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Extended Abstract

Effect of a diesel particle filter on the emission toxicity in lung cells *in vitro*

S. Steiner, J. Czerwinski, P. Comte, A. Mayer, P. Gehr and B. Rothen-Rutishauser

Background

The use of diesel particle filters (DPFs) is inevitable to abide by the exhaust emission restrictions implemented by the EURO VI regulations, one of the great hallmarks of which is the long overdue introduction of a limit number for particle emissions. The rationale behind such stringent emission regulation is the well documented adverse health effects arising by inhalation of diesel exhaust *[1, 2]*. In this regard, a large body of evidence points towards diesel exhaust particles (DEPs) as a key players. It is assumed that via the induction of oxidative stress, DEP inhalation leads to acute inflammatory responses in the respiratory tract, which if persistent for prolonged periods, may result in the raise of respiratory and systemic complications such as asthma, chronic obstructive pulmonary disease, cardiovascular diseases, strokes and cancer *[1, 3, 4, 5]*.

Based on this, it is tempting to hypothesize that the removal of DEPs from diesel exhaust by a diesel particle filter (DPF) will strongly lower or even eliminate the induction of oxidative stress and hence of pro-inflammatory and inflammatory responses, which could basically be considered as exhaust detoxicification. However, whereas it is well described how a DPF influences exhaust composition *[6, 7, 8]*, only very few studies have been performed on how exhaust toxicity is changed. Most importantly, besides particulate matter, diesel exhaust also contains various volatile compounds, most notably nitrogen oxides (NOx), polyaromatic hydrocarbons (PAHs) and nitrated polyaromatic hydrocarbons (NPAHs). These compounds are not retained by a DPF and it is known that they have a profound effect on biological systems. Moreover the composition of the volatile exhaust fraction may be changed by exhaust filtration in an undesirable way if catalytic filter systems are used *[8, 9, 10]*.

In the present study, we compared biological responses to unfiltered and DPF filtered diesel exhaust *in vitro*. We used a non-catalysed DPF, which on one hand reflects a type of exhaust after-treatment system that is currently in use *[7]*, and provides a basic insight into how removal of the particulate exhaust fraction with minimal changes in the composition of the gaseous fraction affects exhaust toxicity on the other hand.

Materials and Methods

Exhaust exposure system: The used exhaust exposure system *[11]* allows exhaust samples to be taken directly at the engine's exhaust outlet. The samples can immediately diluted ten-fold with heated (120°C), filtered ambient air and used for exhaust characterization and cell exposures with a delay of less than 30 seconds. In this way, biased exhaust sample generation or unwanted exhaust aging effects can be ruled out. Wall loss effects of particles have been shown to be negligible. The system disposes of two separate exposure chambers which allows to simultaneously expose identical cell cultures to exhaust samples and filtered air, thereby making a clear differentiation between purely exhaust related effects and exposure system related effects possible.

Test vehicle and vehicle settings: The test vehicle was an Opel Astra X20DTL running on a dynamometer at a constant velocity of 35 km/hr (corresponding to an engine speed 2180rpm) with a force of 66N at the wheel. The originally installed exhaust after treatment system had been removed completely. The vehicle was operated with normal low sulfur diesel (>10mg/kg sulfur, Greenergy) and the recommended lubrication oil (V10.237, Motorex) and the recommended lubrication oil.

For exposures to filtered exhaust, an uncoated wall-flow filter (Peugeot) the oxidative catalyst originally located upstream of which had been removed was installed directly downstream the tailpipe, with no alterations to any other engine setting.

Cell cultures: A triple-cell co-culture model was used for the exposure experiments *[12, 13]*. It consists of a confluent layer of bronchial epithelial cells (16HBE14o- cell line) and the two most important immune cells in the human lung, i.e. macrophages and dendritic cells. Macrophages and dendritic cells were isolated from human blood using CD14+ MicroBeads (Miltenyi Biotec). Macrophages are located at the exhaust sample/filtered air exposed (upper) side of the epithelial cell layer, dendritic cells on the culture medium exposed (lower) side, which reflects the structure of the human airway epithelium, where macrophages are residing in the airway lumen in contact with the inhaled air and dendritic cells reside on the connective tissue and blood vessel exposed side of the epithelium. The great advantage of the used cell culture is that besides acting solely as the model of a physical barrier, it provides a certain set of immunological properties as well as complex cell-cell interaction which cannot be simulated with monocultures. Exposure at the air liquid interface further increases the proximity to the in vivo situation and reduces interactions between exhaust samples and culture medium to a minimum.
Exposure experiments: the cell cultures were exposed to either diluted exhaust or to filtered air for two or six hours, reflecting a low and a high dose, followed by six hours post-incubation. Exposure and post incubation both took place under constant conditions of 37°C, 85% relative humidity and 5% CO2 partial pressure, which simulates the conditions in the human lung.
Exhaust characterization: The size-number distribution, the elemental carbon (EC) content and the total active surface area of the particulate exhaust fraction were measured in the ten-fold diluted exhaust using a Scanning Mobility Particle sizer (Differential mobility analyzer: TSI 3071; Condensation particle counter: TSI 3025 A), a Photoelectric Aerosol Sensor (EcoChem PAS 2000) and a Diffusion Charging Sensor (Matter Engineering LQ1-DC) respectively. Furthermore, the concentrations of carbon monoxide (CO), total gaseous hydrocarbons (HC), nitrogen oxides (NO_x) and nitrogen monoxide (NO) were measured in the ten-fold diluted exhaust using the Horiba MEXA-9400H exhaust gas measuring system. Concentrations of nitrogen dioxide (NO_2) were estimated based on the assumption that NO_x is entirely made up of NO and NO_2. Additionally, particle deposition on the cell cultures was measured by transmission electron microscopy (TEM) of TEM-grids that were place into the exposure chambers during exposure experiments.
Analysis of biological samples: directly after post-incubation, the cell cultures were collected and used for assessment of cellular morphology, cytotoxic effects (necrotic cell death), pro-apoptotic effects (programmed cell death), oxidative stress and inflammatory responses.
Cellular morphology was assessed by laser scanning microscopy of cell cultures stained for nucleic acids and the F-actin cytoskeleton. Cytotoxicity was assessed by quantification of extracellular lactate dehydrogenase (LDH), which is a protein normally kept inside the cell. Its presence in the culture medium indicates damaged cell membranes and hence cytotoxicity. The induction of Apoptotic cell death was assessed by measuring the expression level of caspase 7 (*CASP7*), a gene which is involved in the execution of programmed cell death. The induction of oxidative stress was measured by quantification of the reduced form of glutathione (GSH) an antioxidant molecule which under oxidative conditions becomes oxidized. The cellular response to oxidative stress was assessed by measuring the gene expression level of the oxidative stress-responsive gene heme oxygenase 1 (*HMOX1*). Pro-inflammatory responses were assessed by measurement of the gene expression of the two genes tumor necrosis factor alpha (*TNF*) and interleukin-8 (*IL-8*) and by quantification of their gene product (TNF-α and IL-8) in the culture medium. TNF-α and IL-8 both are pro-inflammatory cytokines, signal molecules which are produced and released by cells in order to induce inflammation.

Results and conclusion

As expected, exhaust filtration removed virtually all particles from the exhaust: unfiltered exhaust contained 4.8 x 10^8 particles/cm^3 resulting in a particle deposition of 1.7 (2hrs) and 7.4 (6hrs) x 10^7 particles/cm^2 in the exposure chamber. Filtered exhaust contained 1.6 x 10^3 particles/cm^3, deposited particles could not be detected. The composition of the gaseous exhaust fraction was not affected by the DPF.
Compared to filtered air exposure, changes in cellular morphology or cytotoxic effects could not be detected, independently on the exhaust type or the exposure duration, which indicates that the results obtained for the other biological endpoints are representative for healthy cells in a normal metabolic state. A weak induction of *CASP7* expression was observable however. Since the extent of this induction was not dependent on the exhaust type, we conclude that diesel exhaust has a certain acute pro-apoptotic effect which does not rely on the particulate fraction.
Independently on the duration of the exposure, reduced GSH was oxidized by both exhaust types to more than 75%, the effect was significantly weaker for filtered exhaust however. In accordance with this finding, *HMOX1* expression was strongly activated by both exhaust types and the effect was stronger for unfiltered exhaust. From this, we conclude that the induction of oxidative stress mainly relies on the presence of oxidative acting gaseous compounds and that particles play a minor role in this regard. Pro-inflammatory responses were activated upon exposure to unfiltered exhaust; compared to the filtered air exposure, an increased transcriptional activity of the genes *TNF* and *IL-8* as well as increased extracellular concentrations of the proteins TNF-α and IL-8 was observed. Exposure for 6 hours resulted in stronger responses than exposure for only 2 hours. In contrast, no such

responses were observed upon exposure to filtered exhaust. This indicates that the removal of particles from the exhaust samples is sufficient to inhibit the induction of pro-inflammatory responses after short term exposure.
In summary, exposure to ten-fold diluted diesel exhaust for two and six hours does not have cytotoxic effects *in vitro* but appears to act slightly pro-apoptotic. The filtration of the exhaust by an uncoated DPF in absence of any catalyst does not change its cytotoxicity or pro-apoptotic properties, but decreases its oxidative and pro-inflammatory potential.
From the present data, it cannot be deduced whether the absence of pro-inflammatory signaling can be considered the result of the decreased levels of oxidative stress. Even though clearly detectable, the observed effect of exhaust filtration on GSH oxidation and *HMOX1* expression is not likely to be biologically relevant, particularly since the cellular pool of reduced GSH was oxidized to a very high degree by both exhaust types. It can therefore also be hypothesized that the absence of deposited particles on the cell cultures is the direct cause for the absence of pro-inflammatory signaling by a mechanism that would yet have to be defined.
The induction of acute inflammation in the respiratory tract is considered to be the starting point for a variety of respiratory but also systemic adverse health effects of diesel exhaust inhalation. The observation that the mere removal of DEPs from the exhaust inhibits the induction of pro-inflammatory responses suggests that the DPF is an efficient technology to detoxify diesel engine emissions. It has to be kept in mind however, that based on the present study this can only be inferred for uncoated filters, used in the absence of any catalytic activity. It cannot be excluded that the use of a DPF in combination with an oxidative catalyst or a fuel-borne catalyst will yield different results, for example because of increased oxidative potential of the exhaust. Further, long term effects have not been assessed in this study, i.e. we cannot conclude that a DPF reduces exhaust carcinogenicity or whether prolonged exposure would have led to the same findings. Future studies including genotoxicity assays and long-term exposures will therefore have to be conducted.

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Effect of a diesel particle filter on the emission toxicity in lung cells *in vitro*

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Air pollution and public health – evidence from epidemiological studies

Contribution of diesel engines to on-road particle emissions (CH):

~ 50%

M. Wyser et al., SAE 1999-01-0116

➡ **Major players: (ultra-)fine particles (< 100nm)**

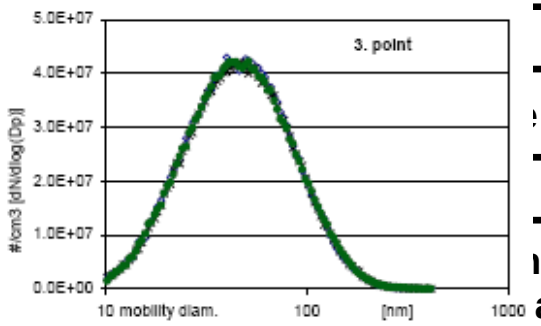
Major players: (ultra-)fine particles (< 100nm)

Particle-ce nteraction

Necrotic cell death (cytotoxicity)

Generation of reactive oxygen species (ROS)

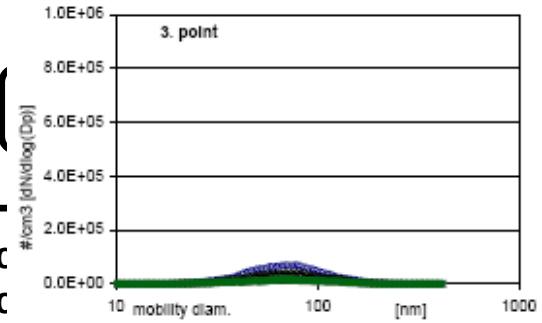
Is the diesel particle filter (DPF) the solution?



stress

DPF

ronic
ation



Liebherr 924 engine, ISO 817 4-cycle, step 3 (50% load, high rpm).
DPF: SiC wall flow filter Pt, Pd coated
Mayer et al. SAE 2010-01-0792

DNA damages

Altered cell proliferation

Apoptosis

Tumor formation

Adapted from Schins *et al.*, Inhalation Toxicology, 2007

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Is the DPF the solution?

Problem: Exhaust toxicity cannot (yet?) be derived from physicochemical exhaust characteristics

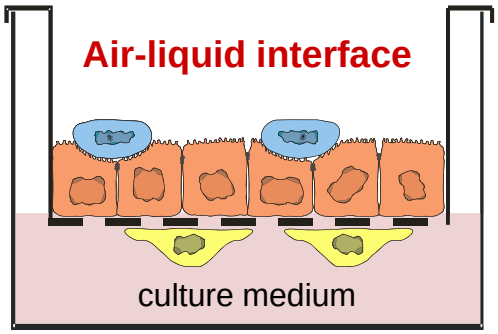
Solution: Experimental studies

Requirements:

1) Suitable biological system
2) Exposure system
3) Biological markers

1) Suitable biological system

In vitro approach



Human epithelial cells

- **16HBE14o$^-$** cell line (bronchial)

Human monocyte-derived **macrophages** (professional phagocytes)

Human monocyte-derived **dendritic cells** (professional antigen-presenting cells)

(Rothen-Rutishauser et al (2005) Am J Respir Cell Mol Biol; Blank et al (2007) Am J Respir Cell Mol Biol.; Muller et al (2010) J R Soc Interface)

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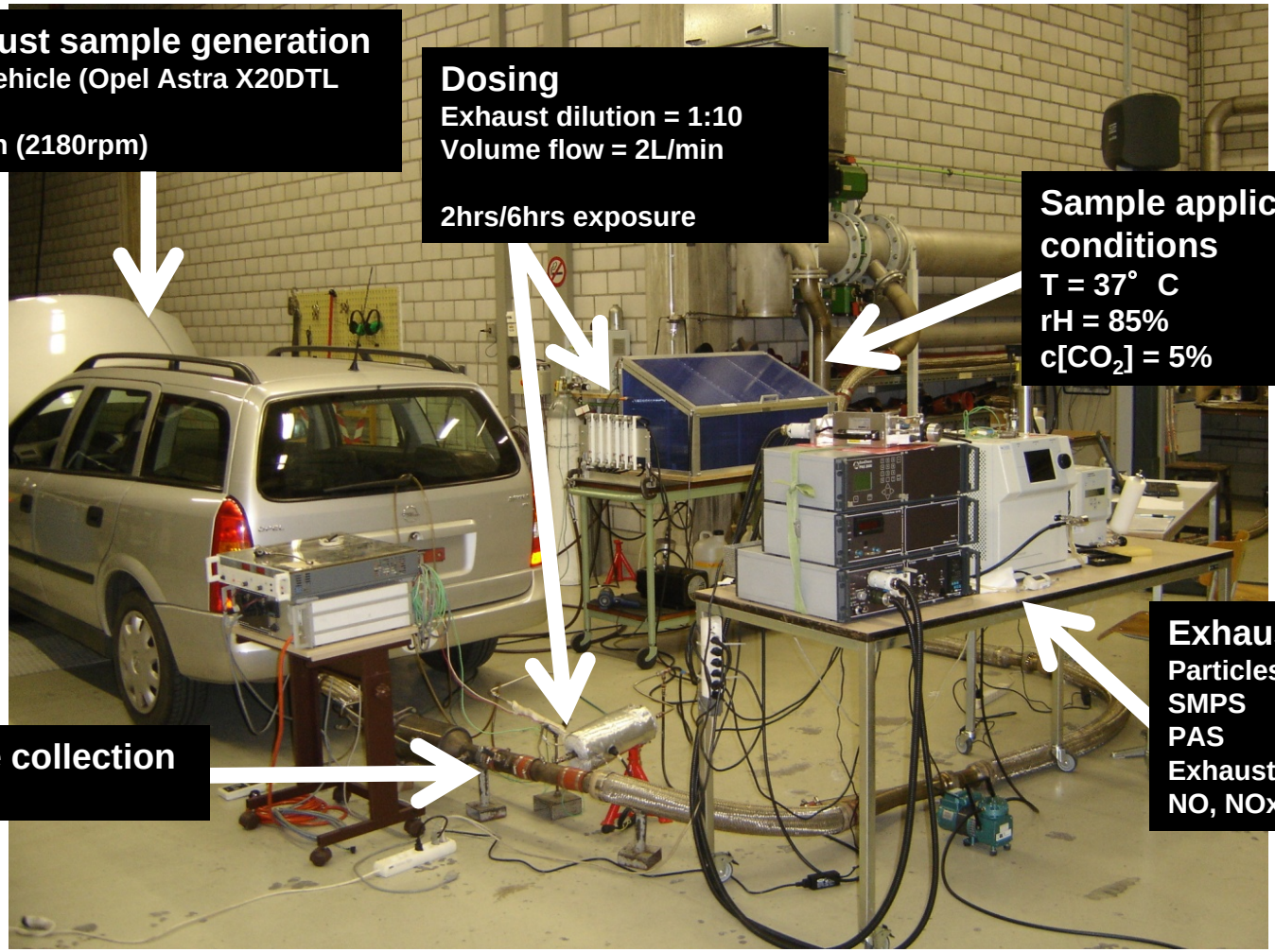
2) Exposure system



Exhaust sample generation
Test vehicle (Opel Astra X20DTL 2L)
35km/h (2180rpm)

Dosing
Exhaust dilution = 1:10
Volume flow = 2L/min

2hrs/6hrs exposure

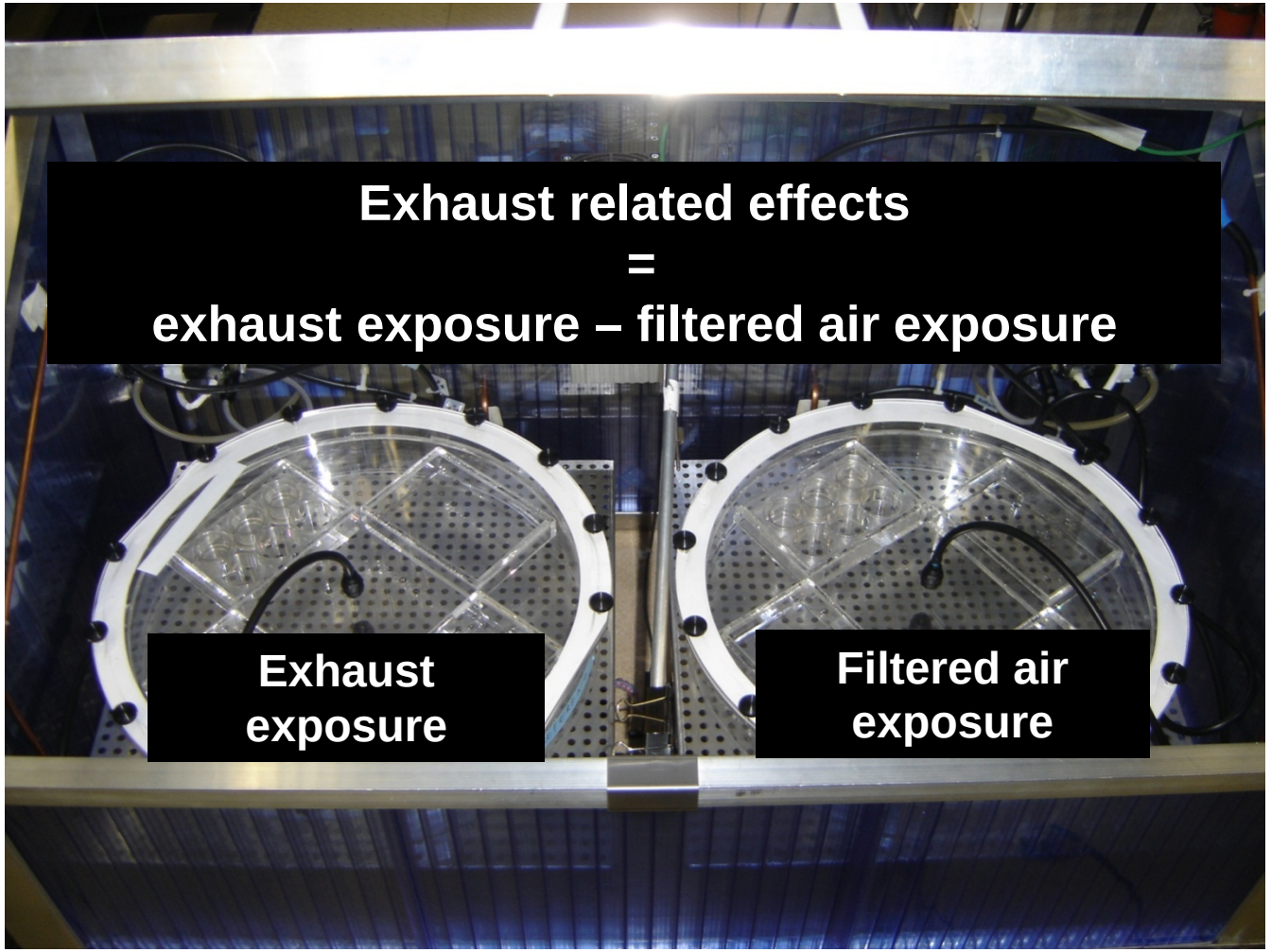
Sample application, exposure conditions
T = 37° C
rH = 85%
c[CO_2] = 5%

Exhaust characterization
Particles:
SMPS
PAS
Exhaust gas:
NO, NOx, CO, HC

Exhaust sample collection
At tailpipe

Located at the Bern University of Applied Sciences, Nidau, Prof. J. Czerwinskis lab

2) Exposure system



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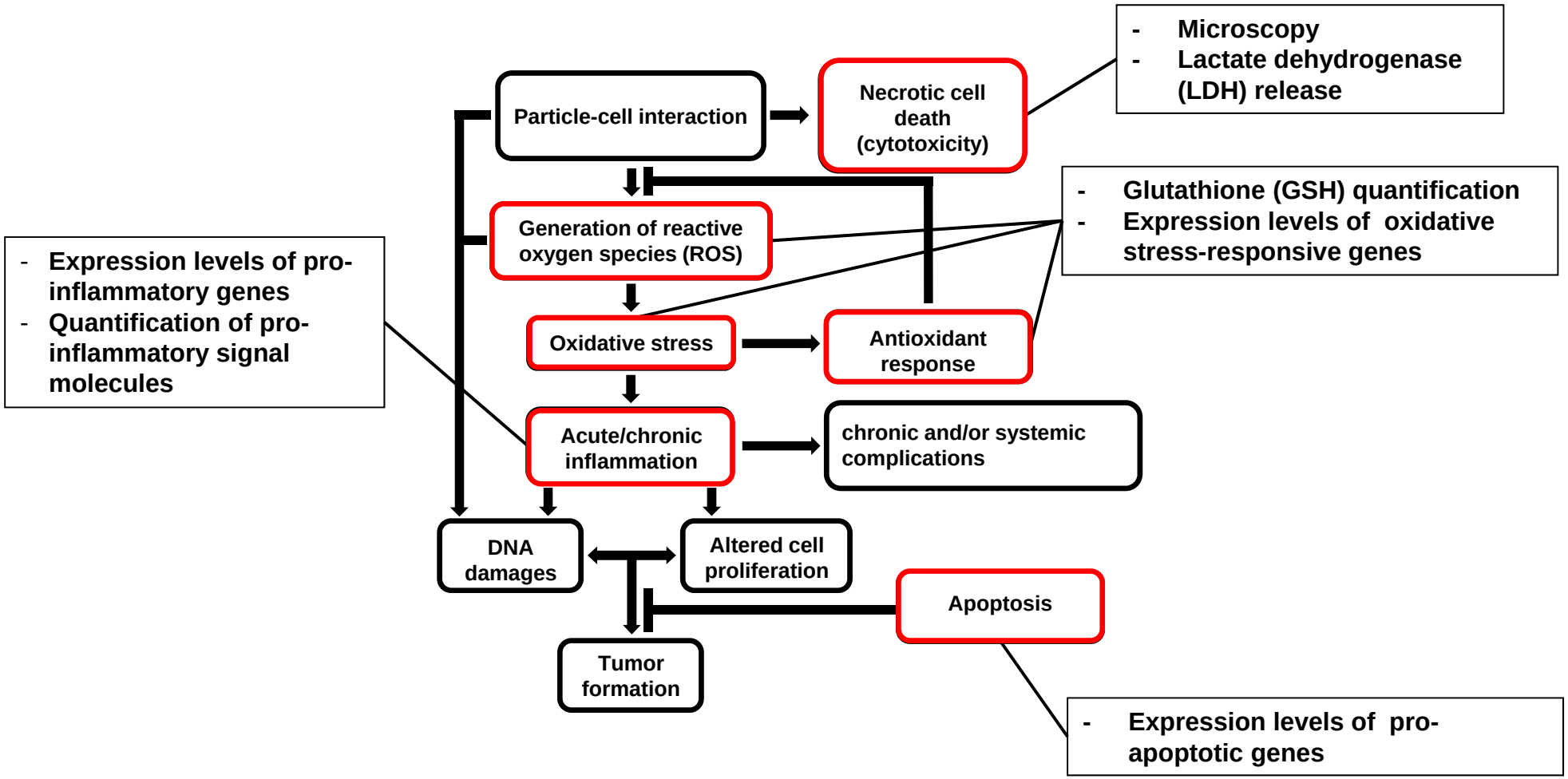
3) Biological markers



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Effects of a DPF on exhaust toxicity - Results

Biological responses to unfiltered exhaust

vs.

Biological responses to DPF-filtered exhaust (6hrs exposure)

Filter type: SiC wall-flow filter (Peugeot), uncoated, no catalyst

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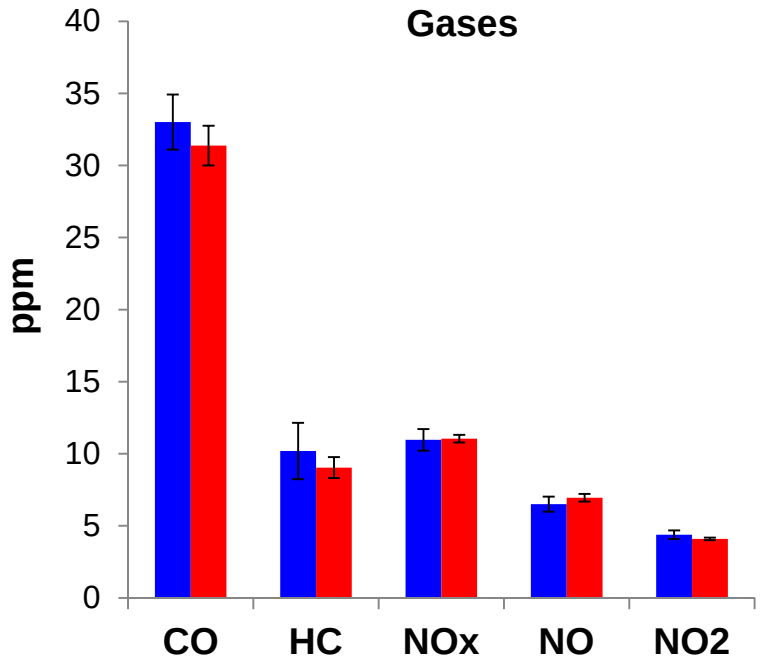
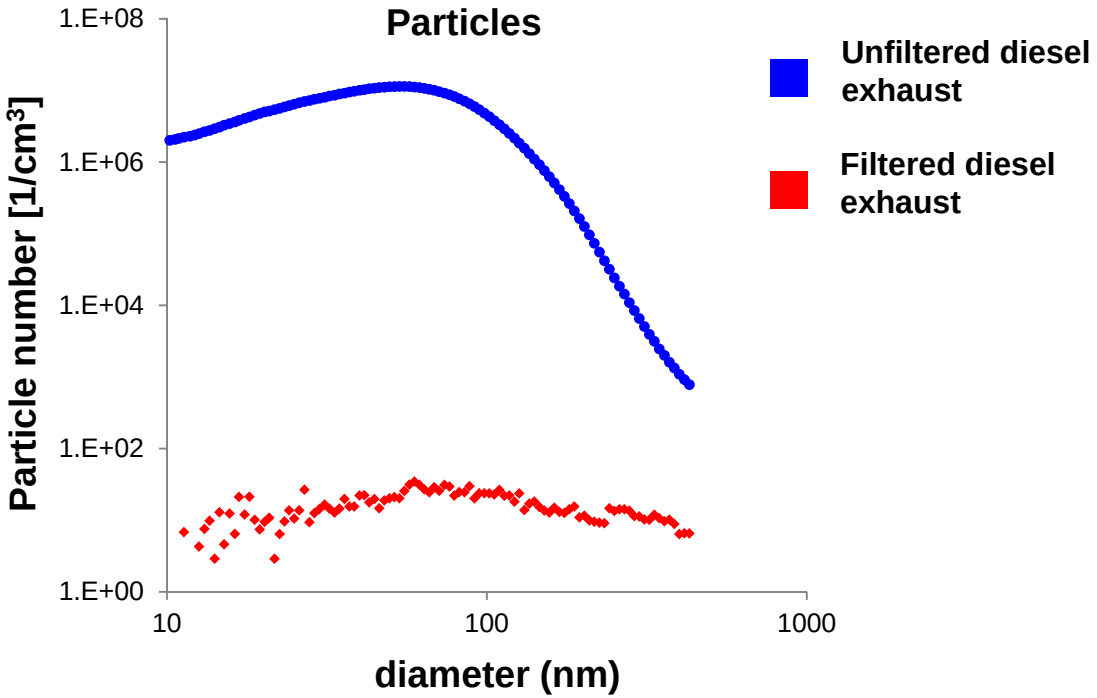
Effects of a DPF on exhaust composition

Unfiltered *vs.* filtered exhaust (SiC wall flow filter, uncoated, no catalyst)





average±SD
Unfiltered: n=12
DPF: n=6

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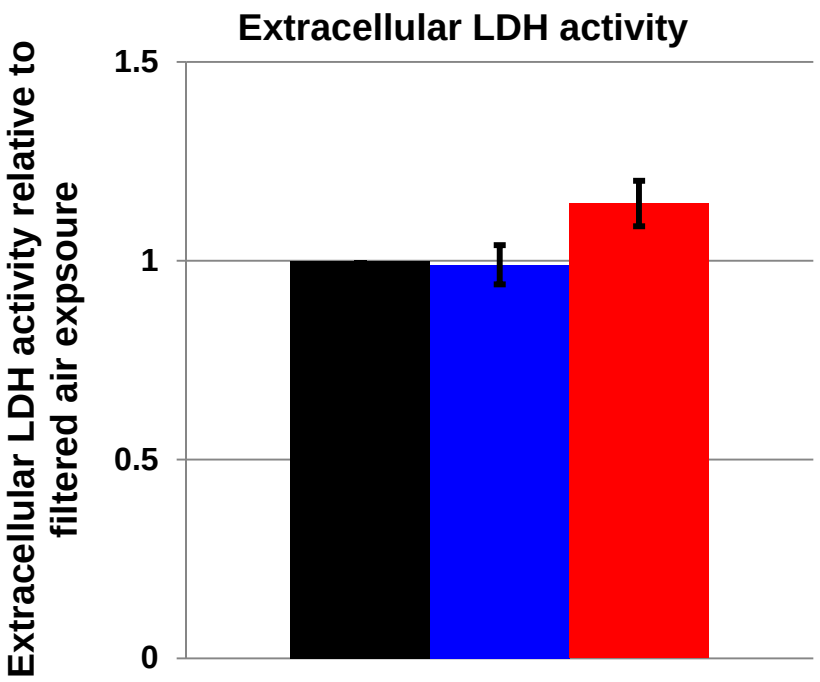
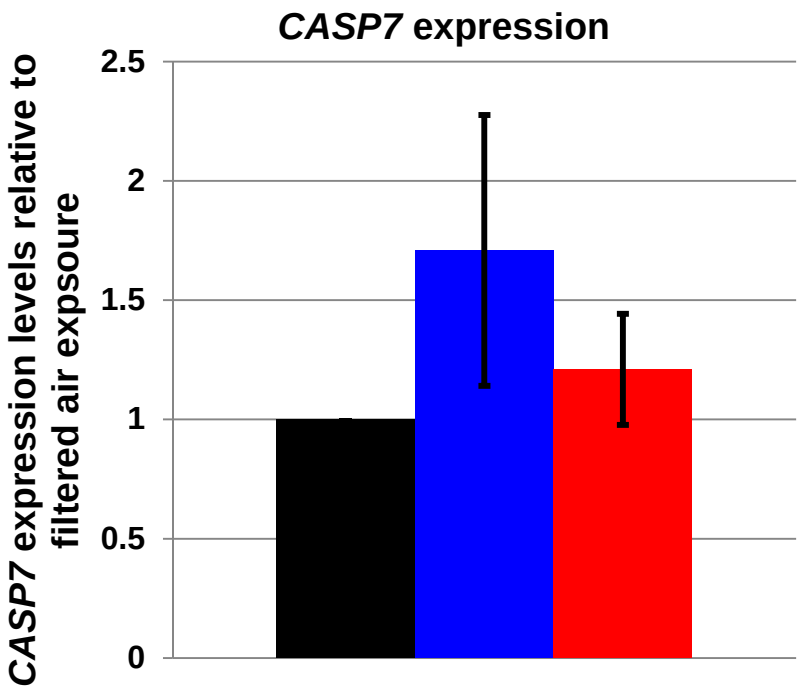
Effects of a DPF on exhaust toxicity

Unfiltered *vs.* filtered exhaust (SiC wall flow filter, uncoated, no catalyst), 6hrs exposure

Cytotoxicity and apoptotis





Filtered air

Unflitered diesel exhaust

Filtered diesel exhaust

LDH: An intracellular protein. Its presence outside the cell indicates damaged cell membranes

CASP7: a protein involved in the execution of apoptosis

Average ± SEM
Unfiltered: n=12
DPF LDH: n=6
CASP7: n=3

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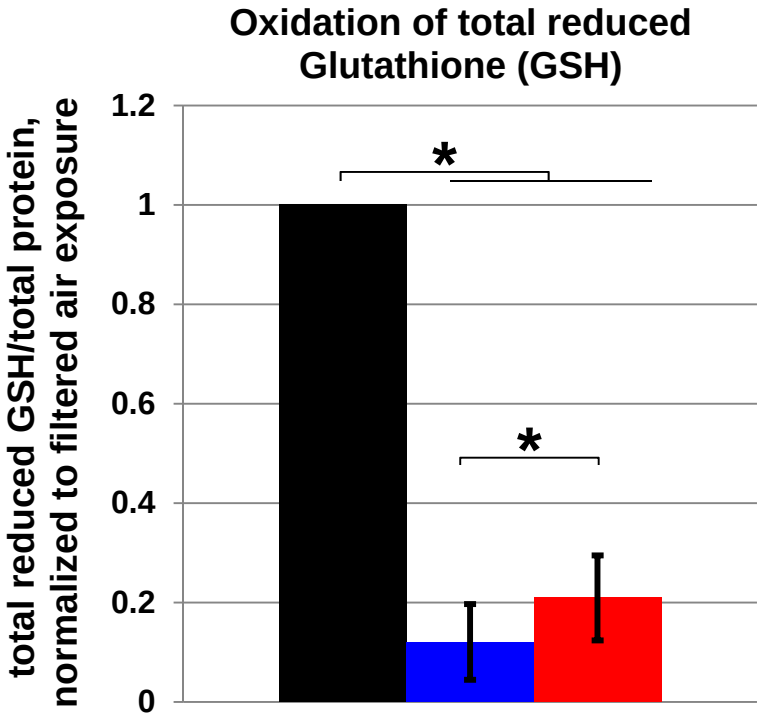
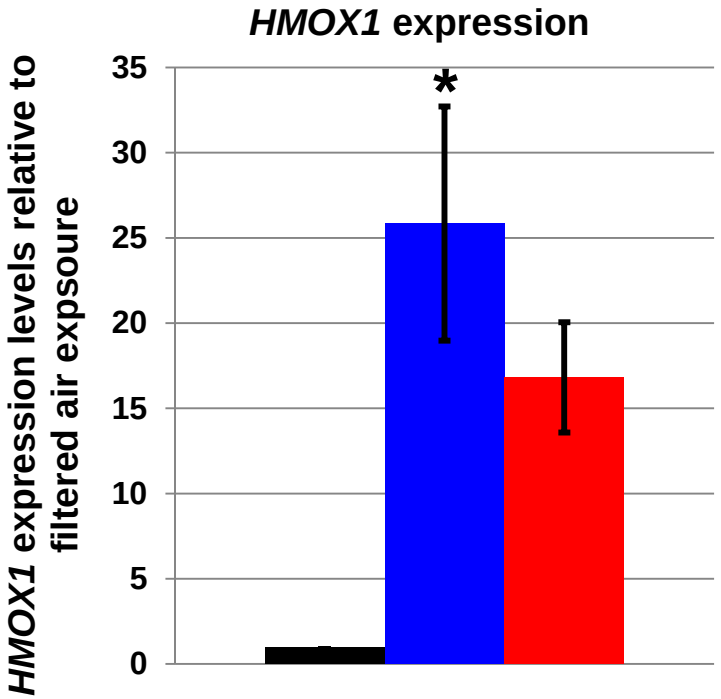
Effects of a DPF on exhaust toxicity

Unfiltered *vs.* filtered exhaust (SiC wall flow filter, uncoated, no catalyst), 6hrs exposure

Oxidative stress and response to oxidative stress





Filtered air

Unflitered diesel exhaust

Filtered diesel exhaust

GSH: an anti-oxidant molecule

***HMOX1*: an oxidative stress-responsive gene**

Average±SEM
Unfiltered: n=12
DPF: GSH: n=6
HMOX1: n=3

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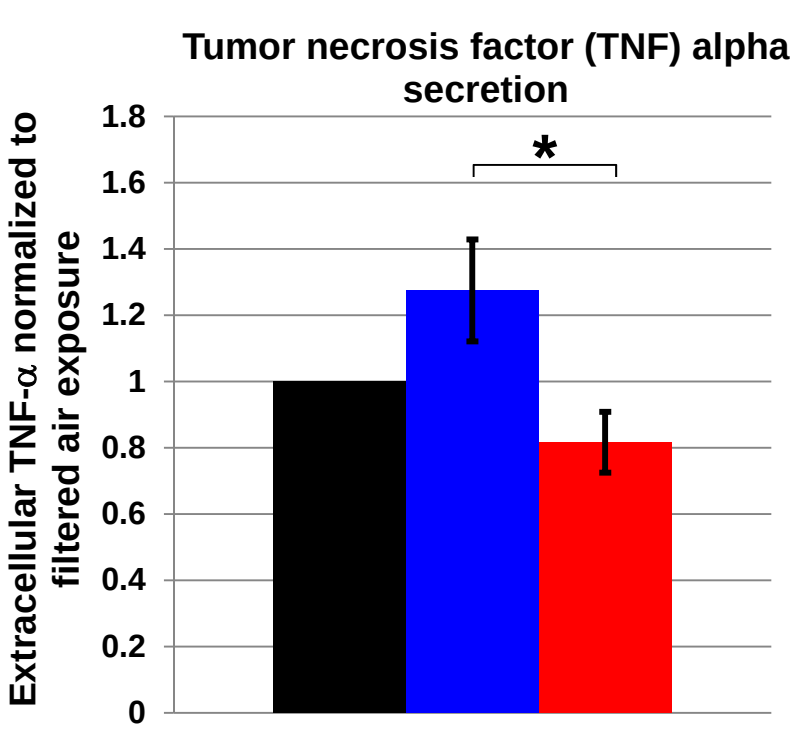
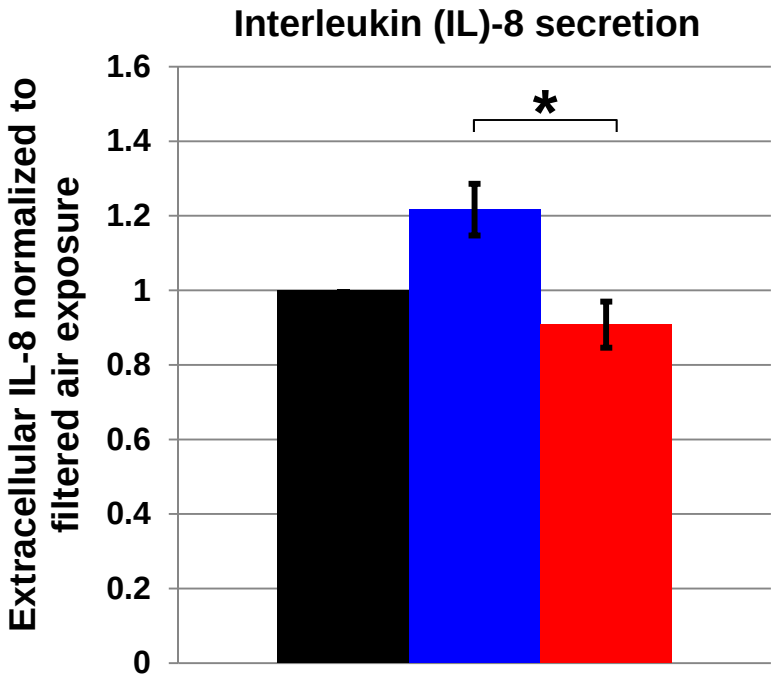
Effects of a DPF on exhaust toxicity

Unfiltered *vs.* filtered exhaust (SiC wall flow filter, uncoated, no catalyst), 6hrs exposure

Inflammatory response





Filtered air

Unflitered diesel exhaust

Filtered diesel exhaust

TNF-α and IL-8: inflammatory cytokines (signal molecules inducing inflammation)

Average±SEM
Unfiltered: n=12
DPF: n=6

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Summary and conclusion

Unfiltered *vs.* filtered exhaust (SiC wall flow filter, uncoated, no catalyst)

No inflammatory responses detectable

Core element of exhaust toxicity disarmed

Why?

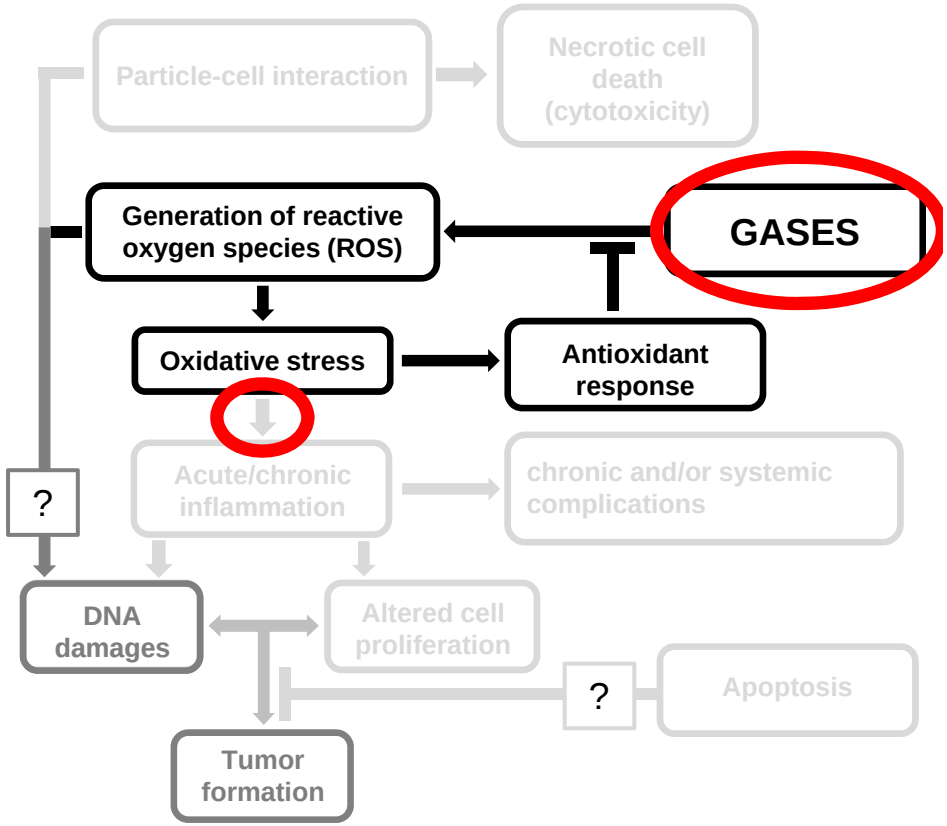
Absence of particles?
Lower oxidative stress?
…

Gas effects are not negligible

Hydrocarbons! (genotoxicity was not tested)

What happens after prolonged exposure?

Filtered exhaust



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