunil et al., 2009), revealed that similar exposures to DE usually results in detectable adverse effects (e.g., hyperresponsiveness and inflammation). Broncheoalveolar fluid was not collected in order to maintain the structural integrity of the lungs for histological analysis. Subsequently, we were unable to accurately quantify levels of lung inflammation induced by DE exposure. Neverthless, histological analysis indicated that DE exposure induced macrophage infiltration into the alveolar and bronchiolar lumina, and the interstitium of the lungs, verifiying that the exposure levels used were sufficient to induce an inflammatory response in the lungs. Furthermore, numerous macrophages contained a dark brown pigment, suggesting that they were laden with diesel exhaust particles, confirming that particles themselves penetrated deep into the alveoli. Previous studies using prolonged exposures to DE focused on either animal models of allergy (Ichinose et al., 2004; Miyabara et al., 1998; Farraj et al., 2010) or bacterial infections (Hiramatsu et al., 2005). In these studies 4 or 8 week exposures to DE with similar or higher PM levels (2–3 $mg/m^3$) had little effect on pulmonary cytokines, chemokines, pathology of the airways, or acute murine mycobacterial infection. Some effects only became evident after 2 months (Hiramatsu et al. 2005), suggesting mice are capable of coping with relatively long periods of exposure. Sunil et al. (2009) suggest that young mice can adapt to DE-induced pulmonary changes, perhaps due to the relatively high pulmonary clearance rates of particles in rodents (Farraj et al., 2010) and this may apply to our study presented here. Finally, another possible explanation for the limited effects of DE in the present study is that a newer engine has been used under a load, whereas previous studies used older engines running under idling conditions. The former results in relatively low particle emissions compared to the gaseous fraction and substantially lower levels of organic carbon (including harmful polyaromatic hydrocarbons) in the

particulate (Maricq, 2007).

While the current study demonstrates that the hazard of the emission from newer engines and fuels may be lower than that reported in studies using older technologies or idling engines, several specific biological parameters were influenced by diesel exhaust (discussed below), highlighting that the exhaust emissions may still have relevant health effects.

### 4.3. Atherosclerotic effects of exposures

There is a large body of evidence showing that levels of particulates within the air are associated with an increase in the size and development of atherosclerotic plaques (Araujo and Nel, 2009; Sun et al., 2010). The proatherosclerotic effect of diesel exhaust, specifically, has not been studied in man. However, a limited number of studies in $ApoE^{-/-}$ mice have suggested that diesel exhaust promotes the growth of atherosclerotic plaques (Campen et al., 2010; Bai et al., 2011). In this study, we found a trend towards an increase in plaque size in animals exposed to DE that was not apparent in DCeE-exposed animals. This trend adds further weight to the findings suggesting that DE exposure may accelerate proatherosclerotic formation. Furthermore, our results also suggest that the particulate fraction of DE, rather than the gaseous component, may mediate the proatherosclerotic effect of DE. This is because exposure to DCeE, which contained a greater proportion of the gases believed to mediate the harmful actions of combustion-derived emissions (e.g., CO, $NO_x$) did not potentiate proatherosclerotic changes.

We reiterate again that the effects seen fell just short of statistical significance. The size and composition of plaques varied considerably between animals, perhaps not surprisingly due to the multi-faceted nature of this disease process. Therefore, in hindsight, greater numbers of animals may have been needed to account for this variability and obtain statistical significance. In this regard, we feel at present it would be unwise to speculate on these findings. However, should the proatherosclerotic effects of DE be a true phenomena, it would be interesting to establish a mechanism for the effects in the absence of a marked inflammatory response. Several biological messengers that were not measured in this study may underlie the process. These include tissue factor (Sun et al., 2008), upregulation of adhesion molecules (Yatera et al., 2008), matrix metalloproteinases (Campen et al., 2010), NAD(*P*)H oxidase (Ying et al., 2009), or altered function of high-density lipoprotein (Araujo et al., 2008). In the current study, after exposure, levels of IL-1β and TNF-α were modulated in the liver raising the possibility that organ-specific changes in certain proinflammatory cytokines may mediate the effects of diesel exhaust. Whether similar changes occur in vascular tissue, and to what extent this influences atherosclerosis, is currently under investigation.

Plaque growth alone does not account for the pathology of atherosclerosis in man and plaque size needs to be considered in parallel to plaque vulnerability to rupture that underlies the clinical consequences of atherothrombosis. Plaques that are rich in lipids and inflammatory cells are believed to be more susceptible to rupture. Neither DE or DCeE exposure altered the percentage of plaque composed of lipids or macrophages/foam cells. The brachiocephalic artery was chosen for an in-depth analysis of plaque development as complex plaques with fibrous layers form at this site and it has been suggested that this represents a site of a previous plaque rupture (Jackson et al., 2007). There was a significant increase in the occurrence of buried fibrous layers in plaques from DE-treated mice, that was not seen in the DCeE-exposed group. The ongoing debate (Jackson et al., 2007; Jackson, 2007; Rosenfeld et al., 2008) as to whether buried fibrous layers represent the formation of a new plaque over a site of plaque rupture, or is merely a feature of the ongoing development of a single plaque cannot currently be answered with this model. Nevertheless, the trend towards a greater size of plaque with a more complicated structural phenotype, suggests that diesel exhaust inhalation promotes atherosclerotic burden, and that these effects may be prevented by the use of cerium-spiked fuels.

### 4.4. CNS effects of exposure

The potential adverse effects of inhaled nanoparticles are not limited to the cardiopulmonary system, but may also affect the CNS. Enhanced neuroinflammation and an altered blood-brain barrier (BBB) has been observed in children and young adults living in cities with high air pollution (Calderón-Garcidueñas et al., 2008). TNF-α and IL-1 are the main proinflammatory cytokines in the brain and chronic neuroinflammation can amplify the generation of these factors. Neuroinflammatory events are associated with the pathology of a number of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Tilleux and Hermans, 2007). Exposure to PM may potentiate the pathogenesis of these disorders by enhancing neuroinflammation (Campbell, 2004). We have previously demonstrated that subacute exposure to DE is associated with increased levels of these cytokines in the striatum of rats (Gerlofs-Nijland et al., 2010a) and in $ApoE^{-/-}$ mice exposed to PM derived from Los Angeles freeways (Campbell et al., 2009). In the present study, standard DE exposure was not accompanied by changes in these cytokines, most likely due to the reasons discussed above. Interestingly, in the DCeE exposed group the levels of TNF were increased in the cerebellum and brain stem when compared to the DE-exposed samples. The absence of Apolipoprotein E compromises the integrity of the BBB (Methia et al., 2001), perhaps allowing $CeO_2$ particles to access the brain parenchyma and induce a local inflammatory response. In normal rats, $CeO_2$ nanoparticles do not bypass the BBB although they have the capacity to alter the levels of the antioxidant enzyme catalase in the hippocampus and cerebellum (Hardas et al., 2010). Therefore, it appears that the direct entry of $CeO_2$ into the brain paranchyma is not necessary for modulation of CNS responses. To what extent the elevation in the cytokines may predispose the brain to adverse effects requires further investigation.

### 4.5. Limitations

The present study design (e.g., use of engine under load, newer engine, moderate exposure dose) was used to take into account OECD guideleines as well as reflect real-world scenarios to some extent, rather than use conditions where we were assured to see clear toxicological effects. Consequently, standard DE had limited detrimental actions and, unfortunately, it is difficult to fully assess the magnitude or preventative effects of the DCeE. At present there are no rodent studies that demonstrate adverse effects of exhaust from $CeO_2$-spiked diesel fuel. This most likely reflects the limited data available on the biological actions of this fuel additive rather than an inherent problem with rodent models. Nevertheless, it is reassuring that, with the exception of the change in proinflammatory cytokines in the cerebellum and brain stem region of the CNS, subacute exposure to DCeE, at $CeO_2$ levels above that likely to be reached following dilution in ambient air, did not result in other harmful effects. Direct extrapolation of our results into human risk are challenging and will need to consider the biopersistance of $CeO_2$ in the body and in the environment. Nevertheless, the limited systemic effects of cerium-spiked diesel exhaust in mice, suggests that controlled studies evaluating the effects of environmentally-relevant levels of this exhaust should be feasible in human subjects.

## 5. Summary & conclusion

In summary, very few detrimental effects were observed following 4 week DCeE exposure providing data for establishing a lowest as well as no observed adverse effect level. Changes in the physicochemical composition of diesel engine exhaust when using the additive cerium oxide may limit the proatherosclerotic effects associated with regular diesel fuel. Further toxicological studies on the use of cerium containing fuel additives are required, especially in regards to the possibility of potential adverse effects in the CNS. However, the general lack of toxicological consequences in this initial study are promising and offer some hope that addition of cerium to diesel fuel may limit the cardiorespiratory effects

of combustion-derived nanoparticles.

## Declaration of interest

The authors declare that they have no competing interests.

## Author contributions

FRC conceived of the study; its design and coordination, as well as drafted and coordinated the manuscript.

AC performed the cytokine analysis and statistical analysis of CNS samples, and helped to draft the manuscript.

AJFB assisted in the design and implementation of the animal exposures, necropsy and pathology, performed the characterization of exposures and contributed to the writing of the manuscript.

SGM assisted with the collection of vascular tissue and performed the histological measurements of atherosclerosis.

RD assisted with the collection and fixation of pulmonary and vascular tissues.

PK performed the analysis of the cerium additive and diesel exhaust particles, the quantification of cerium by ICPMS, and contributed to the writing of the manuscript.

IG wrote the study protocol, arranged ethical approval and contributed to the writing of the manuscript.

MRM carried out the atherosclerosis studies and analysis, participated in the study design, and helped to draft and coordinate the manuscript. All authors have read and approved the final manuscript.

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## Footnotes

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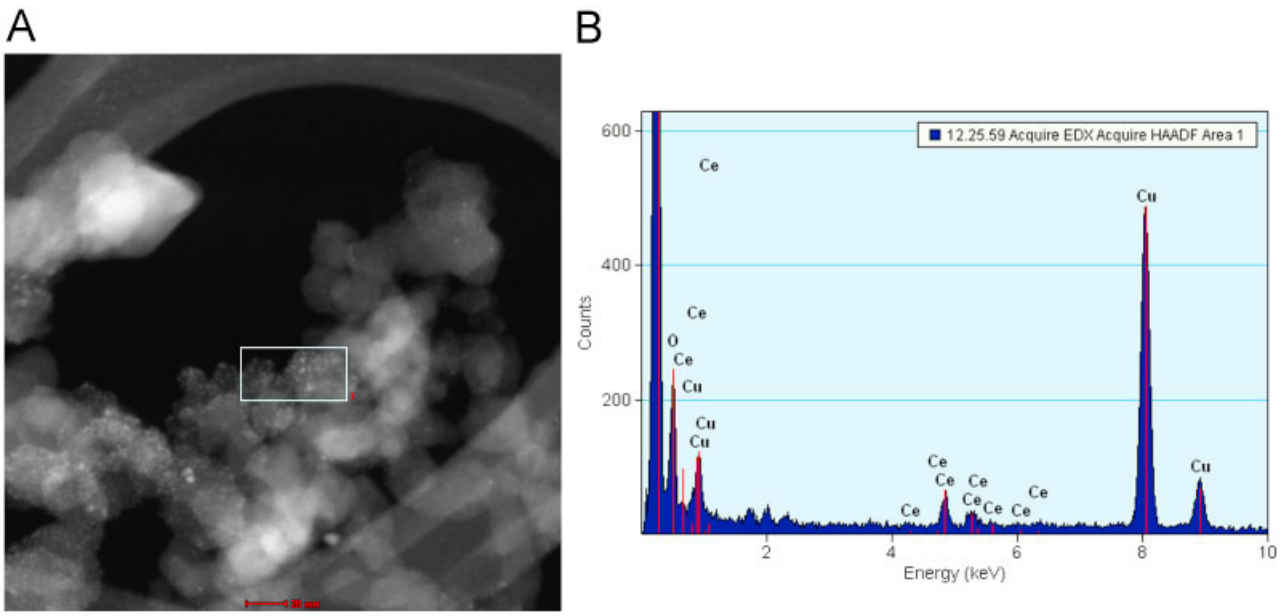
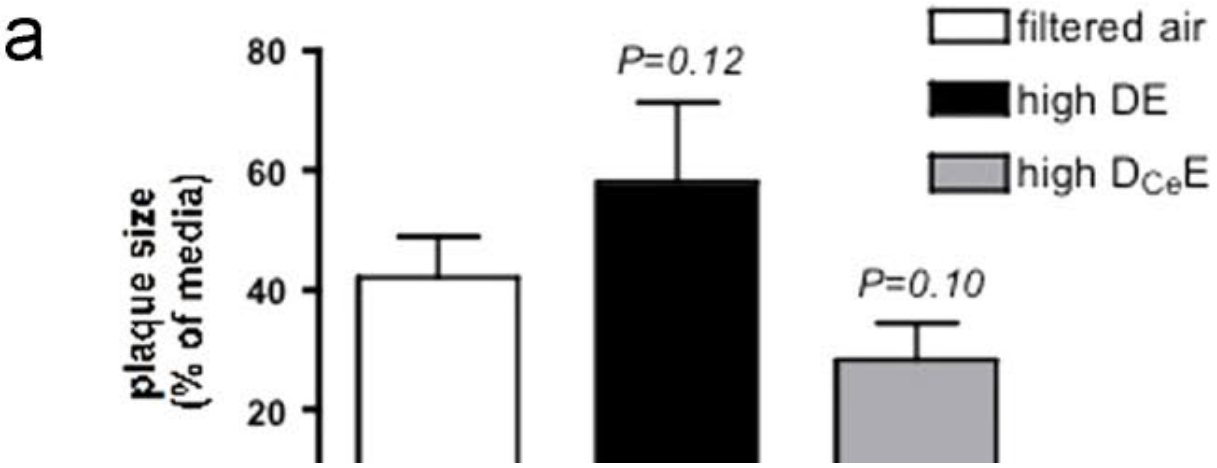
## Figures and Tables

### Fig. 1




Detection of cerium in the soot from the exhaust of an engine run on cerium-spiked diesel. (A) Grey areas are diesel soot. The small white dots are Ce-containing particles. Image obtained by TEM/High Angle Annular Dark Field (HAADF). Typical geometric size of nanoparticles in the scanned area: 4 ± 1 nm. The rectangle represents the area scanned during EDX spectrum acquisition (see (B)). (B) Qualitative identification of Ce and Cu by EDX-spectrum of the marked area.

### Fig. 2

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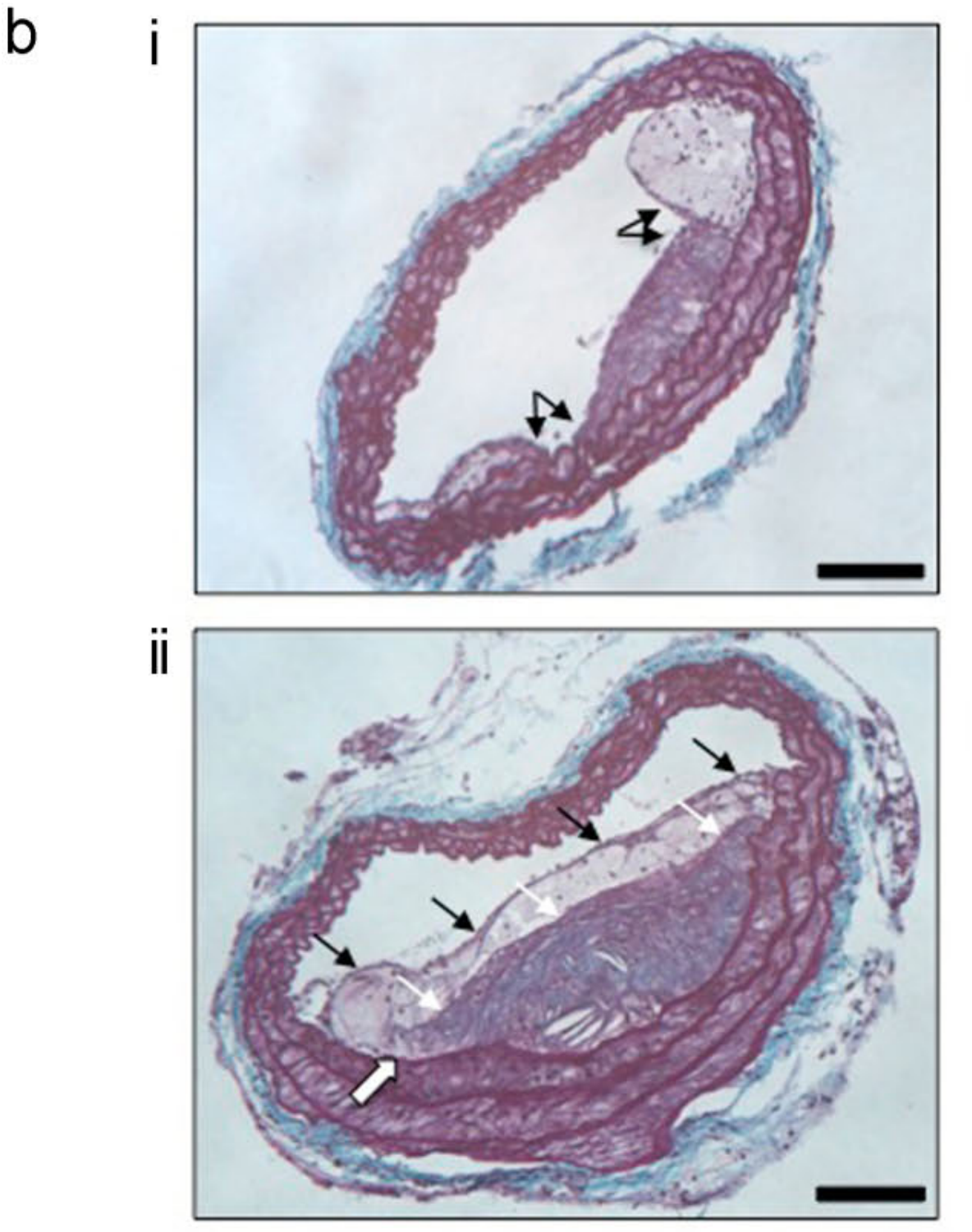
control
high DE
high $D_{Ce}E$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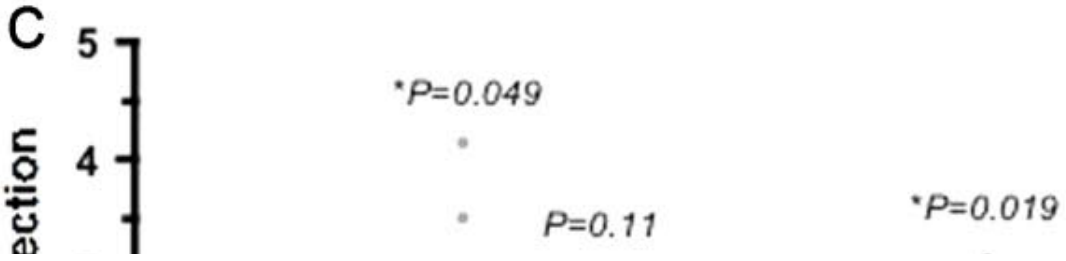
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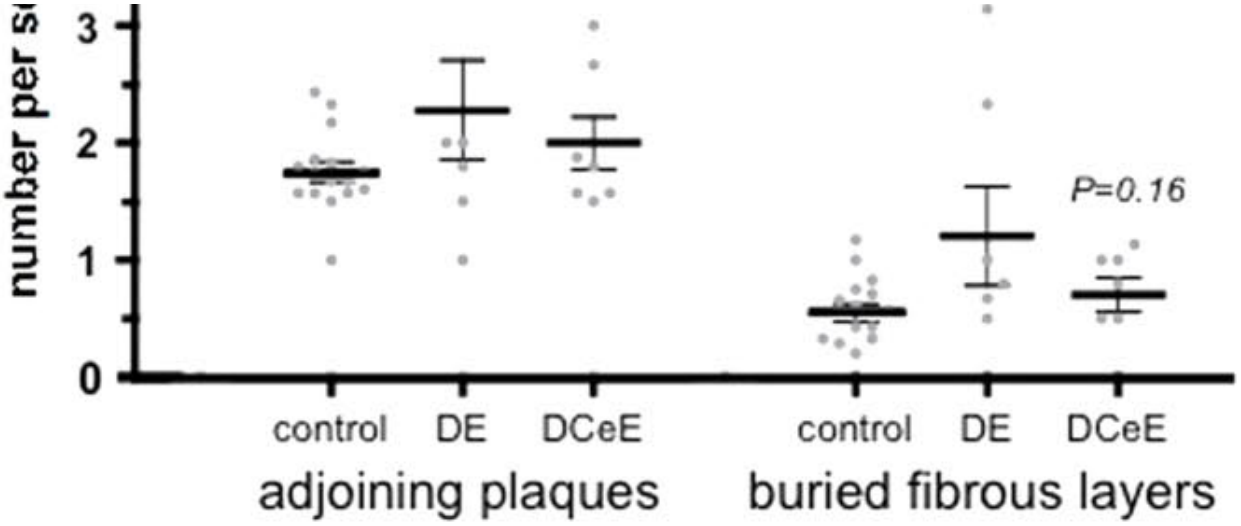
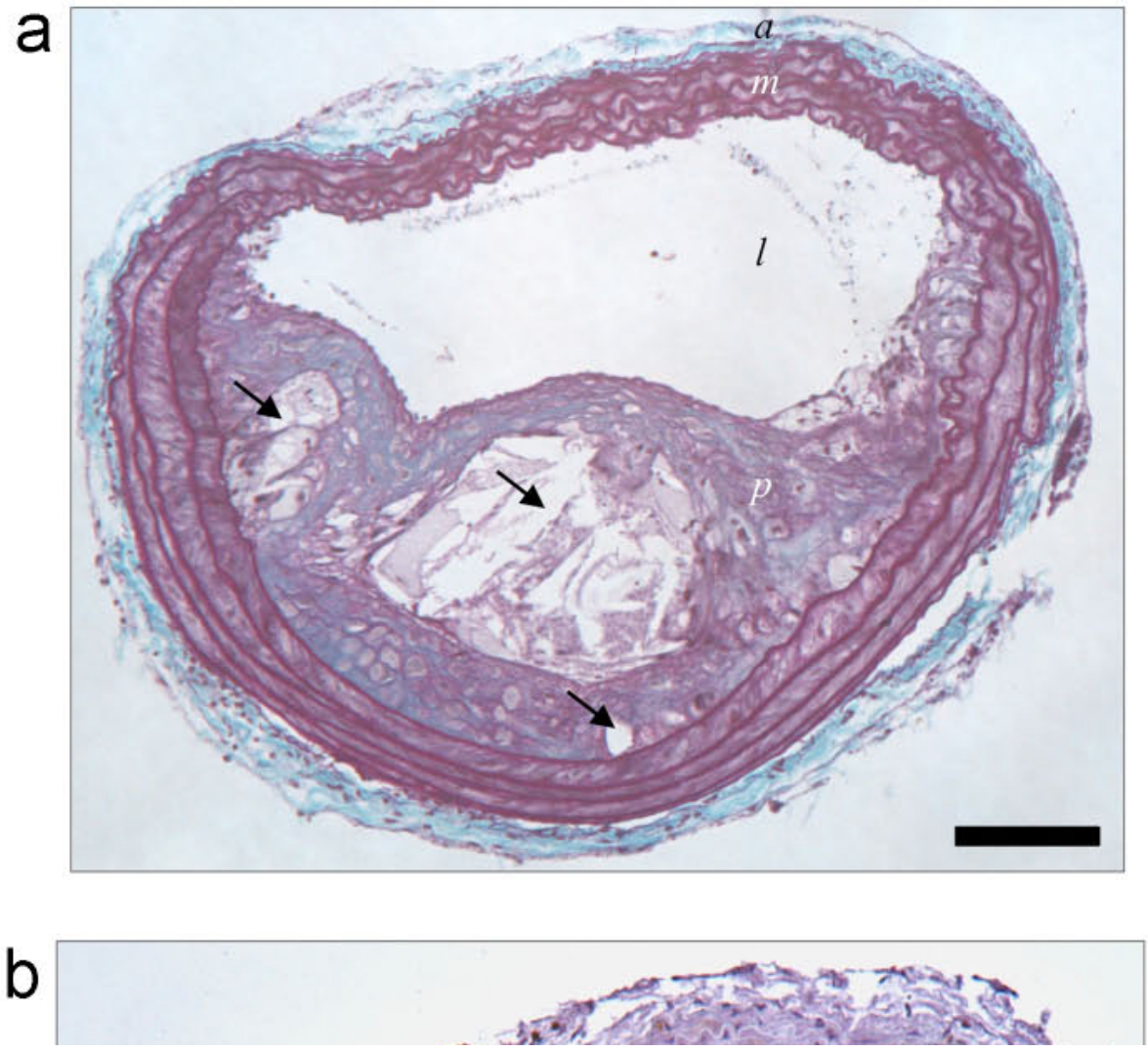
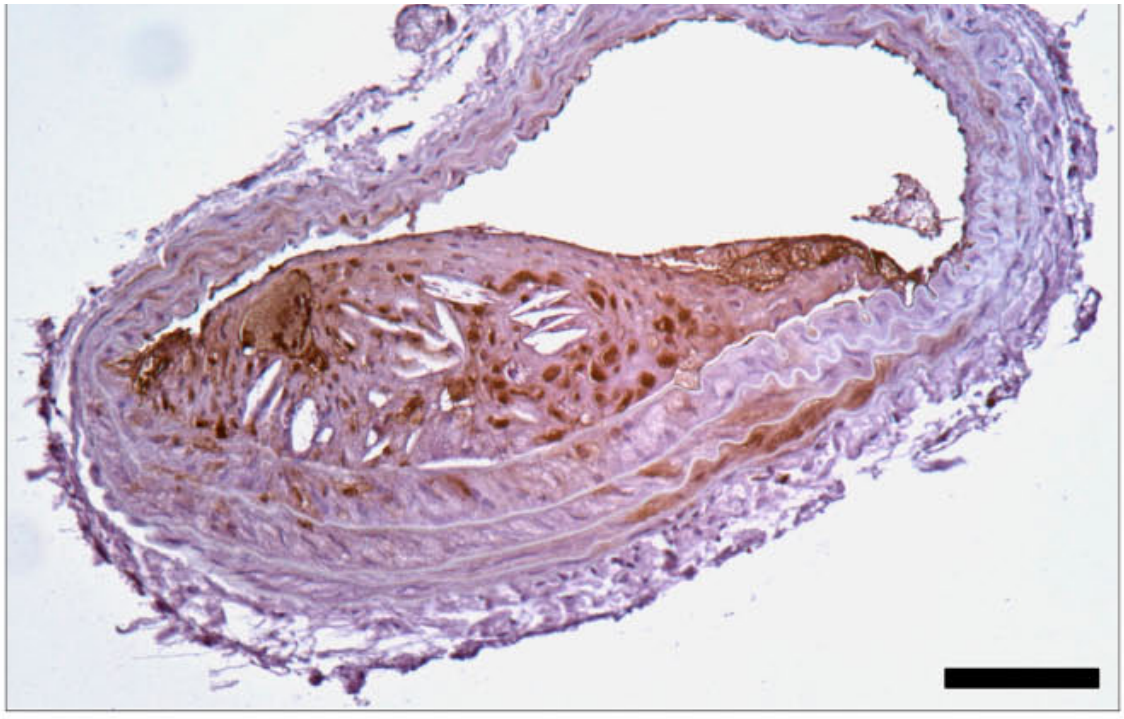
P=0.11
*P=0.019

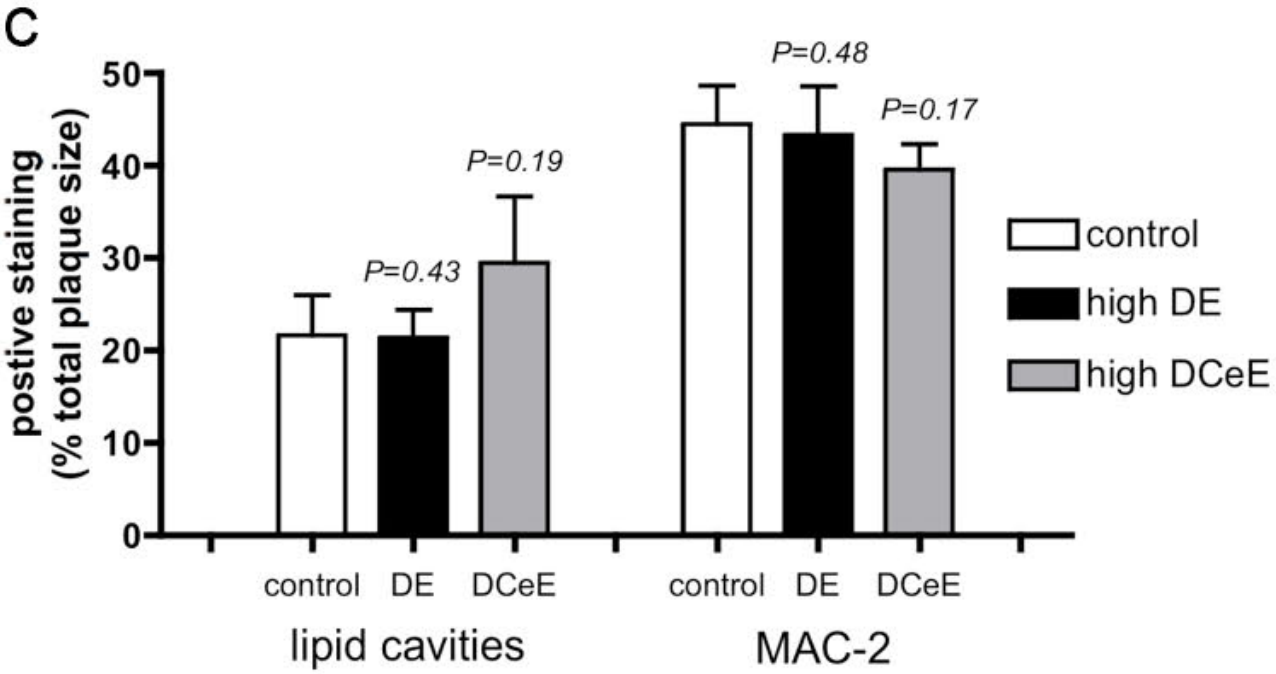



Effect of diesel exhaust, with (DCeE) or without (DE) cerium oxide, on atherosclerotic plaques in the brachiocephalic artery. (a) Plaque size, standardized as a percentage of the area of the media. (b) Example images showing **(i)** three "adjoining plaques" (black arrows) and **(ii)** a "buried fibrous layer" (thin white arrows) with an overlying secondary plaque (thin black arrows). Thick white arrow (with black outline) indicates a potential site of a previously healed plaque rupture. United States Trichrome staining. Black scale bar=100 μm. (c) Plaque "complexity" as assessed by the frequency of adjoining plaques or buried fibrous layers. Mean±S.E.M. ($n$=7−16), $^{*}P<0.05$; unpaired $t$-tests, comparing with control.

**Fig. 3**

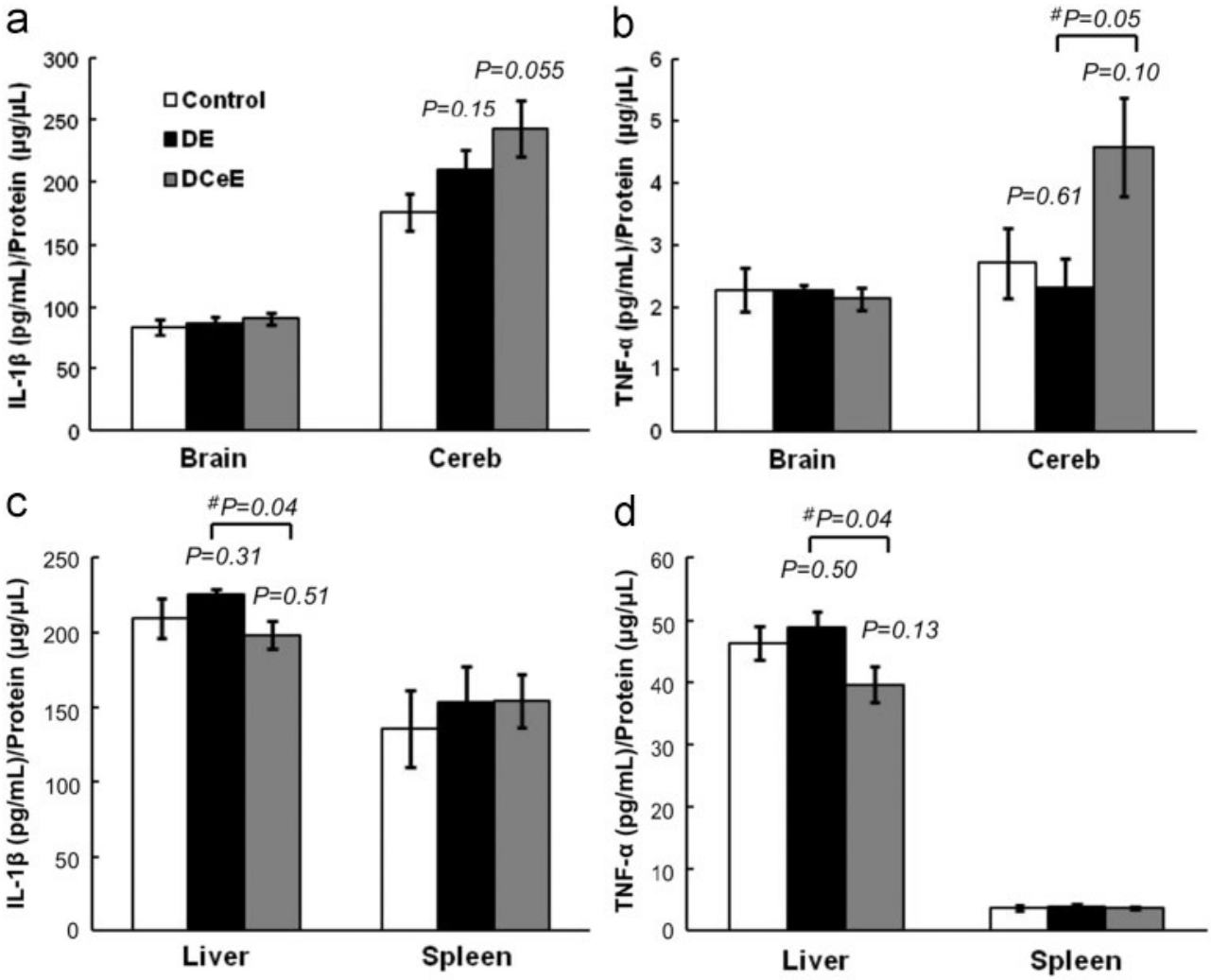







Effect of diesel exhaust with (DCeE) or without (DE) cerium oxide on lipid and macrophage content of atherosclerotic plaques in the brachiocephalic artery of ApoE$^{-/-}$ mice. (a) Lipid cavities (arrows) in plaques stained with United States Trichrome. *a*=adventitia, *m*=media, *l*=lumen, *p*=plaque. (b) Macrophage (brown color) in plaques using MAC-2 immunostaining. Black scale bars on images=100 μm. (c) Exposures did not significantly affect size of lipid cavities or MAC-2-positive content of plaques (% total plaque area). Mean±S.E.M. ($n$=8−11 for lipids, 6−11 for MAC-2), $P$-values shown compare treatment with control groups using unpaired Student's $t$-tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 4**




Effect of diesel exhausts on cytokines in the CNS, liver and spleen. Data from high doses of exhaust from standard diesel (DE; black columns) or diesel containing cerium oxide (DCeE; gray columns) on IL-1β (a),(c) and TNF-α (b),(d) levels in the brain and cerebellum (a),(b) or in the liver and spleen (c,d) of $ApoE^{-/-}$ mice. Control (white columns) is filtered air exposure. Mean±S.E.M. ($n$=7−16). $P$-values shown are unpaired $t$-tests, comparing exposure groups with control values, except when indicated by (No.) where the comparison is between DE and DCeE.

### Table 1

Summary of exposure characteristics.

| | Metric | DE | DCeE |
|---|---|---|---|
| **Particles** | | | |
| Mass (time integrated) | $\mu g/m^3$ | 1741±153 | 1740±162 |
| Mass (continuous) | $\mu g/m^3$ | 1925±79 | 1893±136 |
| Surface area | $\mu m^2/cm^3$ per TB region | 4018±605 | 3636±517* |
| Number counts | No./$cm^3$ ($\times 10^6$) | 5.3±0.1 | 3.6±0.5* |
| Mass median diameter | Nm | 82.0±1.8 | 83.0±1.8 |
| Cerium oxide content | μg Ce/mg soot | 0.2±0.1 | 5.4±4.0* |

| | | | |
|---|---|---|---|
| **Gases** | | | |
| CO | ppm | 10.0±2.1 | 10.6±2.0 |
| NO | ppm | 33.3±10.5 | 36.1±11.0 |
| $NO_x$ | ppm | 35.3±10.8 | 38.2±11.3 |

Abbreviations: DE=diesel exhaust; DCeE=diesel exhaust from fuel spiked with cerium; TB=tracheobroncheolar; CO=carbon monoxide; NO=nitric oxide; $NO_x$=nitrogen oxides; ppm=parts per million. Values are expressed as mean±standard deviation ($n$>3).

*$p$<0.05.

## Table 2

Histopathological observation in $ApoE^{-/-}$ mice after a 4-week exposure.

| | DE | | DCeE | |
|---|---|---|---|---|
| | **Control** | **High** | **Control** | **High** |
| **Total number of animals per group** | **10** | **10** | **10** | **10** |
| **Brain** | | | | |
| Meningeal pigmented cells | 2 | 4 | 2 | 2 |
| **Heart** | | | | |
| Adhesion to thoracic cavity | 0 | 0 | 0 | 0 |
| Foam-cell inflammation | 2 | 0 | 1 | 3 |
| Focal cartilaginous metaplasia | 1 | 0 | 0 | 0 |
| **Kidney** | | | | |
| Basophilic tubules | 0 | 1 | 0 | 0 |
| Hydronephrosis | 0 | 0 | 0 | 0 |
| Mononuclear cell infiltrate | 1 | 1 | 2 | 0 |
| Unilateral pelvic dilatation | 0 | 0 | 1 | 0 |
| **Liver** | | | | |
| Increased hepatocellular vacuolation | 4 | 3 | 3 | 2 |
| Focal hyperemia | 1 | 0 | 0 | 0 |
| **Lungs** | | | | |
| Pigmented macrophages | **1** | **9** | **0** | **10** |
| **Nasal cavity** | | | | |

| | | | | |
|---|---|---|---|---|
| Dermatitis | 0 | 0 | 3 | 3 |
| Mononuclear cell infiltration | 0 | 0 | 1 | 0 |
| **Spleen** | | | | |
| Enlarged | 0 | 0 | 0 | 0 |
| Extramedullary haematopoiesis | 1 | 1 | 1 | 1 |
| Pigmented cells in red pulp | 4 | 3 | 4 | 3 |
| **Adrenals** | | | | |
| Focal zone glomerosa spindle cell hyperplasia | 2 | 4 | 3 | 4 |
| Increased apoptosis in zona reticularis | 4 | 4 | 5 | 4 |
| **Thymus** | | | | |
| Cyst | 0 | 0 | 0 | 0 |
| Cortical decreased cellularity | 0 | 0 | 1 | 0 |
| Increased ductular structure | 0 | 1 | 0 | 0 |
| Increased starry sky appearance | 1 | 1 | 0 | 0 |
| Focal capsular inflammation | 1 | 0 | 0 | 0 |
| **Testis** | (*n*=5) | (*n*=5) | (*n*=5) | (*n*=5) |
| Tubular atrophy | 0 | 1 | 2 | 1 |

Values indicate the number of animals in which the effects were observed. Low and mid dose groups were only examined if abnormalities were seen macroscopically.

Abbreviations: DE=diesel exhaust; DCeE=diesel exhaust from fuel spiked with cerium.

**Table 3**

Hematological analysis of blood from mice following exposure to diesel engine exhaust with (DCeE) or without (DE) cerium oxide.

| | RBC ($\times10^{12}$/L) | WBC ($\times10^{9}$/L) | Hemoglobin (mmol/L) | Hematocrit value (L/L) | Platelets ($\times10^{9}$/L) | MCV (fL) |
|---|---|---|---|---|---|---|
| **DE** | | | | | | |
| Control | 10.5±0.4 | 4.6±1.3 | 8.9±0.5 | 0.48±0.02 | 1111±387 | 45.8±1.2 |
| Low | 10.3±0.3 | 4.9±1.5 | 8.8±0.3 | 0.48±0.02 | 1161±375 | 46.6±1.0 |
| Mid | 10.4±0.4 | 4.6±1.4 | 8.9±0.4 | 0.48±0.02 | 1054±299 | 45.8±0.9 |
| High | 10.6±0.4 | 4.6±1.5 | 9.0±0.3 | 0.48±0.02 | 1027±209 | 45.5±1.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **DCeE** | | | | | | |
| Control | 10.4±0.3 | 5.7±3.4 | 8.8±0.2 | 0.49±0.01 | 1107±581 | 46.8±1.0 |
| Low | 10.2±0.3 | 4.2±2.0 | 8.8±0.3 | 0.48±0.02 | 1351±724 | 47.3±0.6 |
| Mid | 10.0±0.7 | 4.6±1.5 | 8.7±0.5 | 0.47±0.03 | 1164±315 | 46.9±0.8 |
| High | 10.0±0.8 | 5.3±2.4 | 8.6±0.4 | 0.48±0.02 | 1418±803 | 47.7±2.2 |

Values are presented as means±SD ($n$=10). No significant differences were found between DE and DCeE groups (two-way ANOVA).

Abbreviations: DE=diesel exhaust; DCeE=diesel exhaust from fuel spiked with cerium; RBC=red blood cells; WBC=white blood cells; MCV=mean corpuscular volume.

### Table 4

Clinical chemistry of blood from mice following exposure to diesel engine exhaust with (DCeE) or without (DE) cerium oxide.

| | **Albumin (g/L)** | **ALP (U/L)** | **ALT (U/L)** | **Calcium (mmol/L)** | **Glucose (mmol/L)** | **PHOS (mmol/L)** | **Urea (mmol/L)** |
|---|---|---|---|---|---|---|---|
| **DE** | | | | | | | |
| Control | 28.7±3.4 | 122±27 | 30.2±7.3 | 2.28±0.08 | 12.2±2.4 | 2.27±0.44 | 10.59±2.72 |
| Low | 29.2±4.1 | 117±21 | 30.4±3.0 | 2.37±0.07 | 12.3±2.9 | 2.74±0.20 | 9.70±1.53 |
| Mid | 29.8±2.2 | 130±16 | 33.4±7.1 | 2.33±0.08 | 11.7±3.3 | 2.45±0.30 | 8.80±1.12 |
| High | 32.4±1.6 | 137±40 | 22.5±12.7 | 2.44±0.05 | 11.1±2.3 | 2.53±0.25 | 8.56±0.79 |
| **DCeE** | | | | | | | |
| Control | 30.2±2.4 | 126±26 | 29.4±9.2 | 2.33±0.08 | 12.4±2.5 | 2.40± 0.27 | 9.30±0.87 |
| Low | 29.0±2.7 | 116±21 | 41.7±16.1 | 2.12±0.52 | 11.2±1.7 | 2.26±0.29 | 10.00±2.14 |
| Mid | 29.1±3.3 | 111±20 | 35.8±4.7 | 2.34±0.10 | 12.4±1.8 | 2.52±0.50 | 10.27±1.89 |
| High | 29.6±2.6* | 114±18* | 49.6±24.9* | 2.32±0.06* | 12.6±2.2 | 2.59±0.43 | 10.10±2.13 |

| | AST (U/L) | GGT (U/L) | TP (g/L) | TBIL (μmol/L) | Creatinine (μmol/L) | PLIP (mmol/L) | TRIG (mmol/L) |
|---|---|---|---|---|---|---|---|
| **DE** | | | | | | | |
| Control | 120±43 | 5.50±3.60 | 52.4±3.2 | 3.46±1.93 | 9.1±3.9 | 6.91±0.99 | 0.76±0.15 |
| Low | 143±33 | 2.72±2.95 | 54.8±3.8 | 2.42±1.25 | 11.2±3.9 | 7.17±1.16 | 1.15±0.43 |
| Mid | 134±24 | 2.72±2.25 | 52.6±2.3 | 3.16±2.05 | 10.0±4.1 | 7.29±1.49 | 1.05±0.28 |
| High | 149±21 | 5.36±3.48 | 55.6±1.3 | 4.34±2.39 | 8.0±3.6 | 8.31±1.15 | 1.18±0.31 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DCeE** | | | | | | | |
| Control | 135±31 | 3.48±3.00 | 53.8±2.4 | 2.28±1.26 | 10.8±2.4 | 7.97±0.92 | 1.15±0.18 |
| Low | 155±52 | 4.37±2.46 | 53.0±2.2 | 2.51±0.94 | 10.2±3.3 | 7.35±1.10 | 1.17±0.40 |
| Mid | 124±28 | 2.40±1.92 | 52.2±3.2 | 2.67±1.95 | 11.5±3.7 | 7.55±1.02 | 1.14 ±.045 |
| High | 152±63 | 2.32±1.51 | 53.6±2.5* | 2.14±1.74 | 10.4±4.6 | 7.69±1.26 | 1.02±0.29 |

Values are presented as means±standard deviation ($n$=10). Significant differences (*$p$<0.05) were found between DE and DCeE groups (two-way ANOVA). Values for male and female mice are combined and this variable was included in the statistical analysis to assess statistical significance between DE and DCeE.

Abbreviations: DE=diesel exhaust; DCeE=diesel exhaust from fuel spiked with cerium; ALP=alkaline phosphatase activity; ALT=alanine aminotransferase activity; CA=calcium; PHOS=inorganic phosphate; AST=aspartate aminotransferase activity; GGT=gamma glutamyl transferase activity; TP=total protein; TBIL=total bilirubin; PLIP=phospholipids; TRIG=triglycerides.