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Assessment of lung cell toxicity of various gasoline engine exhausts using a versatile *in vitro* exposure system☆

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ABSTRACT

Adverse effect studies of gasoline exhaust are scarce, even though gasoline direct injection (GDI) vehicles can emit a high number of particles.

The aim of this study was to conduct an *in vitro* hazard assessment of different GDI exhausts using two different cell culture models mimicking the human airway. In addition to gasoline particle filters (GPF), the effects of two lubrication oils with low and high ash content were assessed, since it is known that oils are important contributors to exhaust emissions.

Complete exhausts from two gasoline driven cars (GDI1 and GDI2) were applied for 6 h (acute exposure) to a multi-cellular human lung model (16HBE14o-cell line, macrophages, and dendritic cells) and a primary human airway model (MucilAir™). GDI1 vehicle was driven unfiltered and filtered with an uncoated and a coated GPF. GDI2 vehicle was driven under four settings with different fuels: normal unleaded gasoline, 2% high and low ash oil in gasoline, and 2% high ash oil in gasoline with a GPF. GDI1 unfiltered was also used for a repeated exposure (3 times 6 h) to assess possible adverse effects.

After 6 h exposure, no genes or proteins for oxidative stress or pro-inflammation were upregulated compared to the filtered air control in both cell systems, neither in GDI1 with GPFs nor in GDI2 with the different fuels. However, the repeated exposure led to a significant increase in *HMOX1* and *TNFa* gene expression in the multi-cellular model, showing the responsiveness of the system towards gasoline engine exhaust upon prolonged exposure.

The reduction of particles by GPFs is significant and no adverse effects were observed *in vitro* during a short-term exposure. On the other hand, more data comparing different lubrication oils and their possible adverse effects are needed. Future experiments also should, as shown here, focus on repeated exposures.

1. Introduction

It has been shown by many studies that exposure to particulate matter smaller than 2.5 μm ($PM_{2.5}$) correlates with cardiovascular and pulmonary diseases as well as cancer (Brunekreef and Holgate, 2002; Pope et al., 2002). A recent meta-analysis of 110 peer-reviewed time series also showed that a 10 μg/cm^3 $PM_{2.5}$ increment leads to a 1.04% increased risk of death (Atkinson et al., 2014). The pathway by which air pollution affects human health is via inhalation of particles and gases inducing local cellular reactions in the lung tissue, such as oxidative stress followed by (pro-)inflammation, DNA damage, and direct cytotoxic effects. Ambient

Abbreviations: ALI, air-liquid interface; CO, carbon monoxide; CO_2, carbon dioxide; cLSM, confocal laser scanning microscope; CVS, constant volume sampler; ELISA, enzyme-linked immunosorbent assay; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GDI, gasoline direct injection; GPF, gasoline particle filter; HC, hydrocarbons; HMOX1, heme oxygenase 1; CXCL8, interleukin 8; LDH, lactate dehydrogenase; MDDC, monocyte derived dendritic cells; MDM, monocyte derived macrophages; NOx, nitric oxides; NQO1, NAD(P)H dehydrogenase [quinone] 1; PAH, polyaromatic hydrocarbons; PBS, phosphate-buffered saline; PN, particle number; PM, particulate matter; SOD2, superoxide dismutase 2; TNFa, tumor necrosis factor alpha; WLTC, Worldwide-harmonized Light vehicles Test Cycle.

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particles or secondary mediators can also reach the vascular system and other organs, potentially resulting in ischemic heart disease or stroke (reviewed in Risom et al., 2005; Steiner et al., 2016; Stone et al., 2017).

Major contributors to air pollution are traffic related combustion processes, such as diesel and gasoline vehicles. While adverse effects of diesel exhausts have been widely studied (e.g Hashimoto et al., 2001; Steiner et al., 2013; Zarcone et al., 2016), effects from gasoline vehicles are less known. This lack of toxicological data does not reflect the current trends for the use of gasoline vehicles in comparison to diesel cars. In addition, the modern gasoline direct injection (GDI) technology release significantly higher particle number (PN) emissions compared to older gasoline vehicles or modern diesel vehicles equipped with a particle filter (Zhang and McMahon, 2012; Platt et al., 2017). It was also reported that GDI vehicles need a gasoline particle filter (GPF) to comply with the current Euro6 PN-legislation in the future (Czerwinski et al., 2017).

To the best of our knowledge, only one other group investigated *in vitro* pulmonary effects of a GDI vehicle (Maikawa et al., 2016), but with only few biological parameters. More data is needed using a realistic exposure system, that includes freshly produced exhaust and cells at the air-liquid interface (ALI). Additionally experiments with GPF's are needed, as filtration alone has not always been proven to be sufficient for exhaust detoxification (Holder et al., 2007; McDonald et al., 2007; Steiner et al., 2014).

Vehicle exhaust emissions are not only influenced by the engine type, but also by the fuel composition (e.g. ethanol or butanol supplement, octane number), after-treatment system (exhaust gas recirculation, three-way catalyst, and particle filter), ambient conditions, and lubrication oil. It has been shown that lubrication oil significantly contributes to the PN emissions of diesel (Buchholz et al., 2003; Brandenberger et al., 2005) and gasoline (Sonntag et al., 2012) engines, and polyaromatic hydrocarbons (PAHs) have also been shown to be derived (at least partly) from unburnt lubrication oil (Geller et al., 2006). As vehicle mileage increases, the usage of lubrication increases due to engine wear (gasket, valve, and piston condition) (Pedersen et al., 1980; Robert et al., 2007), however, driving style and oil viscosity also influence oil consumption. Despite the known influence of lubrication oil on engine emissions, no regulation is in place for the composition of lubrication oils. Importantly, no study could be found that simulated consumption of different lubrication oils and investigated the *in vivo* or *in vitro* toxicology of such exhausts (or extracts).

We have recently established an *in vitro* exposure system (Muller et al., 2010); the heated system contains two exposure chambers holding up to four 6-well cell culture plates each. The system is versatile and can be directly connected to any engine; this far it has been used with scooter (Müller et al., 2011), diesel (Steiner et al., 2012; Steiner et al., 2013a; Steiner et al., 2013b; Steiner et al., 2014), and gasoline (Bisig et al., 2015; Bisig et al., 2016) vehicles, resulting in reproducible and sensitive data that allowed differentiation between the particle and gaseous fractions in exhaust emissions. Using this set-up we have shown that exhaust emissions, *i.e.* 40% of the particulate fraction, is deposited on inserts which are placed in the 6-well plates, indicating that the exhaust can interact with the cellular fraction (Muller et al., 2010).

Herein we have taken further advantage of the versatility of our exposure system to test the influence of various parameters (Fig. 1). Two different GDI vehicles were used in single acute exposures of 6 h, vehicle GDI1 was driven unfiltered (GDI1 reference) as well as with two different filters (uncoated and coated GPF). A coated GPF was included because it can also remove volatile organic compounds. Vehicle GDI2 was driven unfiltered with normal gasoline (GDI2 reference) and with modified gasoline, *i.e.* two different lube-oils (high ash and low ash content) were added in 2% volume, the one with high ash was additionally driven with a filter (high ash GPF). A GPF was attached because it was hypothesized that the high ash lube-oil would have the highest negative impact on both exhaust emissions and lung-cell cultures, and a filter would diminish some of the effects. Since humans can be constantly exposed to exhaust emissions over a longer period of time, the GDI1 reference was driven three times for 6 h and possible adverse effects of this repeated exposure were assessed in the lung cell cultures to mimic a prolonged exposure.

Lung cell models mimicking the respiratory airway tract were used. The first lung model, the multi-cellular lung model (Rothen-Rutishauser et al., 2005), consisting of a bronchial cell-line supplemented with primary macrophages and dendritic cells, was used for previous hazard identification studies and clearly demonstrated a significant elevation of oxidative stress and pro-inflammatory stress markers upon exposure to unfiltered diesel exhaust, allowing a comparison of the results (Steiner et al., 2012; Steiner et al., 2013; Steiner et al., 2014; Bisig et al., 2015; Bisig et al., 2016). The second model, the MucilAir™ system consists of primary human bronchial epithelial cells, and includes mucus-producing as well as ciliated cells (Huang et al., 2013), two important features in the defense against particulates. The two models complement each other, as one includes immune cells while the other can produce mucus and has functional cilia. It was hypothesized that they will react differently to gasoline exhaust, giving a better overall understanding of the *in vitro* effects of gasoline exhausts.

The major cellular responses induced by traffic-derived air pollutants are oxidative stress and (pro-)inflammation (Xiao et al., 2003; Risom et al., 2005). Therefore, gene expression analysis was performed with genes related to these two endpoints and selected based on their upregulation in other studies with diesel exhaust, diesel exhaust particles only, or gasoline exhaust (e.g Gong et al., 2007; Huang et al., 2011; Wittkopp et al., 2016).

2. Materials and methods

2.1. Test vehicles, exposure system and protocol

Gasoline passenger cars (GDI technology) were driven on a chassis dynamometer following the Worldwide harmonized Light vehicles Test Cycle (WLTC) protocol for 6 h (ten cycles), which included one cold start.

A modern flex-fuel car (Euro5b, 2012, mileage during exposure 15271–21608 and 27693–28600 km), herein defined as GDI1, was previously tested with different ethanol-gasoline fuel blends (Bisig et al., 2016). The GDI1 car was driven unfiltered (GDI1 reference) as well as with two different filters on conventional unleaded gasoline. The uncoated GPF (as used in Bisig et al., 2015) had a pore size of 19 μm and 50% porosity while the coated GPF had a smaller pore size (14 μm) and higher porosity (55%). Both GPFs (cordierite, 200 cells per square inch) were installed approximately 60 cm downstream from the original three-way catalyst.

As the GDI1 vehicle was not available during the lubrication-oil experiments and also to compare different GDI technologies, a second GDI car was used. The second car (GDI2, Euro6b, 2014, mileage during exposure 27912–31345 km) was driven unfiltered on normal gasoline (GDI2 reference) as well as with two lubrication-oil-enriched fuels to simulate high oil consumption. The oils with equal viscosity were each mixed with gasoline (2% V/V). Ash and metal content differed in the two used oils, high-ash (≤1.2%) and low-ash oils (≤0.5%) were used. It was hypothesized that the high-ash oil would have an impact on both emission and biological endpoints, therefore a freshly thermally-regenerated, uncoated GPF (as used in Steiner et al., 2013) was mounted at the

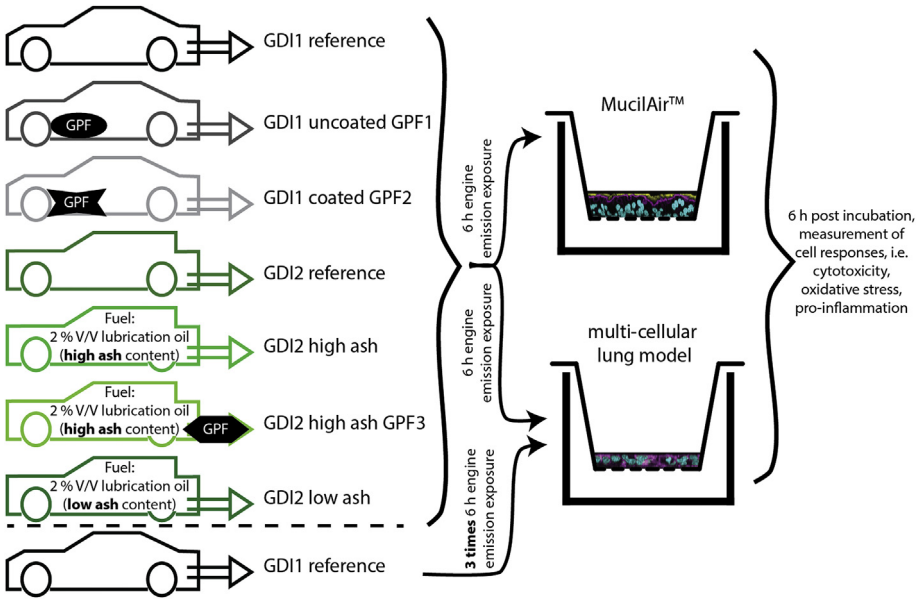

Fig. 1. Schematic overview of the exposure setup. A total of seven different GDI exhausts were applied to two lung cell models for 6 h driving the Worldwide harmonized Light vehicles Test Cycle (WLTC) protocol, which included one cold start per 6 h. Three different GPFs and three different fuels were tested. Additionally, the GDI1 reference was applied repeatedly (three times, 6 h each) to one lung cell model. The original three-way catalyst was used, and the fuel used was commercially available unleaded gasoline unless otherwise stated. GDI = gasoline direct injection, GPF = gasoline particle filter.

tailpipe and run with high ash oil (high ash GPF).

The exposure system was used as described previously (e.g Muller et al., 2010; Steiner et al., 2013). Briefly, the exhaust was tenfold diluted, which represents the exposure of a person working/standing for 6 h approximately one meter from the exhaust pipe on a sidewalk (Bisig et al., 2016). The exhaust was subsequently humidified (app. 80% rH), carbon dioxide (CO_2)-enriched (5%), and applied (2 L/min, 6 h) to an exposure chamber containing the cell cultures at 37 °C. In parallel, filtered ambient air with similar humidity, CO_2 content, and flow was applied to identical cell cultures (cells from the same origin) as a control. The protocol for the repeated exposure was designed identical, but the same cell cultures were added on three consecutive days to the engine emissions.

2.2. Exhaust characterization

The exposure experiments were performed at the exhaust gas control laboratory of the Bern University of Applied Sciences in Nidau, Switzerland. Exhaust sample characterization was performed on-line and in parallel to the exposure experiments. The PN was measured in the tenfold-diluted exhaust using a condensation particle counter (TSI 3790). Particle size distributions were measured at constant speed (95 km/h) in a separate experiment using a scanning mobility particle sizer (SMPS, differential mobility analyzer, TSI 3081). Furthermore, the concentrations of carbon monoxide (CO), CO_2, total gaseous hydrocarbons (HC), and nitric oxides (NOx) were measured from the constant volume sampler (CVS) emissions tunnel using a Horiba MEXA-9400H exhaust gas measuring system. At least six WLTC were evaluated every day, including the cold start WLTC. For each analyte, averages were calculated over the whole volume concentration for each day of exposure.

2.3. Cell cultures

A co-culture model composed of cells mimicking the human bronchial tissue barrier was used and compared to commercially available reconstituted 3D human airway epithelia.

The multi-cellular lung model composed of epithelial lung cells (16HBE14o-human bronchial cells), monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC) is described in detail elsewhere (Rothen-Rutishauser et al., 2005; Blank et al., 2007; Steiner et al., 2013). Briefly, 16HBE14o-cells were grown on a PET-insert (10^6 cells/six-well insert or 2.4×10^5 cells/cm^2, 3.0 μm pore size) for five days before addition of MDDC on the basolateral side of the insert (1 h incubation time allowed for attachment of MDDC) and MDM on the top of the 16HBE14o-monolayer. One day after this composition, the medium was removed and 1.2 mL fresh supplemented RPMI (Roswell Park Memorial Institute medium with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin) was added on the bottom. After 24 h acclimatization time at ALI the exposures were performed.

Additionally, primary human airway epithelial cells (MucilAir™) of bronchial origin were purchased from Epithelix (Epithelix Sàrl, Plan-les-Ouates, Switzerland) and treated according to the provided protocol with the provided medium. Briefly, cells arrived at our laboratory one week before the experiments. Upon arrival, cells on 24-well inserts were transferred to supplemented MucilAir™ serum-free medium (supplemented with 0.5 μg/mL posaconazol) and subsequently cultured at ALI (0.7 mL medium on the bottom). The medium was changed every two to three days.

2.4. Cell sample analysis

After the 6 h exposure to exhaust or filtered air and an additional 6 h post-incubation time, the cells and supernatants from the two human lung models were collected and processed as described for the specific assays. Supernatants from the basal media were stored in the fridge or freezer for later analysis.

2.4.1. Confocal laser scanning microscopy (cLSM)

Microscopy images were taken to evaluate cell morphology after

the different exposures. Samples were fixed in 3% paraformaldehyde for 10 min and stored at 4 °C in phosphate-buffered saline (PBS) for later staining. Before staining, samples were washed with PBS, permeabilized with 0.25% Triton X-100 for 15 min, and washed again. The staining was performed with Phalloidin Rhodamine (F-Actin stain) and 4′,6-diamidino-2-phenylindole (DAPI, nuclei stain) for 1 h. MucilAir™ cultures were additionally stained for alpha-tubulin (monoclonal mouse anti-alpha tubulin clone DM 1A, Sigma). After a last washing step, samples were mounted on objective slides in Glycergel® (Dako, Denmark). Image acquisition was performed on a Zeiss LSM 710. Image restoration was done with the IMARIS software (Bitplane 7.4, Zürich, Switzerland).

2.4.2. Quantitative reverse transcription real-time polymerase chain reaction (RT-qPCR)

PET-membranes with cells were stored in RNAprotect buffer (Qiagen) in the fridge. After up to 10 min vortexing to detach the cells from the membrane, the membrane was removed and discarded. RNA isolation was performed with the RNeasy Plus kit (Qiagen), and RNA concentration was measured using a NanoDrop 2000 (ThermoFisher). cDNA was produced with the Omniscript RT system (Qiagen), Oligo dT (Microsynth), and RNasin Inhibitor (Promega) as recently described (Bisig et al., 2015). RT-qPCR was performed with SYBR-green (Applied Biosystems) on a 7500 Fast Real-Time PCR (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the standard gene. Heme oxygenase 1 (*HMOX1*), superoxide dismutase 2 (*SOD2*), and NAD(P)H dehydrogenase [quinone] 1 (*NQO1*) were the genes used to assess oxidative stress. For pro-inflammatory responses, genes for tumor necrosis factor α (*TNFa*) and interleukin-8 (*CXCL8*) were measured. The primer sequences for all assessed genes can be found in SI Table S1.

2.4.3. Positive controls for oxidative stress and pro-inflammatory stimulus

Different positive controls were used to show responsiveness of the multi-cellular lung model (16HBE14o-/MDM/MDDC). *HMOX1* and *NQO1* expression was upregulated by hydroquinone (Sigma), 100 μM hydroquinone (stock solution 10 mM in PBS, further dilution in medium) was applied to the basolateral side (1.2 mL) for 12 h (Cheah et al., 2013). Hydroquinone also induced CXCL8 expression (data not shown). Interestingly, *SOD2* was upregulated by 6 μg/mL R-848 (1.2 mL basolateral) for 12 h (stock solution 1 mg/mL in DMSO, Enzo Life Sciences, Lausen, Switzerland.). Addition of 0.1 ng/mL interleukin 1 beta obtained from an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems) induced both *TNFa* and *CXCL8* expression in the multi-cellular human lung model after basolateral (1.2 mL) exposure of 12 h. MucilAir™ cultures were incubated with TNFa (15 ng/mL; 700 μL on the bottom and 20 μL on the top) for 12 h, this increased gene expression of almost all assessed genes and is shown here. *tert*-Butyl hydroperoxide (Sigma, 1–5 mM) either did not induce oxidative stress or was cytotoxic (no mRNA yield and high lactate dehydrogenase (LDH)-level).

2.5. Number of repetitions and data processing

2.5.1. Number of repetitions

Gasoline exhaust was produced on four (for GDI1) or three (for GDI2) different days, and on each day two different cell cultures were used (*i.e.* different passage number for 16HBE14o-cells, and MDM and MDDC from different donors) resulting in sample repetitions of eight (for GDI1) and six (for GDI2). An exception is the exposure with the coated GPF, where on each day only one cell culture was exposed (n = 4). Fewer exposures (n = 3) were performed with the MucilAir™ cultures.

2.5.2. Repeated exposure

The exhaust of vehicle GDI1 was produced on four subsequent days, cells of four different cell cultures were exposed either from day 1–3 or from day 2–4, resulting in a sample repetition of seven (one mRNA isolation failed due to human error). Of note, the medium was changed every morning before exposure started, due to this frequent medium change no ELISA or LDH measurements were performed.

2.5.3. Data normalization and presentation

To account for the different baseline levels in the various cultures and for the continuous potentially stress-inducing airflow, data of exhaust-exposed cell cultures were normalized to data of the filtered air controls (Steiner et al., 2013). RT-qPCR data was calculated with the $2^{-\Delta\Delta Ct}$ method, using *GAPDH* as the housekeeping gene and filtered air as the control. Positive controls were normalized to untreated incubator controls. The normalized data are presented as single data points with mean, the Y-axis is plotted in Log2 scale (as data represent fold changes). In the text, fold changes relative to the control (filtered air or untreated control) are presented as mean ± SEM. Statistics were performed with GraphPad Prism using two-way and one-way ANOVAs.

3. Results

3.1. Exhaust emissions of two GDI cars

3.1.1. Particle number count

The GPFs attached to vehicle GDI1 diminished the PN by 94% (uncoated) and 78% (coated), thus significantly reducing the amount of PN (Fig. 2A). PN emissions were identical in the two exposure weeks in GDI1 reference. Comparing the two GDI cars without filters, GDI1 and GDI2 reference emissions showed PN concentrations in the same order of magnitude ($1.5*10^5$ and $3.5*10^5$ #/cm^3, respectively). The different lube-oil additions increased the PN value and the attached GPF removed almost all particles (99.8%) (Fig. 2A). In particular the small particle fractions (nuclei-mode) were increased while the accumulation-mode was comparable to GDI2 reference (SupFig. 1). Differences in ash and metal content of the two oils only slightly changed particle size distribution and PN (SupFig. 1).

3.1.2. Volatile exhaust emissions

The legislated volatile exhaust components, *i.e.* HC, NOx, and CO were measured in the CVS tunnel (Fig. 2B–D).

The two filters installed in GDI1 vehicle not only lowered PN concentrations, but also HC and NOx levels, CO levels remained unchanged. The second measurements with GDI1 reference yielded higher HC and NOx but similar CO concentrations (Fig. 2B and C).

Comparing the two reference vehicle emissions, increased HC-emissions were observed in GDI2 (9 ppm) compared to GDI1 (5 ppm) reference (Fig. 2B), while NOx remained low in both (Fig. 2C). CO levels were higher in GDI1 reference (25 ppm) than in GDI2 reference 2 (16 ppm).

The unfiltered lubrication oils did not alter HC and NOx emissions in GDI2, but the high-ash GPF increased HC and NOx levels (Fig. 2B and C).

CO levels were found to increase with lubrication oil addition. All three settings increased CO levels compared to GDI2 reference, the highest values being found in GDI2 high-ash filtered (69 ppm) and low-ash (61 ppm), as illustrated in Fig. 2D.

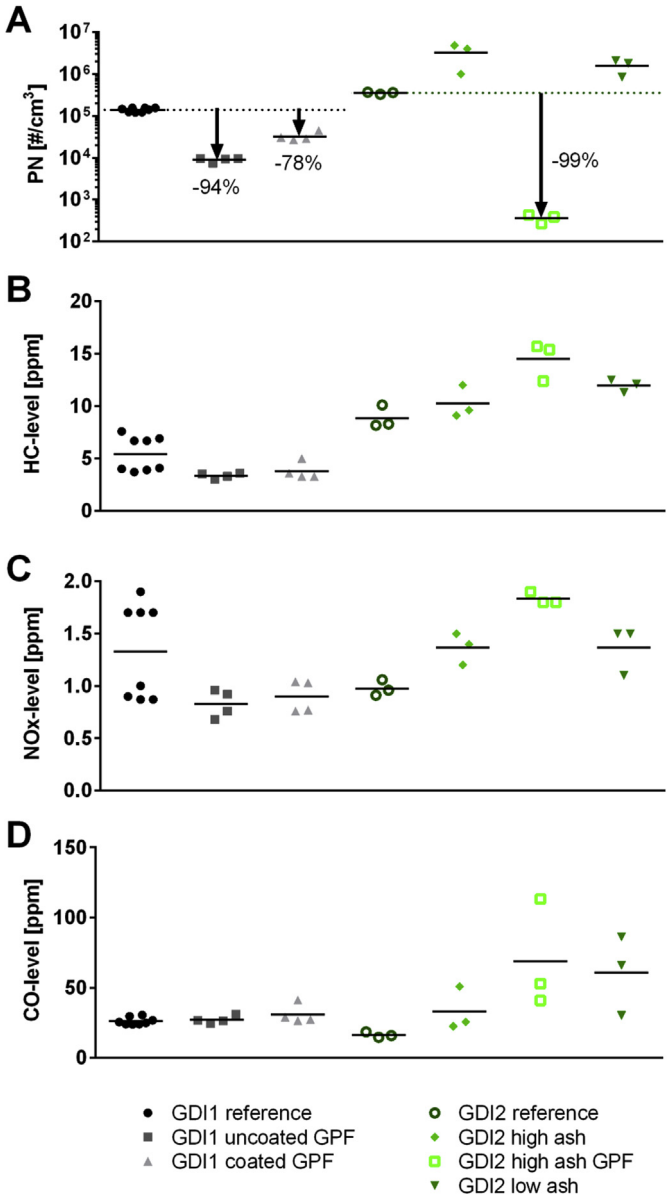

Fig. 2. Exhaust emissions of two gasoline direct injection (GDI) cars with different settings. PN concentrations were measured in the diluted exhaust (A), while the volatile emissions, *i.e.* HC (B), NOx (C), and CO (D), were measured in the CVS-tunnel. The three filters reduced (and lube-oil addition increased) PN concentrations, the reference cars had similar PN emissions. The three filters (GPFs) had particle count filtration efficiencies of 94%, 78%, and 99.8%. One data point on the graph represents one exposure day, each is an average of at least 6 WLTC (including cold-start). Values are shown diluted as they reach the cell cultures.

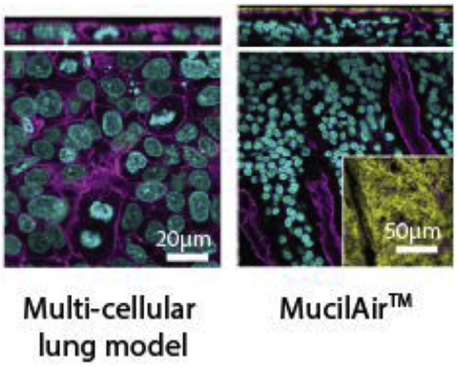

Fig. 3. Representative images acquired by laser scanning microscopy. Cell nuclei are shown in cyan, the F-Actin cytoskeleton in magenta, and the cilia in the MucilAir™ cultures in yellow. Both pictures represent XY (lower image) and XZ projections (upper image).

3.2. Biological effects in two lung cell culture models after exposure to exhaust aerosols

3.2.1. Morphology and cytotoxicity

The 6 h exhaust exposure and 6 h post-incubation period applied to the different exhausts did not produce any visible morphological changes in the co-cultures with the 16HBE14o-cells and were comparable to the filtered air or the incubator control (Fig. 3 and SupFig. 2). MucilAir™ also showed no morphological changes upon exhaust exposures (Fig. 3 and SupFig. 3). A pseudostratified epithelium is observed, closely mimicking the bronchi/upper respiratory tract regions. Additionally, cilia are shown in a separate box (SupFig. 3). Cytotoxicity, as measured with the LDH assay, was not enhanced for all tested exhaust and cell culture conditions. The highest variation was found in the high-ash GPF exposure (SupFig. 4), however, this was not statistically significant. Overall, no morphological changes or increases in cytotoxicity were observed, this is crucial to exclude non-specific cytotoxic effects in the further gene expression analysis.

3.2.2. Gene expression of three oxidative stress-related genes

HMOX1 expression was unchanged in both cell cultures upon gasoline exhaust exposures, while the positive controls induced 6.6 ± 1.3 and 6.7 ± 2.7-fold in the multi-cellular lung model and MucilAir™, respectively (Fig. 4A). Additionally, lysates of GDI2 exhaust treated cells of the multi-cellular lung model were used to evaluate the HMOX1 protein level by ELISA. No increase in protein expression was observed confirming the results of the gene expression analysis (data not shown).

mRNA of *SOD2* was not increased after exposure to the different exhausts, while the positive controls induced *SOD2* expression 1.8 ± 0.1-fold (multi-cellular lung model) and 2.2 ± 0.2-fold (MucilAir™) (Fig. 4B).

NQO1, the third oxidative stress marker assessed, was also unchanged in both cell culture systems, while the positive control induced an oxidative stress response (2.6 ± 0.4-fold). The MucilAir™ cultures were only marginally increased (1.5 ± 0.2-fold) (Fig. 4C).

3.2.3. Measurement of two pro-inflammatory cytokines at the gene and protein level

There was no increase in *CXCL8* (both systems) or *TNFa* (multi-cellular lung model) when the cells were exposed to filtered and unfiltered GDI1 car exhausts (Fig. 5). Vehicle GDI2, when driven with high-ash oil and a GPF, showed a moderate but not significant increase of *CXCL8* expression in the multi-cellular lung model compared to filtered air controls, but not in MucilAir™ cultures (Fig. 5). This increase is accompanied by a high variability, similar to that seen in the LDH level. Protein measurements in the supernatants confirmed the results of gene expression analysis (SupFig. 5).

3.3. Repeated exposure to the multi-cellular lung model

As with the one-time exposures, no morphological changes in the cells were observed (SupFig 6). In the gene expression analysis, a significant increase in *HMOX1* (Fig. 6A) and *TNFa* (Fig. 6B) was observed compared to the respective filtered air exposure, but this was not significant in comparison to the single exposure.

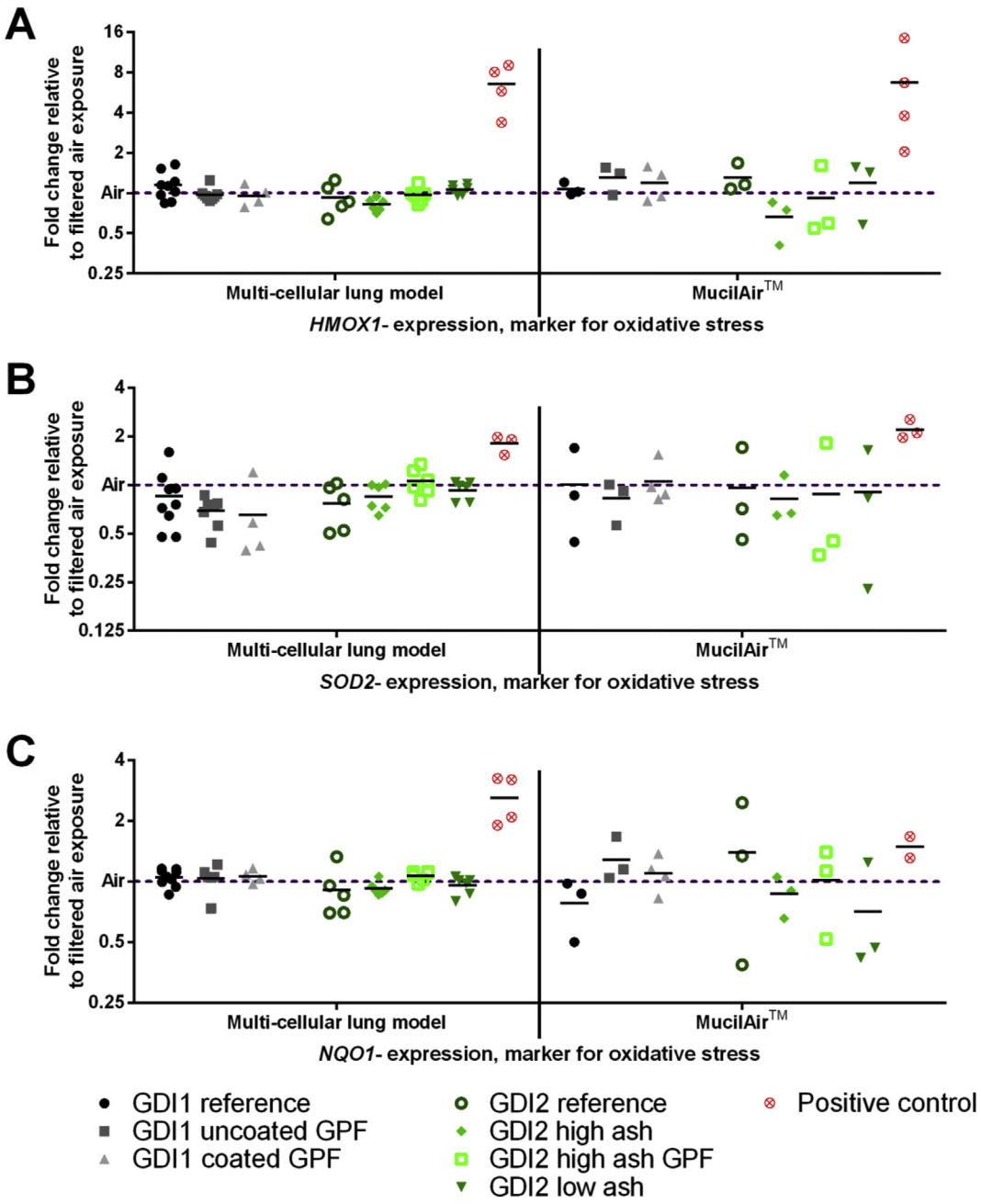

Fig. 4. Oxidative-stress-related gene expression in two different cell culture models and seven different gasoline direct injection (GDI) exhausts. Three different genes were measured, *HMOX1* (A), *SOD2* (B), and *NQO1* (C). No induction of oxidative stress was observed. Data for the GDI1 car with the two filters (GPFs) are displayed in black to grey, while the effects of GDI2 car are shown in green shapes. Positive controls are shown in red circles (see materials and methods for detailed information).

4. Discussion

Two different GDI vehicles, *i.e.* GDI1 and GDI2, were driven on a chassis dynamometer. The exhaust emissions were variied by application of two GPFs (GDI1) or by addition of two lubrication oils to the fuel in combination with a GPF (GDI2). The exhausts were applied to two human lung cell culture models at ALI and endpoints like cell viability, oxidative stress, and pro-inflammation were assessed (Fig. 1).

The analysis of the exhaust revealed an efficient filtration of the applied GPFs. The GPF on GDI2 had the highest efficiency with 99.8% removed particles, even during the first cycles on the first day (data not shown), where the GPF was freshly cleaned. This is interesting, as usually GPFs only efficiently remove particles when soot-loaded.

The coated GPF was installed after the three-way catalyst and included in this study to target potentially carcinogenic volatile compounds and reduce CO and HC levels. However, no CO-reduction and only low HC-reduction was observed in the filtered exhaust. This could be due to low levels of oxygen reaching the GPF, as they were used up by the three-way catalyst, or because CO (and HC levels) were already low and further reduction could not or only partially be achieved. Further analysis would be necessary to fully understand the catalytic action.

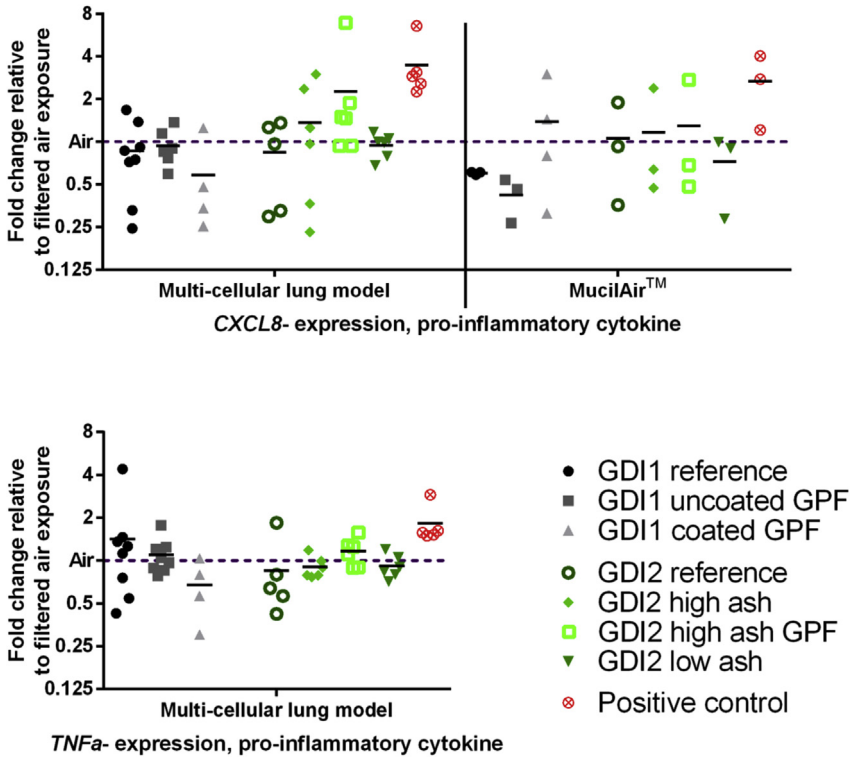

Fig. 5. Pro-inflammatory cytokine gene expression in the multi-cellular lung model (left) and MucilAir™ after exposures to gasoline direct injection (GDI) exhausts. *CXCL8* (A) and *TNFa* (B) were measured. A slight induction in *CXCL8* in the GDI2 high ash GPF exhaust is observed due to one high value. Data for the GDI1 car are shown in black to grey shapes, those for the GDI2 car are shown in green colors. Positive controls are shown in red circles (see materials and methods for detailed information).

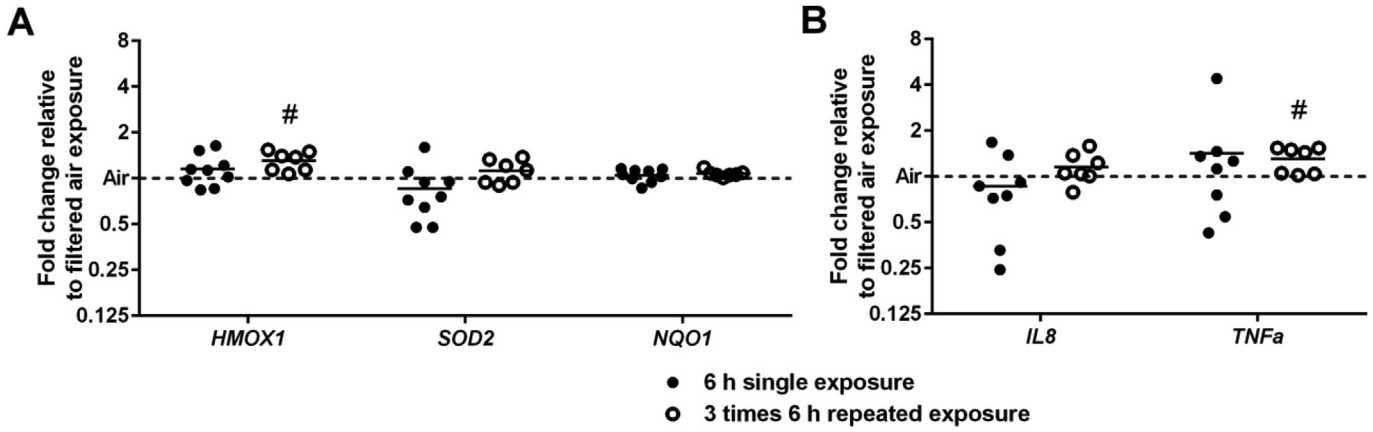

Fig. 6. Gene expression analysis of (A) oxidative-stress-responsive genes and (B) pro-inflammatory cytokines in the multi-cellular lung model. GDI1 reference vehicle exhaust was applied on one (black dots) or three consecutive days (black circles) for 6 h each. There is no significant difference between the 6 h and the three times 6 h exposure. However, small but significant increases in *HMOX1* and *TNFa* expression levels were observed in the repeated exposure (compared to filtered air control). Two-way ANOVA, $p > .05$.

Addition of lubrication oils in the fuel changed the PN size distribution, a dominant fraction between 10 and 60 nm is observed, as typically seen in two-stroke engines (Rijkeboer et al., 2005), where metals act as seed crystals for particles.

The oxidative potential of the different gasoline exhausts was evaluated by measuring the mRNA levels of three genes, *i.e. HMOX1, SOD2,* and *NQO1.* HMOX1 catalyzes the rate-limiting step in the conversion of heme to bilirubin and is an important protein related to oxidative stress (Yachie et al., 1999; Clark et al., 2000). SOD2 converts superoxide to hydrogen peroxide, which can further be catalyzed to water and oxygen, one of the first proteins in the response against oxidative stress (Fridovich, 1978). NQO1 is a phase II detoxifying enzyme induced after oxidative stress and PAH exposure (Jaiswal, 1991; Ross et al., 2000; David et al., 2003). All three oxidative-stress-related genes revealed no upregulation upon a 6 h exposure to any of the exhaust samples, neither in the multi-cellular lung model nor in MucilAir™ cultures (Fig. 4). In an earlier study published by this group, we found an upregulation of *HMOX1*, *SOD2*, and *NQO1* in unfiltered (but not filtered) exhaust, however, a different gasoline car was used (Bisig et al., 2015). Measured PN emissions in the previous study were comparable to those from GDI1 and GDI2 herein (PN $2.3*10^5$ #/cm^3 versus $1.5*10^5$ and $3.5*10^5$

#/cm^3) (Bisig et al., 2015). The differences between the current study and the study published in 2015 could be caused by the possible different semi-volatile organic compound emissions (e.g. PAH). PAH-concentrations, including genotoxic PAHs, in gasoline engine exhaust emissions can vary significantly, depending on engine load and vehicle type (Alves et al., 2015; An et al., 2016; Muñoz et al., 2016), and further studies are required to analyze these exhaust components. In another study, different ethanol-gasoline fuel blends with vehicle GDI1 did not induce oxidative stress (Bisig et al., 2016) as was reported in this work, indicating that adverse effects of exhaust emissions are vehicle-dependent and cannot be generalized. Only one other study on oxidative stress induced by gasoline exhaust was found: Maikawa et al. reported that murine precision-cut lung slices showed upregulated *HMOX1* expression after exposure to unfiltered (1.63 ± 0.03-fold) and filtered (1.55 ± 0.04-fold) GDI exhaust (Maikawa et al., 2016). Again, the *HMOX1* expression was not upregulated here, and differences in exposure setup and biological models have to be taken into account.

Two cytokines were measured to assess pro-inflammation, *i.e. CXCL8* and *TNFa.* CXCL8 is produced by various cell types and attracts neutrophils to the site of injury (Jundi and Greene, 2015). TNFa is involved in inflammation, immunity, and apoptosis, is primarily expressed by monocytes, fibroblasts, and endothelial cells, and acts on epithelial and endothelial cells in the airway (Mukhopadhyay et al., 2006; Sabio and Davis, 2014). Since no immune cells were present in the MucilAir™ system, we only analyzed the expression of *CXCL8* in these cultures. So far, studies with the same exposure system and multi-cellular lung system only showed an upregulation of pro-inflammatory cytokines when diesel cars were used (Steiner et al., 2013), but not in the two studies with gasoline engine exhaust (Bisig et al., 2015, 2016). A study by Cheng et al. also showed no increase in CXCL8 protein expression of A549 cells at the ALI after exposure to gasoline exhaust (Cheng et al., 2003). To mimic a pro-inflammatory condition they treated A549 with TNFa prior to gasoline exhaust exposure and found significant increases in CXCL8 secretion. No information on the engine type was provided in this study, also no exhaust-analysis of the volatile fraction is given and only PN values were reported in the range from 1 to 2 $*10^6$ #/cm^3 (Cheng et al., 2003), which is about ten times higher than our results.

Both models mimic the human bronchi, one comprises an epithelial monolayer combined with primary immune cells, the other is a pseudostratified epithelium with cilia and mucus. In neither model a response upon a short-term exposure to gasoline engine exhaust in any condition was observed. The use of two cell culture models is a strength of this study, however, no recommendation can be given on which model should be used in future, though it is evident that long term experiments (weeks to months) can only be performed with MucilAir™.

The herein presented (as well as previously published) results were performed in an acute scenario over 6 h exposure, however, humans may be exposed to air pollution daily and chronic exposures could give important additional insight into possible adverse effects of gasoline exhaust. To test the feasibility of a prolonged exposure, a repeated exposure of three 6 h exposures was performed with the multi-cellular lung model and one vehicle (GDI1 reference). Only one cell model was used due to shortage of available space in the exposure chambers, *i.e.* the multi-cellular lung model was chosen because the first results of the study pointed towards similar reactions to gasoline exhaust and the model was readily available. This repeated exposure led to a significant increase of *HMOX1* and *TNFa* each by 1.3-fold. We have shown that diesel engine emissions can induce a 41-fold and 1.7-fold upregulation of *HMOX1* and *TNFa*, respectively, applying exactly the same exhaust exposure system and multi-cellular lung model (Steiner et al., 2013), thus our results herein show relatively low inductions. Nevertheless, this indicates that repeated gasoline exhaust emissions do cause mild responses in our *in vitro* cell culture model.

5. Conclusion

Few experimental studies on gasoline exhaust and the effects of GPFs have been reported to date, even though GDI engines can emit a substantial number of particles. To the best of our knowledge, this is also the first study including a worst-case scenario with direct lubrication oil addition into the gasoline fuel. No increases in oxidative stress or pro-inflammation were measured after acute exhaust exposure (unfiltered, GPFs, and lubrication-oil addition) to two different human cell culture models. The limitations of the present study are, however, that only a limited number of genes and proteins have been analyzed. However, we have observed a significant decrease of PN for both GPFs, and even without proof of adverse effects an implementation of particle filters also for gasoline cars is highly recommended.

It was hypothesized that the lubrication oils will have different adverse effects when mixed with the fuel, and as no adverse effects were measured, no recommendation from a toxicological point of view can be given at this point. Again, it would be important to study more genes and proteins in future studies. In addition, we have only focused on short-term effects, studies with chronic exposure will be needed to better understand the potential long-term effects. The multi-cellular lung model is viable in exposures for up to three days, and such an exposure to the GDI1 reference indicated that oxidative stress and pro-inflammation is induced, which is valuable information for future experiments.

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Conflicts of interest

Andreas Mayer is the owner and general manager of “TTM Andreas Mayer”, Switzerland, an emission consulting company. Neither he nor the other authors declare a conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.061.

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