Review

In vitro exposure to complete engine emissions – a mini-review

Pavel Rossner Jr. [a,*], Tereza Cervena [a], Michal Vojtisek-Lom [b]

[a] *Department of Nanotoxicology and Molecular Epidemiology, Institute of Experimental Medicine of the CAS, Videnska 1083, 142 20, Prague, Czech Republic*
[b] *Centre of Vehicles for Sustainable Mobility, Faculty of Mechanical Engineering, Czech Technical University in Prague, Technicka 4, 160 00, Prague, Czech Republic*

ARTICLE INFO

ABSTRACT

Outdoor air pollution is classified as carcinogenic to humans and exposure to it contributes to increased incidence of various diseases, including cardiovascular, neurological or pulmonary disorders. Vehicle engine emissions represent a significant part of outdoor air pollutants, particularly in large cities with high population density. Considering the potentially negative health impacts of engine emissions exposure, the application of reliable test systems allowing assessment of the biological effects of these pollutants is crucial. The exposure systems should use relevant, preferably multicellular, cell models that are treated with the complete engine exhaust (i.e. a realistic mixture of particles, chemical compounds bound to them and gaseous phase) at the air-liquid interface. The controlled delivery and characterization of chemical and/or particle composition of the exhaust should be possible. In this mini-review we report on such exposure systems that have been developed to date. We focus on a brief description and technical characterization of the systems, and discuss the biological parameters detected following exposure to a gasoline/diesel exhaust. Finally, we summarize and compare findings from the individual systems, including their advantages/limitations.

1. Introduction

The pollution of ambient air significantly affects the health of the human population worldwide, contributing to increased incidence of pulmonary, cardiovascular or neurological diseases (An et al., 2018; Combes and Franchineau, 2019; Killin et al., 2016). Outdoor air pollution was classified by the International Agency of Research on Cancer (IARC) as carcinogenic to humans (Group 1, (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans and International Agency for Research on Cancer, 2015)). The biological impacts of air pollutants have been widely studied for more than five decades using numerous toxicological approaches, including tests *in vitro* in cell models of various origin, *in vivo* animal studies, as well as epidemiological investigations in human populations. Although the assessments conducted in human organisms provide the most reliable information on the health effects of air pollutants, such experiments are time-consuming, costly and limited by ethical considerations. Thus, animal tests have been commonly used in accordance with the OECD guidelines (Hayes and Bakand, 2010), and the results extrapolated to human organisms. However, the application of the 3R concept (replacement, reduction, refinement) in animal experimentation and differences between human and animal organisms (e.g. lung anatomy, inhalation capacity, or populations of immune cells) necessitate alternatives to *in vivo* tests using cell cultures (Rehberger et al., 2018; Romeo et al., 2020; Upadhyay and Palmberg, 2018).

In vitro testing of air pollution is complicated by a complex character of ambient air that contains a mixture of gaseous pollutants [e.g., ozone, SO_2, CO, NO_x, volatile organic compounds (VOC)], particulate matter (PM) of various size and compounds bound to it, including e.g., polycyclic aromatic hydrocarbons (PAHs) and metals. Depending on the aerodynamic diameter, PM is classified as coarse (PM10, $\leq 10\ \mu m$), fine (PM2.5, $\leq 2.5\ \mu m$) or ultrafine (PM0.1, $\leq 0.1\ \mu m$) fraction. In the lungs, fine particles penetrate into alveoli and can interfere with the gas exchange between inhaled air and blood (Almetwally et al., 2020), while the ultrafine fraction enters individual cells including subcellular structures, and can be distributed to distant tissues and organs via the blood stream. The size of the particles, along with the chemical composition of the complex mixture, determines the possible adverse biological effects of air pollutants. In the organism, the effects mediated by PM are linked to both the physical properties of PM and the chemical

Table 1

An overview of exposure systems allowing treatment of relevant tissues/cells with complete engine emissions and results of studies in which the systems were first used.

System	Tissue/cellular model	Fuel	Exposure conditions	Results	First report
1	Rat lung tissue	Diesel	1 h exposure + 1 h, 24 h recovery	Decreased ATP, GSH levels and SOD activity; no effect on CAT and GPx	(J. P. Morin et al., 1999a)
2	Human bronchial epithelial cells (BET-1A)	Diesel	Up to 14 h exposure	Decreased [^{3}H] thymidine incorporation; increased production of IL-8, but not IL-6, IL-10 and TGF-1β protein to supernatant; induction of *IL-6*, *IL-8*, *TGF-1β*, but not *IL-10* mRNA expression	(Abe et al., 2000)
3	Human bronchial epithelial cells (HFBE 21)	Diesel	1 h exposure + 2 h post-exposure	Decreased cell viability (WST-1, cell number)	(Aufderheide et al., 2002; Knebel et al., 2002)
4	Human bronchial alveolar carcinoma cells (A549)	Diesel Gasoline	Up to 6 h exposure	Increased interleukin-8 (IL-8) production for some conditions	(Cheng et al., 2003)
5	Human bronchial epithelial cell line (16HBE14o)	Diesel	6 h exposure + 20 h post-exposure	Increased IL-8 production linked with particles presence	(Holder et al., 2007)
6	Human bronchial alveolar carcinoma cells (A549)	Diesel	1 h exposure + 9 h post-exposure	Increased LDH and IL-8 production when compared with power off	(de Bruijne et al., 2009)
7	Triple co-culture (A549, macrophages, dendritic cells) Triple co-culture (16HBE14o-, macrophages, dendritic cells)	Gasoline (two-stroke)	1 h, 2 h exposure + 0-24 h post-exposure	No significant effects observed Increased LDH production after 2 h exposure and 8 h post exposure; elevated IL-8 and TNF-α production after 2 h exposure followed by 12 h post exposure	(Müller et al., 2010)
8	Human bronchial alveolar carcinoma cells (A549)	Diesel	3h exposure + 21 h post-exposure	No effect on AK activity, IL-8 and IL-6 promoter induction and IL-8, IL-6, MCP-1, IL-1beta production	(Oostingh et al., 2013)
9	Human bronchial alveolar carcinoma cells (A549)	Diesel	15, 30 and 60 min	Cell viability (ATP, MTS, NRU assays) decrease depending on air flow and time of exposure	(Joeng et al., 2013)
10	Human primary bronchial epithelial cells	Diesel	1 h – 6.25 h exposure	Decreased TEER, increased *HMOX-1*, *NQO-1, CHOP, GADD34, CXCL8* expression and IL-8 production No effect on LDH production and expression of *XBP1spl* and *BiP*	(Zarcone et al., 2016)
11	Human bronchial alveolar carcinoma cells (A549) Human bronchial epithelial cell line (BEAS-2B)	Diesel (ship engine)	4 h exposure	A comprehensive transcriptome, proteome and metabolome analyses: deregulation of inflammation (e.g. IL-8, IL-6, IL-1) and xenobiotic metabolism (e.g. CYP1A1) markers and pathways related to cell motility, stress response, proliferation or cell death	(Oeder et al., 2015)
12	Human bronchial alveolar carcinoma cells (A549) Human bronchial epithelial cell line (BEAS-2B)	Gasoline (motorcycle)	1 h exposure 30, 60 and 90 min	0.2 μm-filtered and non-filtered exhaust: increased cytotoxicity (WST-8, LDH), increased ROS production, more pronounced effects in non-filtered samples 0.2 μm-filtered exhaust only: significant cytotoxicity (WST-8) after 60 and 90 min of exposure	(Yu et al., 2017)
13	Human bronchial epithelial cell line (BEAS-2B) A commercial 3D model	Gasoline+20% ethanol	2 x 1 h exposure (1 day) 2 x 1 h exposure (repeated, 5 days)	Increased LDH, mucin production and H2AX phosphorylation, but no other biologically significant changes (AK production, micronuclei formation, changes of gene expression, lipid peroxidation, immunomodulatory markers) Changes of TEER and decreased *CYP1A1*, increased *HSPA5* expression; no other biologically significant changes (LDH, AK, mucin production, weak impact on lipid peroxidation and proinflammatory cytokines production)	(Rossner et al., 2021, 2019)

composition of the compounds adsorbed to it. The presence of particles themselves contributes to the activation of the immune system, potentially resulting in the production of reactive oxygen species (ROS) by the immune cells. ROS cause oxidative damage to macromolecules, including nucleic acids, inducing mutations, and thus increasing the risk of tumorigenesis (Klaunig, 2019). Additionally, ROS act as signaling molecules affecting the expression of genes involved in metabolic regulation or stress response (Sies and Jones, 2020). Some of the compounds bound to PM, notably polycyclic aromatic hydrocarbons (PAHs), are possibly carcinogenic, or carcinogenic to humans. The effect of PAHs is exerted upon desorption in the organism and metabolic activation to reactive intermediates, that bind to macromolecules, including DNA, and form bulky adducts that affect the function of macromolecules and contribute to the induction of mutations (Moorthy et al., 2015). PAH reactive intermediates may also be metabolized into *o*-quinones, that enter redox cycling and cause oxidative stress by the formation of ROS (Moorthy et al., 2015). The presence of transition metals in PM further contributes to ROS generation and the oxidative damage of macromolecules (Moller et al., 2014). Although the effect of gaseous components of air pollution on human health is less significant than that of PM (Hamanaka and Mutlu, 2018), they further potentiate pro-oxidant and pro-inflammatory properties, as well as the overall carcinogenicity of polluted air. It has been demonstrated that complete diesel emissions induce the production of interleukin (IL)-8, a pro-inflammatory cytokine. The removal of volatile and semi-volatile organic compounds from the exhaust greatly reduced the release of this molecule, suggesting that VOC are important factors modulating the inflammatory response caused by diesel exhaust (Holder et al., 2007).

Overall, the toxicity of polluted air arises from a complex mixture of various components. Therefore, for a comprehensive evaluation of the biological effects of air pollutants, the application of exposure experiments that involve a complete mixture of ambient air pollutants (i.e. a complex of gaseous pollutants and PM with adsorbed compounds in the form they exist in the atmosphere) is crucial. Despite this fact, studies investigating the *in vitro* toxicity of ambient air are often limited to the exposure of monocellular cultures to organic or water-based extracts from PM, or PM separated from the ambient air using filters or other methods (Zavala et al., 2020). The application of these approaches results in the selective investigation of a group of pollutants (e.g. organic compounds bound to PM), their physical alteration (e.g. agglomeration of particles during their collection and resuspension in cell culture media) and the omission of aerosol aging, secondary pollutant formation, and the interactions between complex mixtures of air pollutants and the organism.

For many years, toxicological experiments relied on testing in submerged *in vitro* monocellular models. However, for inhalation toxicity
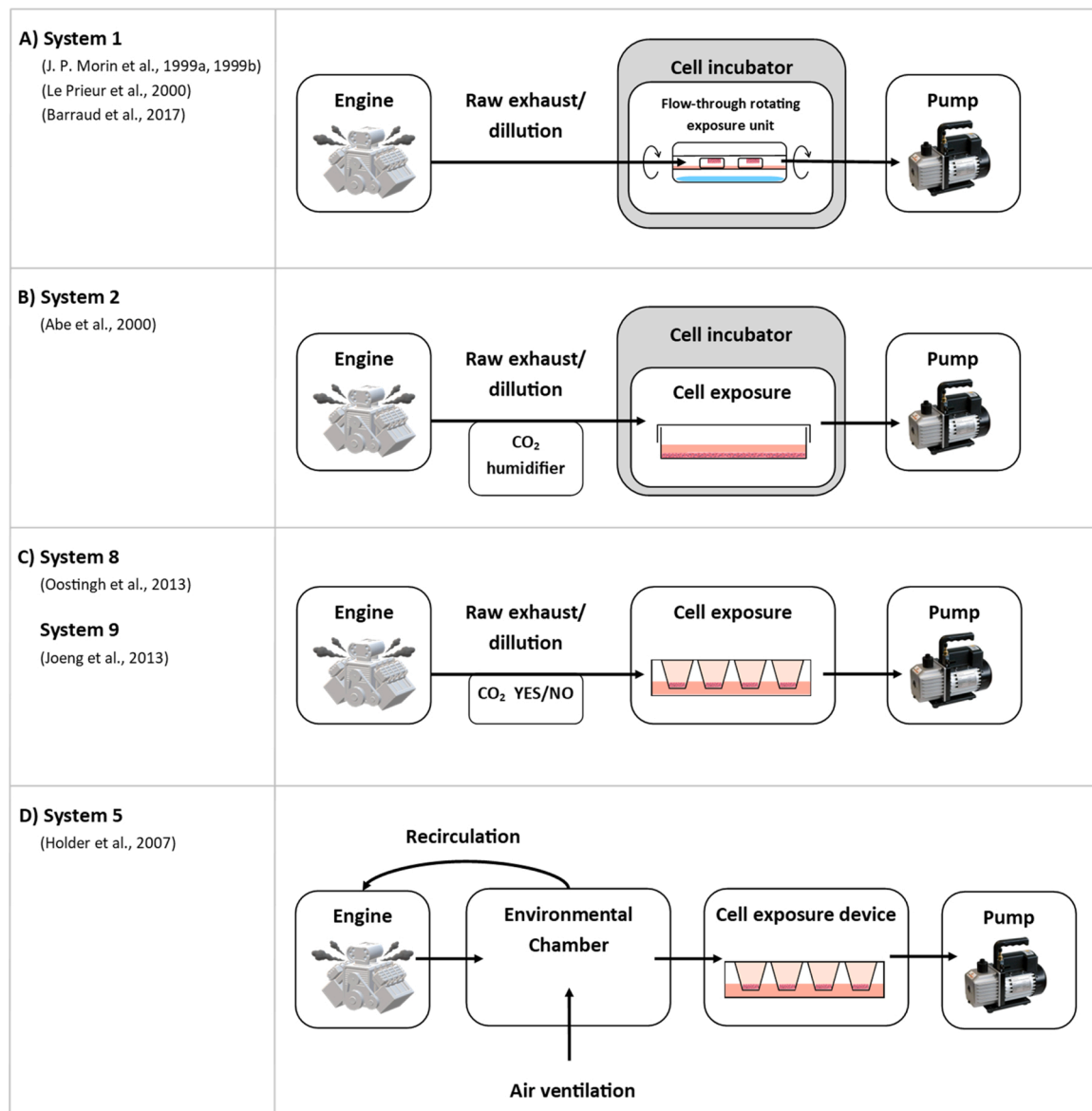
Fig. 1. A schematic overview of exposure systems used with complete engine emissions (part 1).

A) System 1 used rotating chambers to expose rat lung slices; B) In System 2 cells were treated in the culture dishes at the ALI; C) System 8 and 9 used Transwell inserts to which emissions were not routed by a dedicated distributor head (indirect exposure); D) System 5 contained an environmental chamber and a cell incubator in which samples were treated in Transwell inserts by undirect exposure.

experiments such cell cultures are not optimal, as they lack the physiological features of airway mucosa that consist of more than 40 different cell types (Upadhyay and Palmberg, 2018). Among other cells, the airway epithelium contains ciliated cells interspersed with goblet cells that produce mucous, as well as the immune cells such as macrophages, neutrophils or dendritic cells. To avoid the generation of misleading results and inaccurate conclusions, the experiments in submerged conditions have been replaced by other approaches in which tested pollutants are delivered to the cell cultures either via bubbling through the culture medium, exposing the cells incubated on collagen gels or microporous membranes, or cell cultures grown at the air-liquid interface (ALI) (Aufderheide, 2005). The latter approach is considered the most advanced, providing the data most relevant to real-world exposure scenarios.

As demonstrated in this mini-review, in most experiments particles are deposited by diffusion and/or gravitation. The ALI exposure method is considered superior to the application of particles in suspension as it exhibits a similar response at a significantly lower dose, and allows avoiding artifacts caused by the collection of the particles and their suspension in a culture media. Additionally, the number of particles deposited in the ALI cultures is similar to that in the bronchoalveolar region, although the particles tend to be of a larger size (Holder et al., 2008). Resuspended particles have been shown to lack carbonyl compounds compared to direct aerosol exposure, suggesting that the toxicity of such particles is underestimated (Lichtveld et al., 2012). Several types of ALI exposure systems have been developed, including primary cell-based models, co-culture systems, and diseased ALI cultures (reviewed in (Upadhyay and Palmberg, 2018)).

In general, the ALI systems can be used for cell exposure to aerosolized particles, including nanoparticles, gases or complex mixtures of both types of pollutants. Their application has been reported e.g. for cigarette smoke, e-cigarette vapors, environmental airborne chemicals, drugs delivered by inhalation or respiratory viruses and bacteria, as summarized in the recent review (Cao et al., 2021). These systems can be obtained commercially, or developed in-house. The following text specifically focuses on the description of the exposure systems, most of them
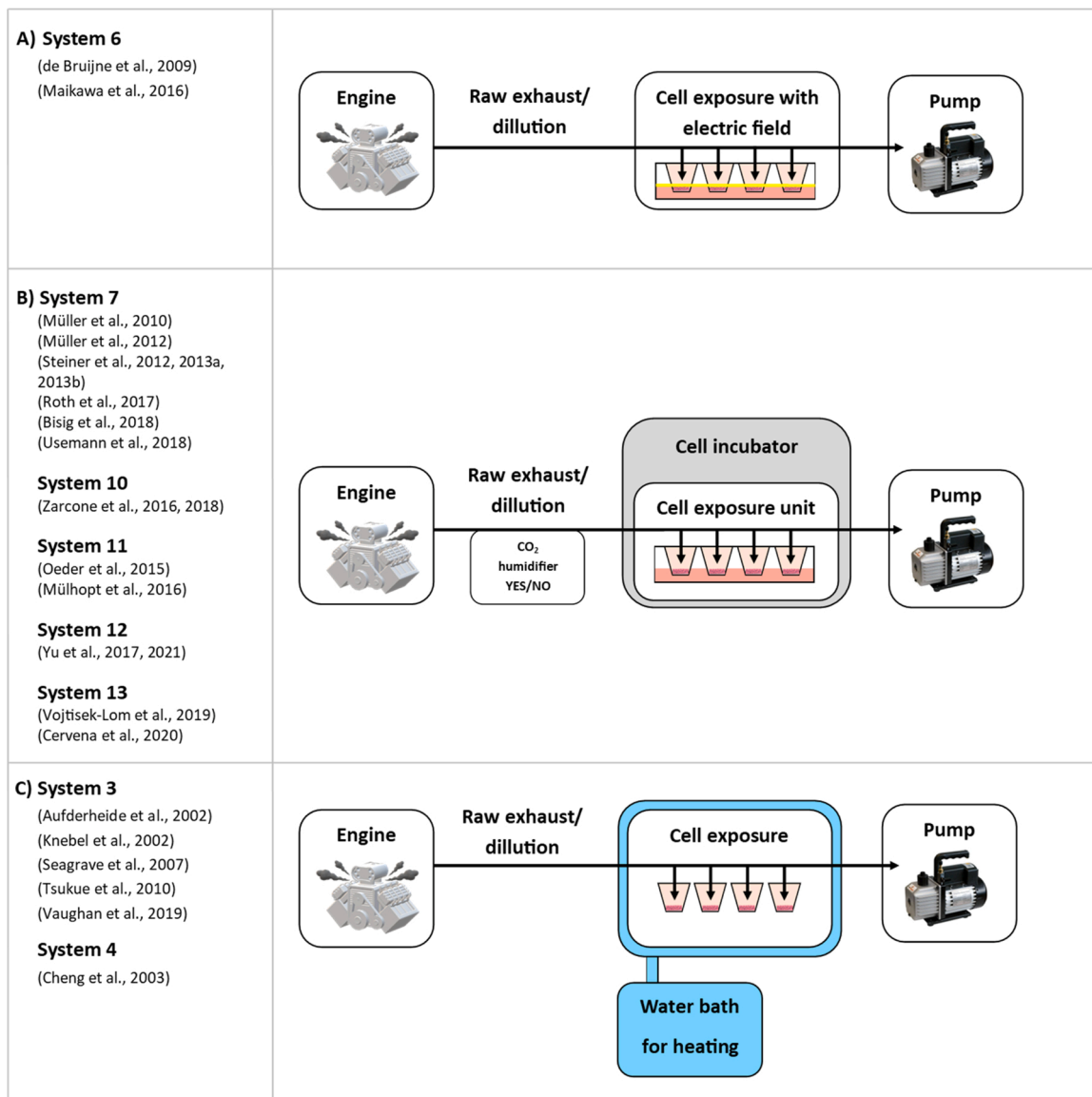
Fig. 2. A schematic overview of exposure systems used with complete engine emissions (part 2).
A) In System 6, exhaust was directly routed to cell inserts (direct exposure) and electric field was applied; B) System 7 includes various units, either commercial, or in-house fabricated, all involving direct exposure; C) Systems 3 and 4 (an in-house fabricated) are characterized by direct exposure and application of water bath for heating the samples.

based on the ALI cultivations, used to assess the toxicity of complete engine emissions. A brief description and technical parameters of these systems is provided, and exposure experiments along with the analyzed biological markers are discussed.

2. An overview of systems allowing exposure of cell cultures to complete engine emissions

Over the years, attempts have been made to develop exposure systems that would allow the treatment of relevant cells/tissues to complete engine emissions (Cheng et al., 2003; de Bruijne et al., 2009; Holder et al., 2007; Knebel et al., 2002; Morin et al., 1999a; Müller et al., 2010; Oostingh et al., 2013; Vojtisek-Lom et al., 2019; Zarcone et al., 2016). A chronological list of the studies in which given systems were first described is provided in Table 1. A schematic overview of individual exposure systems grouped according to the basic characteristics of their construction (e.g., samples in rotating units, culture dishes, or Transwell inserts; emissions routed indirectly, or via a distributor head; the

application of an electric field to improve particles deposition; heating with a water bath or in an incubator), is shown in Figs. 1 and 2. The following text is a comprehensive overview of the experiments performed using the exposure systems that summarizes the main findings and compares individual test approaches. Although technical solutions for individual exposure systems are briefly mentioned, the main focus of this minireview is on biological impacts of engine emissions exposure in relation to treatment conditions (type of fuel used for emissions generation, time of exposure) and cell models tested in individual studies.

3. A description of exposure systems and main biological findings

3.1. Rat lung slices exposed to diesel emissions (System 1)

Arguably the first *in vitro* system designed to investigate the impacts of exposure to complete diesel exhaust on lung tissues was reported by Morin et al. (1999a, 1999b) (Fig. 1A). The system consisted of rotating

chambers with a controlled atmosphere (20% O_2, 5% CO_2, 85-90% humidity), in which rat lung slices were positioned in rolling inserts that allow the access of emissions to the tissues. The authors confirmed the viability of the cells in the system for at least 48 h, and showed that exposure of the tissues to 5x diluted diesel emissions (a continuous flow of 2.5 L/min) decreased intracellular ATP and GSH levels, and reduced superoxide dismutase (SOD) activity after 1 h exposure. Interestingly, no effect of complete emissions on the activities of other antioxidant enzymes [(catalase (CAT), glutathione peroxidase (GPx)] was detected. The decreased levels of GSH and SOD suggest the induction of oxidative stress that was compensated by antioxidant mechanisms. This study was unique by the application of real animal tissues that retained cell-cell interactions, biochemical and histological properties, although the preparation of such samples was time-consuming, and the stability and reproducibility of the experiments might be limited. In the follow-up study, five concentrations (10, 15, 25, 60 and 85%) of diesel exhaust were used to treat rat lung slices for 3 or 6 h (Le Prieur et al., 2000). In the exposed samples, DNA integrity (detection of nucleosomes and apoptosis induction) and cytokine production [tumor necrosis factor (TNF-α), IL-1β] was assessed. The authors observed the effects manifested by increased nucleosome levels, and elevated TNF-α concentration in the culture medium after 3 h treatment and apoptotic response after 6 h exposure.

The exposure system was later used to expose standard cell cultures grown at the ALI to diesel engine emissions, for which various after treatment strategies were applied (diesel oxidation catalysis, diesel particulate filter) (Barraud et al., 2017). In the experiments, human alveolar basal epithelial cells (A549) were treated with emissions from standard diesel fuel and rapeseed methyl ester-diesel blends (7, 30%) for 3 h, followed by an analysis of cytotoxicity, oxidative stress and DNA damage markers. The treatment induced cytotoxicity, measured as lactate dehydrogenase (LDH) release. The authors did not detect any significant differences between the tested emissions when ROS production, glutathione levels, catalase and superoxide dismutase activities were assessed. The exposure to the standard diesel emissions induced oxidative DNA damage, that was reduced after the application of a catalysts/particulate filter. The tested emissions also caused DNA breaks, detected as histone H2AX phosphorylation.

3.2. A human bronchial epithelial cells monolayer exposed to diesel emissions in an in-house system located in a cell incubator (System 2)

In this exposure system, the first one to apply the ALI incubation conditions (Fig. 1B), the emissions generated by a diesel engine were used to treat BET-1A cells, a simian virus 40-transformed human bronchial cell line. The diesel engine was operated at a speed of 1050 rpm and 80% load, and the emissions were diluted with temperature- and humidity-controlled clean filtered air at 1:8 ratio. The exhaust was characterized for particle concentrations and their distribution, as well as the content of gases, including CO, NO_2 and SO_2. It was introduced at a constant flow of 5 L/min into the exposure system, consisting of a polystyrene container placed in the incubator with stable conditions (5% CO_2, 37 °C) (Abe et al., 2000). The biological impacts of diesel emissions exposure lasting 0.5, 1, 2, 4, 8 and 14 h were investigated using several, mostly immune, response-related parameters. To assess cell survival, the incorporation of [³H] thymidine was analyzed. The authors detected a time-dependent decrease of cell viability. The detection of mRNA and protein expression of IL-6, IL-8, IL-10 and TGF-1β, revealed elevated levels of IL-6, IL-8 and TGF-1β mRNA and IL-8 protein; other changes were not observed. Filtration of the exhaust eliminated IL-8 expression modulation, suggesting the important role of particles in the induction of immune response-related processes (Abe et al., 2000).

3.3. Human bronchial epithelial cells exposed to diesel emissions without an incubator (System 3)

The commercial cell cultivation system became the basis for construction of an exposure device that allowed 1 h exposure of human bronchial epithelial cells (HFBE 21) independent of an incubator (Fig. 2C). This setup simplified exposure conditions, however, cell treatment might be limited to a shorter period of time, as a controlled atmosphere (O_2, CO_2, humidity) was not delivered to the cell cultures. The system, which could be positioned close to the tested engine, was used to evaluate the effect of both diluted (1:10, 1:100) and undiluted complete diesel exhaust, generated under different engine operating conditions. Simultaneously, online monitoring of the particle concentrations and analysis of selected atmospheric compounds was performed. The authors performed cell viability measurements (WST-1 assay, cell counting) and observed a decrease of both parameters, particularly mitochondrial dehydrogenase activity in a dose-dependent manner (Aufderheide et al., 2002; Knebel et al., 2002). Although these results indicate the negative biological effects of exposure to complete diesel emissions, the measured parameters are too general to make any conclusions on the mechanisms of the biological effects of exposure.

This system was later used in more advanced biological experiments involving 3D cultures derived from three donors exposed for 3 h to diluted (1:50) diesel exhaust, followed by a 1 h or 21 h post-incubation period. In the cell cultures, transepithelial electrical resistance (TEER), macromolecular permeability, parameters of cytotoxicity, mucin secretion, alkaline phosphatase activity, oxidative stress markers (glutathione, heme oxygenase protein expression) and the production of twelve cytokines were monitored. The exposure impacted TEER, macromolecular permeability, cytotoxicity, and glutathione levels after 1 h post-exposure, while the production of IL-4 and IL-6 increased after 21 h post-incubation period. For some parameters, substantial interindividual variability was observed (Seagrave et al., 2007). This comprehensive study conducted on a relevant model system indicated that diesel exhaust induced negative biological responses, most of which were resolved during an extended post-incubation period.

Another application of the system, in which diesel exhaust was introduced through a dilution tunnel to treat A549 cells, was reported by Tsukue et al. (2010). The cells were exposed for 1 h to the diluted exhaust (1:10, 1:100), RNA for gene expression analyses extracted 3 h, and cell viability evaluated 22 h, after the exposure finished. Depending on the treatment conditions, the authors observed decreased cell viability and increased expression of *HO-1* and *IL-1β*. DNA microarray analysis identified deregulation of 2552 common genes, these included those related to cancer and immunity. Although this study could provide potentially valuable data, the validity of the results is limited by the cell model used in the experiments. Furthermore, the authors did not provide a detailed description of the microarray data.

Vaughan et al. used the system to investigate the cellular effects of emissions generated from diesel fuel containing various fractions of coconut oil (0, 10, 15, 20%) (Vaughan et al., 2019). The authors treated human bronchial epithelial cells (16HBE) for 30 min (flow rate of 0.16 L/min) and assessed the parameters of cytotoxicity (WST-1, LDH tests), markers of inflammation (IL-6 and IL-8 protein secretion), oxidative stress (*SOD1*, *SOD2*, *HO-1* mRNA expression), apoptosis (*BCL-2*, *CASP-3* mRNA expression) and xenobiotic metabolism (*CYP1A1* mRNA expression). Despite the short exposure time, conventional diesel emissions affected cytotoxicity, induced cytokine production, affected antioxidant response and expression of *CYP1A1*. The cellular response was also observed for coconut oil-diesel blends, although the effects were dependent on the content of the alternative component of the fuel.

3.4. A monolayer culture exposed to diesel and gasoline emissions without atmosphere control (System 4)

A direct air-cell interface deposition technique was used by Cheng

et al. (Fig. 2C), to monitor the production of IL-8 by A549 cells grown at the Transwell membranes after their exposure to diluted (10:1 – 15:1) gasoline and diesel (high-sulfur, low-sulfur) exhaust particles for up to 6 h. The cell exposure chamber was water-heated; however, constant atmosphere conditions were not maintained, nor monitored in this system. Apart from the cell exposure, the system allowed size and chemistry measurement of the exhaust particles. Although reported by the authors as particle exposure system, the apparatus seemed to mediate complete emissions treatment (Cheng et al., 2003). The exposure to gasoline and high-sulfur diesel caused similar effects, characterized by elevated IL-8 production; low-sulfur diesel caused a generally weaker response. As IL-8 is a chemokine that stimulates chemotaxis of target cells to the site of infection, as well as respiratory burst, it can be concluded that both types of engine emissions contribute to the induction of processes associated with oxidative stress. The value of the data is, however, limited by the selection of the cell model, consisting of a single cell type of tumor origin.

3.5. A bronchial cell monolayer exposed to diesel exhaust in an environmental chamber (System 5)

The treatment of an immortalized human bronchial epithelial cell line (16HBE14o), in an instrumented environmental chamber with whole, filtered and hydrocarbon denuded diluted diesel exhaust, was performed by Holder et al. (Holder et al., 2007) (Fig. 1D). The chamber consisted of a room equipped with a controlled ventilation system that supplies filtered outside air and allows the dilution of diesel exhaust. The measurement of flow rate, ventilation, temperature and relative humidity, as well as particle size distribution and concentration of NO_x, CO, and SO_2 within the chamber, was performed during the exposure. The cell cultures grown in the Transwell plates were placed in a stainless-steel box that allowed passive deposition of the particles during exposure lasting 6 h, followed by a 20 h post-exposure time. Similar to the previous study, the exposure induced IL-8 production suggesting the role of oxidative stress in the toxic response to the exposure, mostly mediated by the particles. This is supported by the fact that IL-8 concentration decreased after the removal of a gas phase, and a portion of hydrocarbon in the particle phase (Holder et al., 2007).

3.6. A system for improved particle deposition efficiency applied in a monolayer culture exposed to diesel exhaust and mice lung slices exposed to gasoline emissions (System 6)

As unassisted deposition of particles in the air-liquid interface (ALI) settings is rather limited (Oldham et al., 2020), and attempts have been made to improve the particle deposition efficiency from an air stream onto cells. Among them, a system that used the application of an electric field (Electrostatic Aerosol in Vitro Exposure System, EAVES) was developed by de Bruijne et al. (2009) (Fig. 2A). The apparatus was based on an electrostatic aerosol sampler, modified by the installation of devices that helped to maintain the desired temperature, and by the fabrication of a well in which a titanium dish containing tissue culture media during exposure was placed. The EAVES system was housed in a tissue culture incubator operated at 37 °C. The tested emissions or control air mixed with CO_2 to a final concentration of 5%, were pulled through the device at a constant flow rate of 1 L/min. The system could hold a maximum of four tissue culture inserts. The application of the EAVES alone using clean, control air had no significant adverse effects on the A549 cells, when compared with the cells grown at the standard conditions. The exposure to diesel exhaust for 1 hour in the EAVES significantly increased LDH and IL-8 production measured 9 h post-exposure, when compared with treatments in which EAVES power was off. However, no effects of exposure were detected for the samples treated in the device with the power off. Although the application of an electric field clearly affects the biological impacts of particle exposure, the question remains how this approach compares with the deposition of

particles in human lungs in real-world scenarios. A recent study suggested a large interindividual variability in respiratory tract deposition efficiency, that depends on the breathing patter and structural and functional properties of the lungs (Rissler et al., 2017). The EAVES system was later modified to eliminate the effects of oxidant by-products generated by corona discharge, and used in an experiment in which mice lung slices were exposed to gasoline direct injection engine exhaust (Maikawa et al., 2016). The tissues were exposed to diluted (1:100) exhaust for 1 h at conditions corresponding to a steady-state highway cruise conditions, followed by a 3 h post-treatment incubation in a cell culture incubator. Detailed information on polycyclic aromatic hydrocarbons in the particle and gas phase of the exhaust was available. The expression of *CYP1A1* and *CYP1B1* genes was induced following complete emissions exposure; heme oxygenase (*HMOX-1*), an oxidative stress marker, was modulated by both complete and gas fraction of the engine exhaust (Maikawa et al., 2016). The results indicate the combined contribution of particles and PAHs, adsorbed onto them in biological response to complete emissions exposure.

3.7. Bronchial-immune cell co-cultures and/or a 3D model exposed to diesel and gasoline emissions (System 7)

In this exposure system, more complex cellular models and a commercial 3D culture were exposed. As discussed above, such a setting has the potential to generate more biologically relevant results. The system was originally developed to evaluate the toxicity of emissions generated by two-stroke scooter engines (Müller et al., 2010), but later used for other studies (Bisig et al., 2018; Müller et al., 2012; Roth et al., 2017; Steiner et al., 2013a, 2013b, 2012; Usemann et al., 2018) (Fig. 2B). It consists of two exposure chambers placed in a box to which exhaust emissions diluted 1:100 were routed at a flow rate of 2 L/min, CO_2 concentration adjusted to 5% and relative humidity to 85%. The exhaust was heated before entering the exposure chambers. The following parameters of the diluted exhaust were monitored: temperature, relative humidity, CO_2 and CO concentration. The exhaust entered the culture medium by passive diffusion. In the system, triple co-culture models consisting of lung cells (A549, 16HBE14o-), monocyte-derived macrophages and monocyte-derived dendritic cells were exposed for 1 h or 2 h, followed by 0 – 24 h post-exposure. When compared with the control samples exposed to clean air, no significant effects were observed in the cultures derived from A549 cells. In the system containing the 16HBE14o- cell line, increased LDH production was detected after 2 h exposure and 8 h post-exposure. Concentrations of inflammatory markers (TNF-α, IL-8) were elevated in the culture media of these cells after 2 h exposure and 12 h post exposure.

Another study investigated the toxicity of diesel exhaust (with and without the application of diesel particle filter) in a multi-cellular human lung model, consisting of 16HBE14o-cell line, macrophages, and dendritic cells (Steiner et al., 2013a). Diesel exhaust was diluted 10-fold and used at a constant flow of 2 L/min at 37 °C, 5% CO_2 and 80% relative humidity. The exposure lasted 2 or 6 h and was followed by a 6 h post-exposure period. The biological parameters related to oxidative stress, apoptosis and immune response were assessed to evaluate the potentially toxic properties of both exhaust types: LDH production, glutathione levels, expression of *HMOX1, SOD1, TNF, IL-8, CASP7, FAS* and production of TNFα and IL-8 to a culture media. Although changes in the levels of most of these parameters were noted, the significant response was only observed for reduced GSH levels, *HMOX1* and *IL-8* expression, highlighting the role of reactive oxygen species and inflammation in biological response to diesel exhaust. The filtered exhaust was characterized by a lower pro-inflammatory response (mRNA and protein expression of TNFα and IL-8) (Steiner et al., 2013a). This multi-cellular model was later used to compare the cytotoxicity (LDH release) and pro-inflammatory response (TNF-α, IL-8 production) of a two-stroke and four-stroke scooter and diesel car engine emissions (Müller et al., 2012), to evaluate the effects of co-exposure of CeO_2

nanoparticles and diesel exhaust (assessment of LDH activity, glutathione levels, expression of *HMOX1*, *SOD1*, *TNF-α* and *IL-8*, as well as TNF-α and IL-8 protein production) (Steiner et al., 2012), to investigate the impact of the application of diesel exhaust particle filters to oxidative stress parameters (glutathione levels, expression of *HMOX1* and *SOD1*) and inflammatory markers (gene and protein expression of TNF-α, IL-8) (Steiner et al., 2013a), and to analyze the biological response induced by diesel and biodiesel fuel emissions (parameters of cytotoxicity, oxidative stress and inflammation mentioned above, as well as the expression of pro-apoptotic genes) (Steiner et al., 2013b).

A co-culture model consisting of a bronchial 16HBE14o- cell line and natural killer cells obtained from the peripheral blood of healthy volunteers, was developed and tested with the exposure system to study the toxic effects of pure gasoline and an ethanol-gasoline blend (85% ethanol, 15% gasoline). The parameters of cytotoxicity, oxidative DNA damage, expression of bronchial cell surface receptors and expression of genes encoding surface proteins (ULBP2, MICA) and chemokines (IL-8, IP-10) were assessed in the co-cultures and bronchial cells alone, but no major toxic effects were observed (Roth et al., 2017). A similar cell model and toxicity markers design was used in a later study analyzing the effect of gasoline exhaust emissions generated by a car with and without a gasoline particle filter (GPF). The application of the GPF reduced oxidative DNA damage in bronchial cell monocultures, but no other significant effects were detected (Usemann et al., 2018).

Exhaust from gasoline direct injection engines was tested in the multi-cellular human lung model and a 3D model of primary human airway (Bisig et al., 2018). The effects of regular gasoline and fuel containing ash oil, with or without particle filter, were evaluated. For the exposure, the Worldwide harmonized Light vehicles Test Cycle (WLTC) protocol was used, including one cold start. The treatment was conducted either as a single 6 h exposure, or as a repeated 3 times 6 h experiment. Cytotoxicity measured as LDH production to a cell culture media, was not affected by any exposure condition. RT-qPCR analysis of oxidative stress (*HMOX-1*, *SOD2*, *NQO1*) and immune response (*TNFα*, *CXCL8*) related genes, showed no significant effects after a short exposure. The repeated dose resulted in an elevated expression of *HMOX-1* and *TNFα* expression in the multicellular model, suggesting a response comparable with diesel exhaust exposure. However, no significant effects were detected in a physiologically more relevant 3D system, indicating the generally weak impacts of gasoline exhaust exposure on analyzed parameters.

3.8. Immune response parameters in a bronchial cell monolayer exposed to diesel exhaust (System 8)

In the cell exposure system designed by Oostingh et al. (2013) (Fig. 1C), diesel exhaust generated by an engine operating at a constant speed, was used to analyze the toxic effects on A549 cells. The aerosol was diluted to soot particle concentration of about 10^5 particles/cm^3, supplied with CO_2 to a concentration of 5% and used for treatment at a flow rate of 2 l/minute at temperature 37 °C. The treatment was performed in exposure chambers placed in a Plexiglas container with temperature controlled at 37 °C. In each exposure chamber, two 6-well Transwell plates could be placed. The cells were exposed for 3 h followed by 21 h post-exposure period. Cell viability by measurement of adenylate kinase activity, IL-8 and IL-6 promoter induction in transfected cell lines, and cytokines (IL-8, IL-6, MCP-1, IL-1beta) production was then assessed. The response of the cells to the treatment was generally weak and no significant results were reported.

3.9. A dynamic direct exposure method applied in human bronchial alveolar carcinoma cells (System 9)

This exposure system, based on a commercial product (dynamic exposure chambers by Harvard Apparatus, Inc., USA) (Fig. 1C), used either a truck (laboratory tests), or a passenger car (a field study) diesel engine. Apart from biological parameters, the concentration of gases, including CO, CO_2, NO, NO_2, Cl_2 and O_2 in the exhaust was also monitored and an organic and elemental carbon analysis performed. Prior to the exposure the engines were stabilized by idling for 15 min, and the exhaust was then delivered to the exposure chambers using negative pressure pumps at variable flow rates (25, 37.5 and 50 mL/min). The human bronchial alveolar carcinoma cells (A549) were exposed for 15, 30 and 60 min, and the impact of diesel emissions was evaluated by three tests of cytotoxicity (ATP, MTS, NRU). A significant decrease in cell viability was detected after 15, 30 and 60 min exposure in the laboratory, and after 60 min exposure in the field settings, both at the 37.5 mL/min air flow (Joeng et al., 2013).

3.10. Human primary bronchial epithelial cells exposed to diesel exhaust in a commercial product-based exposure unit (System 10)

An exposure system based on commercial exposure units (Fig. 2B) was used to investigate the effects of emissions produced by a diesel-fueled generator, a representative non road mobile machinery (Zarcone et al., 2016) and diesel exhaust generated by a city bus engine (Zarcone et al., 2018). In the first study, the emissions produced by the engine were first diluted 9 times and then transferred using stainless steel tubing to a central tank in a laboratory where it was either diverted to the unit, or further diluted to mid and low diesel exhaust concentrations. Each exposure unit contained three Transwell inserts with the mucociliary differentiated human primary bronchial epithelial cells grown at the ALI to which the emissions were supplied at a flow rate of 5 mL/min. The exposure units were kept at 37 °C in the central tank temperature (23.35 °C), and relative humidity (20.16%) was monitored. Particle size distribution was used to calculate the PM mass concentrations. For the cell cultures, delivered and deposited PM doses were estimated, and the physical-chemical composition of the mixtures reported. In the system, the cells were exposed for 1 h – 6.25 h and the toxicity parameters, including TEER, lactate dehydrogenase (LDH) release, mRNA expression of selected genes (oxidative stress-related *HMOX-1*, *NQO-1*; unfolded protein response-linked *XBP1spl*, *BIP*, *CHOP*, *GADD34*; inflammatory response-associated *CXCL-8*) and production of IL-8 was performed. The exposure caused the induction of oxidative stress-related markers (*HMOX-1*,*NQO-1*), and general stress response parameters (expression of *CHOP*, *GADD34*, *CXCL8*; concentration of IL-8 in basal media) (Zarcone et al., 2016). In the second study, the same cell line was exposed to the whole diesel exhaust generated by a Euro V bus engine followed by treatment with inactivated *Haemophilus influenzae* bacteria, to mimic microbial exposure. Diesel exhaust was first diluted 17-fold and collected in the central tank as described above. It was then either directly routed to the unit, or first further diluted before its use for exposure, lasting 6 h, 2.5 h or 1 h, at a flow rate of 5 mL/min. The TEER measurement and LDH release analysis was conducted, along with RT-qPCR detection of oxidative stress, unfolded protein and innate immune response. Diesel exhaust exposure induced the expression of oxidative stress related genes (*HMOX1*, *NQO1*), and inhibited expression of human beta-defensin-2 (*DEFB4A*), and the chaperone *HSPA5/BiP*. Expression of the stress-induced *PPP1R15A/-GADD34* and the chemokine *CXCL8* was increased. No effect of the cold engine start was noted.

3.11. Human lung cells exposed to diesel emissions using a mobile system based on multiple commercial modules (System 11)

This complex system consisted of multiple components, including a size-selective inlet that excluded particles larger than 2.5 μm, a mass flow controller that regulated the sampling flow rate, a reactor that kept relative humidity of the exhaust at 85% and temperature at 37 °C, and commercial modules where cell exposure was performed (Fig. 2B). Samples for gravimetric filter sampling or mobility spectrometry were also collected. The modules allowed simultaneous exposure of up to 18

inserts (organized in a 6-well format) at a flow rate of 100 mL/min, with an option to increase the deposition efficiency by the application of an electric field. The control samples could be supplied by a separate gas stream passed through a HEPA filter and conditioned to 85% humidity at 37 °C. All exposure parameters could be controlled by a Lab View acquisition system (Mülhopt et al., 2016). The exposure system was applied to expose human bronchial alveolar carcinoma cells (A549), and a human bronchial epithelial cell line (BEAS-2B), to ship engine emissions generated from heavy fuel oil (HFO) or diesel fuel (DF) (Oeder et al., 2015). The cells were treated with diluted (1:100 for HFO, 1:40 for DF) emissions for 4 h and viability tests, as well as a comprehensive analysis of transcriptome, proteome and metabolome, were conducted. The authors reported the quantification of 42 205 mRNA transcripts, 6192 proteins, and 400 metabolic molecules, and observed a significantly different response between the tested fuels. The deregulated biological pathways included e.g. those involved in pro-inflammatory signaling, xenobiotic metabolism and oxidative stress, with affected genes such as *IL-8, IL-6, IL-1, CXCL1, CXCL2* or *CYP1A1*. Combined proteome and transcriptome data showed that the emissions might affect cell motility, stress response, response to organic chemicals, proliferation, and cell death. Interestingly, DF emissions also impacted histone acetylation pathways, which may be linked with epigenetic changes in the cells. These emissions also significantly reduced glucose flux into lactic acid, but increased carbon flux into glycine, a process associated with tumorigenesis. Although the authors collected a lot of information on the biological effects of ship engine emissions, the validity of the data is limited by the selected monocellular cell systems, of which A549 cells are of tumor origin and their application for genomic analyses is not optimal.

3.12. Exposure of cell monolayers to motorcycle emissions in a commercial device (System 12)

A commercial exposure system (Fig. 2B) was applied to investigate the toxicity of motorcycle emissions in human model lung cell monolayers (A549, BEAS-2B) treated at the ALI. Additionally, the impact of the application of a 0.20 μm particulate matter filter on the biological response was studied (Yu et al., 2017). The motorcycle exhaust generated from a regular gasoline was characterized for particle size distribution, particle number, surface area and mass concentration. The concentration of gaseous components (CO, CO_2, VOC, total hydrocarbons and NO_x) was also assessed. The cell cultures grown on the Transwell inserts in the exposure device were treated with the filtered or non-filtered exhaust for 1 h at a 25 mL/min/well flow rate (A549 cells), or for 30, 60 and 90 min at 15 mL/min/well (BEAS-2B cells). Filtered clean air served as a control. The biological impacts of motorcycle engine exhaust exposure were determined using parameters of cytotoxicity (WST-8 reduction, LDH release) and ROS generation. The authors observed an increased cytotoxicity induced by both filtered and non-filtered emissions in A549 cells, the effect of the non-filtered samples being more pronounced. Similar results were observed for the ROS production. In BEAS-2B cells, where different exposure conditions were applied (several time points, lower emissions flow rate), cytotoxic effects were found upon longer (60, 90 min) exposure periods. ROS production was not measured for this cell line.

In a follow-up study, the effect of motorcycle engine emission dilution on cell viability was analyzed in BEAS-2B cells (Yu et al., 2021). The emissions were diluted with clean air at a 1:20, 1:10, 1:5 and 1:2 ratio and used for a 1 h ALI exposure of the cells at a flow rate of 25 mL/min/well. Cell viability was assessed based on WST-8 reduction. The authors observed a significant dose-dependent decrease of cell viability for dilutions 1:10 and lower.

Overall, the results of these studies provide relatively minor information on the toxicity of engine exhaust, as monolayer cell cultures were used and a limited number of endpoints were analyzed. Nevertheless, the role of particulate matter and the tested dose in the biological

response was highlighted.

3.13. Human bronchial epithelial cells and a 3D model exposed to gasoline exhaust in a portable exposure system (System 13)

The development of a portable exposure system, suitable for toxicity tests of complete engine emissions in cells grown at the ALI, has recently been reported (Vojtisek-Lom et al., 2019) (Fig. 2B). The system was based on a standard 24-well plate that could house up to 8 Transwell inserts. The exhaust was routed via an in-house fabricated stainless-steel distributor head fitting over the plate, that divided air flow into eight paths with equivalent geometry. The bottom plate of the divider served as an impactor that removed large particles, water droplets and debris from the sample. The entire system was housed in a polycarbonate airtight box that contained a stainless-steel plate on the bottom. It could be easily transported to exposure sites. The exposure to engine emissions was realized via inlet and outlet connectors. The system was designed for a maximum flow rate of 25 mL/min per well. For the exposure, the chamber was placed in a toxicological incubator to which diluted exhaust was routed and that maintained a temperature of 37 °C. CO_2 concentration was measured at the outlet of the dilutor and adjusted so that the concentration at the sample was 5%. Before entering the incubator, the exhaust was split into a stream diverted to instrumentation measuring exhaust characteristics (including particle mass and number concentrations and mean particle size distribution), and a stream used for cell treatment (flow rate 0.2 L/min) that was stabilized for desired temperature and humidity.

This system was recently used for a comprehensive evaluation of the toxic effects of complete gasoline engine exhaust, generated by a standard (E5) and alternative fuel containing 20% (v/v) ethanol (E20). In the study, human bronchial epithelial cells (BEAS-2B) and a commercial 3D model grown at the ALI were used (Cervena et al., 2020; Rossner et al., 2021, 2019) and a panel of toxicity markers was analyzed, including TEER, LDH, adenylate kinase (AK) and mucin production measurement, extracellular reactive oxygen species (ROS) detection, DNA damage analysis (micronuclei induction, histone H2AX phosphorylation), 16 lipid peroxidation and 22 inflammation markers and the evaluation of mRNA expression of 370 selected relevant genes. The exposure scheme consisted of 1 h emissions treatment using 2 WLTC cycles, 1 h incubation in the cell incubator, followed by another 2 WLTC cycles exposure. The cell cultures were then either harvested and used for biological analyses, or incubated overnight and repeatedly exposed to engine emissions for up to 5 days.

Despite the depth of this investigation, the biological effects were relatively minor, generally limited to the induced LDH activity and mucin production in BEAS-2B cells. In the 3D model, TEER values and expression of genes encoding xenobiotic metabolism (*CYP1A1*, *GSTA3*) and a heat-shock protein (*HSPA5*) were affected. However, it is important to note that the standard gasoline emissions induced proinflammatory cytokines production, an effect not observed for ethanol-gasoline blend emissions, suggesting greater toxicity of the ordinary gasoline fuel.

4. The biological effects of complete engine emissions in model cellular systems – data overview and interpretation

The presented overview indicates that various technical solutions, testing approaches, cell/tissue types, as well as biological parameters, have been applied in the exposure systems used for complete emissions exposure of cellular models. This fact complicates the generalization of the data and formulation of conclusions on the impacts of engine emissions in biological systems.

In total, we identified thirteen exposure systems developed between 1999 and 2019. Five of them were based on modified commercial products (Aufderheide et al., 2002; Joeng et al., 2013; Knebel et al., 2002; Oeder et al., 2015; Yu et al., 2017; Zarcone et al., 2016), while

others used various in-house technical solutions (Abe et al., 2000; Aufderheide et al., 2002; Cervena et al., 2020; Cheng et al., 2003; de Bruijne et al., 2009; Holder et al., 2007; Joeng et al., 2013; Knebel et al., 2002; Morin et al., 1999a; Müller et al., 2010; Oeder et al., 2015; Oostingh et al., 2013; Rossner et al., 2019; Vojtisek-Lom et al., 2019, p.; Zarcone et al., 2016). In general, the systems differed by the approach used for cell cultivation (rolling chambers, standard cultivation dishes, Transwell inserts), by the method of exhaust delivery to the cells (an indirect approach without any specific routing to cell cultures, or a direct exposure using a dedicated distributor head) or other specific modifications, as e.g. a humidifier, application of electric field to enhance particle deposition, or water bath for cell cultures heating.

The aspects of particles deposition and dose estimation in the model exposure systems are particularly important as in the human pulmonary system inhaled particles are unevenly distributed with some regions being more prone to particles accumulation. The lowest particle concentrations are usually detected in the largest airways and their number increases as the airways narrow (Churg and Brauer, 2000). In the respiratory bronchioles the numbers are commonly 25-100 times greater than in the mainstream bronchus. Similarly, substantial particle deposition is observed in the carinal mucosa (Churg and Brauer, 2000). Additionally, a wide range of retained particle concentrations in the lungs of the general population was described, with age being an important factor affecting deposition efficiency (Churg and Brauer, 2000; Oldham and Robinson, 2006). These factors suggest a significant interindividual variability in potential negative health impacts of particles inhalation. Interestingly, ultrafine particles below 10 nm efficiently deposit in the upper airways, specifically in olfactory mucosa from where they may potentially translocate to brain and contribute to the development of neurodegenerative disorders (Manigrasso et al., 2017). Thus, the region of the respiratory system from which the cells/tissue of the model system originate is a key factor that affects the quality of the experimental data in terms of their possible extrapolation to real-world exposures. In this context, rat lung slices exposure represents a perspective approach for engine emissions testing, as most of the particles are deposited in these parts of the respiratory system (Morin et al., 1999a). The authors of some exposure systems discussed in this mini-review proved experimentally that a significant number of particles generated by an engine were deposited on the membrane of the insert (de Bruijne et al., 2009; Knebel et al., 2002; Müller et al., 2010), or used estimates based on previous measurements and/or calculations (Cheng et al., 2003; Rossner et al., 2019). Importantly, Holder et al. compared the number concentration of particles deposited at the ALI with the modeled deposition in human lungs and found it to be similar, although larger particles tended to be accumulated at the ALI (Holder et al., 2008). Some authors used deposition efficiency of the exposure system, experimental deposited dose and the exposure time and compared it with the real-life breathing parameters, including breathing rate, the pollution level, the total surface area of human lungs and the estimated deposition rate in the lungs (Oeder et al., 2015; Zarcone et al., 2016). For several exposure systems reported in this mini-review the issue of particles deposition was not addressed (Abe et al., 2000; Joeng et al., 2013; Oostingh et al., 2013; Yu et al., 2017).

The results of experiments in which these systems were involved were reported in 31 publications. The effect of diesel engine emissions was investigated in nineteen studies (Abe et al., 2000; Barraud et al., 2017; de Bruijne et al., 2009; Holder et al., 2007; Joeng et al., 2013; Knebel et al., 2002; Le Prieur et al., 2000; Morin et al., 1999a; Mülhopt et al., 2016; Oeder et al., 2015; Oostingh et al., 2013; Seagrave et al., 2007; Steiner et al., 2013a, 2013a, 2013b, 2012; Tsukue et al., 2010; Vaughan et al., 2019; Zarcone et al., 2018, 2016), ten reports focused on gasoline emissions (Bisig et al., 2018; Cervena et al., 2020; Maikawa et al., 2016; Müller et al., 2010; Rossner et al., 2021, 2019; Roth et al., 2017; Usemann et al., 2018; Yu et al., 2021, 2017); in two publications both types of engines were compared (Cheng et al., 2003; Müller et al., 2012). Despite the limited biological significance of the monocellular

cultures discussed above, sixteen studies used this type of lung cells as a single model in the experimental design (Abe et al., 2000; Barraud et al., 2017; Cheng et al., 2003; de Bruijne et al., 2009; Holder et al., 2007; Joeng et al., 2013; Knebel et al., 2002; Mülhopt et al., 2016; Oeder et al., 2015; Oostingh et al., 2013; Tsukue et al., 2010; Vaughan et al., 2019; Yu et al., 2021, 2017; Zarcone et al., 2018, 2016). The application of more advance co-cultures of lung and immune cells was reported in eight studies (Bisig et al., 2018; Müller et al., 2012, 2010; Roth et al., 2017; Steiner et al., 2013a, 2013b, 2012; Usemann et al., 2018, p. 201). As representative 3D models, two commercial products were tested in five reports (Bisig et al., 2018; Cervena et al., 2020, p. 202; Rossner et al., 2021, 2019; Seagrave et al., 2007), the experiments being conducted along with a cell co-culture or a cellular monolayer to compare the response of two different model systems. The use of lung slices, either rat, or mice, was a unique approach performed in three studies (Le Prieur et al., 2000; Maikawa et al., 2016; Morin et al., 1999a).

Most of the experiments focused on the analysis of a limited number of biological parameters, these usually included the immune response- or oxidative stress-related markers (e.g., production of cytokines or antioxidant molecules). As ROS are believed to be a major factor mediating the toxicity of complete emissions in the organisms, the selection of these markers is plausible, although more comprehensive analyses would be needed to gain a deeper insight into the molecular mechanisms of the response. The increased production of cytokines and/ or cytokine-encoding genes expression seems to be a common response detected in many, but not all, studies regardless of the cell model or the fuel tested (Abe et al., 2000; Bisig et al., 2018; Cheng et al., 2003; de Bruijne et al., 2009; Holder et al., 2007; Le Prieur et al., 2000; Müller et al., 2012, 2010; Oeder et al., 2015; Rossner et al., 2021; Roth et al., 2017; Seagrave et al., 2007; Steiner et al., 2013a, 2013a, 2013b, 2012; Tsukue et al., 2010; Usemann et al., 2018; Vaughan et al., 2019; Zarcone et al., 2018, 2016). Among the affected molecules, the production of IL-8, a cytokine with pro-inflammatory properties acting as a potent neutrophil attractant (Amin and Rahmawati, 2017), should be mentioned. Markers of oxidative stress included ROS (Cervena et al., 2020; Rossner et al., 2019; Yu et al., 2017), small molecules with antioxidant properties (e.g., glutathione; (Barraud et al., 2017; Morin et al., 1999a; Seagrave et al., 2007; Steiner et al., 2013a, 2013a, 2012)), expression and/or activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, heme oxygenase, quinone reductase) (Barraud et al., 2017; Bisig et al., 2018; Morin et al., 1999a; Steiner et al., 2013a, 2013a, 2013b, 2012; Tsukue et al., 2010; Vaughan et al., 2019; Zarcone et al., 2018, 2016), oxidative DNA damage (Barraud et al., 2017; Roth et al., 2017; Usemann et al., 2018) and products of lipid peroxidation (derivatives of arachidonic and linoleic acid) (Rossner et al., 2021). The results mostly confirmed the pro-oxidant properties of complete emissions as was evident from the decreased glutathione levels and elevated expression of genes encoding antioxidant enzymes, although the response cannot be generalized (e.g. (Barraud et al., 2017; Cervena et al., 2020; Rossner et al., 2019; Yu et al., 2017)). Additionally, the impact of exposure on lipid peroxidation was minimal. Interestingly, the removal of particles using the filters significantly reduced immunotoxicity of emissions, underscoring the importance of these components of the exhaust in the biological impacts of engine emissions (Abe et al., 2000; Barraud et al., 2017; Steiner et al., 2013a, 2013a; Usemann et al., 2018).

Many authors focused on the general markers of toxic response (e.g. cytotoxicity assessment. TEER changes, mucin production, cell numbers) with variable results probably reflecting specific exposure conditions; i.e. the selected time and the tested dose (Barraud et al., 2017; Cervena et al., 2020, p. 202; de Bruijne et al., 2009; Joeng et al., 2013; Knebel et al., 2002; Le Prieur et al., 2000; Müller et al., 2012; Oeder et al., 2015; Rossner et al., 2019; Roth et al., 2017; Seagrave et al., 2007; Steiner et al., 2013b, 2012; Tsukue et al., 2010; Vaughan et al., 2019; Yu et al., 2021, 2017; Zarcone et al., 2018, 2016). While these parameters provide some information on the effect of complete

emissions exposure, they are not designed to reveal the mechanisms of the biological response and their value is therefore limited.

Due to the presence of PAHs and their derivatives in the engine exhaust, the analysis of the expression of xenobiotic metabolism genes is another relevant approach for the toxicity assessment of complete emissions. In four studies, the effect of engine emissions on genes encoding CYP genes was evaluated. While in the mice lung slices and human lung cell lines (A549, BEAS-2B, 16HBE), the expression of these genes was induced by short treatments (hours) (Maikawa et al., 2016; Oeder et al., 2015; Vaughan et al., 2019), in the 3D model a decrease was detected following 5-days exposure protocol (Cervena et al., 2020; Rossner et al., 2019).

Several authors aimed to investigate the complex biological response of the target cell models by the application of genomics/proteomics approaches (Cervena et al., 2020; Oeder et al., 2015; Rossner et al., 2019; Tsukue et al., 2010). In two of these studies, whole genome gene expression changes were assessed (Oeder et al., 2015; Tsukue et al., 2010) using the Agilent microarray platform. Although the analyzed endpoints were identical and the effects of diesel emissions were investigated in both studies, the sources and chemical composition of the engine exhaust differed: emissions containing a various content of PM and NO_2 generated by an engine dynamo vs. exhaust from a ship engine running on either common heavy fuel or diesel fuel. These conditions most likely contributed to the fact that many significantly affected genes differed between, and even within, the studies. However, the processes linked with oxidative stress and immune response were often found in test scenarios. While these results could potentially reveal details on the mechanisms of toxicity of complete engine emissions, study designs did not involve advanced relevant 3D models. Instead, A549 (Tsukue et al., 2010) and A549 + BEAS-2B (Oeder et al., 2015) monolayers were exposed. In this context, it should be mentioned that data interpretation from genomic analyses of A549 is problematic, as the cell line is of tumor origin and thus genetically unstable. These facts limit the biological significance of the results.

Although a custom panel of a limited number of genes was used to perform genomics analyses in Cervena et al., 2020 and Rossner et al., 2019, these studies arguably represent the most comprehensive evaluation of biological effects of engine emissions. A wide range of markers, including LDH and AK activity, TEER measurements, mucin production, DNA damage, lipid peroxidation, immunomodulatory proteins production, and the expression of relevant genes, was evaluated in the commercial 3D model and BEAS-2B monolayer cultures. Gasoline emissions from the standard fuel impacted the integrity of the 3D model and caused LDH leakage and mucin production in BEAS-2B cells, especially after longer exposure periods. Histone H2AX phosphorylation was induced after 5 days exposure in the 3D model; no DNA damage was observed in BEAS-2B cells. Changes of *CYP1A1* and *GSTA3* expression levels were detected in the 3D model, but no significant results were found in BEAS-2B cells. The emissions also significantly modulated pro-inflammatory cytokine production in the 3D model. The emissions from gasoline-ethanol blend fuel minimally impacted the cytotoxicity markers in both cell models, apart from LDH and mucin production by BEAS-2B cells and decreased TEER values after extended treatment. However, histone H2AX phosphorylation in BEAS-2B cells was induced, suggesting the formation of double strand DNA breaks. Micronuclei frequency analyzed in BEAS-2B cells was not affected after either treatment. Similar to E5 emissions exposure, mRNA expression changes were limited to the 3D model, specifically to *CYP1A1* and *HSPA5* deregulation. E20 emissions had a minimal effect on the expression of immunomodulatory proteins and production of lipid peroxidation markers in both cell models.

The studies discussed in this mini-review were mostly focused on the investigation of complete emissions in the ALI systems and a comparison with the effects of organic and/or water extracts was not assessed. Such comparative reports are important as they help to identify the different biological impacts of individual components of engine exhaust. In a

recent publication by Rossner et al. lipid peroxidation and inflammatory markers were analyzed in two cell models (a 3D model and a standard cell culture) exposed at the ALI to both the complete emissions and organic extracts from PM (Rossner et al., 2021). The results particularly revealed a more pronounced pro-inflammatory response induced by complete emissions when compared with the extracts. The response was characterized by the production of cytokines (interleukin 1 receptor agonist, IL-6, TNF-α), chemokines [interferon gamma-induced protein 10, IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha, or regulated upon activation, normal T cell expressed and presumably secreted] and growth factors (granulocyte-macrophage colony stimulating factor, and leukemia inhibitory factor), most of them not being affected by the treatment with extracts. Interestingly, in mice inhalation studies, elevated levels of pro-inflammatory cytokines and chemokines (including TNF-α, IL-6, IL-13, MCP-1) were detected in the lung tissues of the animals upon exposure to diesel exhaust (Liu et al., 2018; Shvedova et al., 2013), supporting the validity of the above-mentioned *in vitro* data. Thus, these examples indicate that traditional experimental approaches utilizing organic extract exposure may result in inaccurate data generation and misleading conclusions on the toxic effects of engine emissions. They also highlight the importance of testing strategies based on the advanced 3D models.

5. Summary and future directions

To investigate the potential toxicity of outdoor air pollution, a reliable testing approach should be used. Current research indicates that a complex character of engine emissions requires a specific experimental design, that ideally involves the treatment of 3D cellular models grown at the ALI with the complete emissions. Additionally, realistic exposure conditions (e.g., standardized test cycles, exposure times and exhaust dilution) should be used. The application of such settings ensures the generation of the data that could be extrapolated to real human exposures. Despite these facts, cellular monolayers were mostly treated in the exposure systems discussed in this mini-review, thus limiting the value of the data. The results of the reviewed studies clearly show that exposure effects in the more complex commercial systems, representative 3D cultures, were generally weak and/or were resolved during post-incubation periods, while the effects in cell monolayers were more pronounced, yielding most of the significant data. Although the positive observations may be regarded as desirable in the testing of engine emissions that are known to contain toxic compounds, the limited effects probably better reflect the response in the lungs, that need to cope with a variety of external and internal stimuli while keeping a functional gas-exchange system. Therefore, to further explore the toxicity of engine emissions in realistic settings, more studies are needed that involve lung 3D models. Moreover, a battery of relevant biomarkers, including whole-genome gene expression and regulatory pathway analysis, as well as a comprehensive assessment of immunomodulatory and oxidative stress-related protein production, should be included.

Despite the advantages of engine emission testing in 3D models grown at the ALI, there are still some limitations that need to be considered when evaluating the experimental data and extrapolating them to the human organism. Anatomic features are an important aspect that differentiates the *in vitro* models from the organism. The existence of the trachea lined with epithelium consisting of several types of cells, including e.g. ciliated or secretory cells, affects the fate of particles that enter the respiratory system. Importantly, there are differences in the anatomical features even between the human and established animal model systems (e.g. mouse). The ALI cellular/tissue models significantly differ not only from conventional testing approaches, but also between their individual types. The co-culture systems are generally easier to obtain and maintain, but they lack many tissue characteristics. The 3D tissue models are usually more expensive and provide a limited number of cells for laboratory analyses, as subculturing is not possible, but they

Table 2

A comparison of experimental approaches for engine toxicity testing.

Experimental system	Cost	Cultivation/care requirements	Sample availability	Ethical considerations	Relevance
Cellular monolayer	Low	Low	Very high	N/A/Low	Low
Co-culture system	Medium	Medium	High	N/A/Low	Medium
3D tissue model	High	High	Medium	Low	Medium/High
Tissue slices	Very high	Very high	Low	Medium	Medium/High
Animals	Very high	Very high	High	Medium	High
Human volunteers	N/A	N/A	High	Very high	Excellent

Individual parameters scaled from low to excellent; N/A – not applicable for the given parameter. Cost – funds needed to obtain and maintain the system; cultivation/care requirements – how laborious/time consuming maintaining the system and preparing the samples is; sample availability – number of samples/amount of material that can be obtained from the experimental system for subsequent laboratory analyses; ethical considerations – potential ethical issues linked with obtaining the material and processing the samples; relevance – how physiological relevant the data are, whether they could be extrapolated to the human organism.

originate from real tissues thus generating more realistic data. Rat lung slices consist of real tissues and thus represent a specific category of the exposure systems, although the experiments are laborious and exposure is time-limited (see Table 2 for a comparison). Therefore, even an advanced 3D model should be regarded as an artificial system that needs to be thoroughly tested before the generated data can be extrapolated to the human organism. Consensus should therefore be reached on validation studies for the ALI cellular/tissue models so that they can then be applied in inhalation toxicology for regulatory purposes (Lacroix et al., 2018).

In summary, several systems for investigation of complete engine emissions toxicity have been fabricated, and the toxicity of diesel and gasoline engine exhaust assessed in monolayer and 3D cell cultures. Generally, the observed biological effects were rather limited, particularly in the 3D models. To gain more knowledge on the impacts of complete engine emissions, comprehensive studies with relevant experimental design need to be conducted.

Funding

CRediT authorship contribution statement

Pavel Rossner: Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition. **Tereza Cervena:** Writing - review & editing, Visualization. **Michal Vojtisek-Lom:** Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

References

Abe, S., Takizawa, H., Sugawara, I., Kudoh, S., 2000. Diesel Exhaust (DE)–Induced Cytokine Expression in Human Bronchial Epithelial Cells: A Study with a New Cell Exposure System to Freshly Generated DE *In Vitro*. Am J Respir Cell Mol Biol 22, 296–303. https://doi.org/10.1165/ajrcmb.22.3.3711.

Almetwally, A.A., Bin-Jumah, M., Allam, A.A., 2020. Ambient air pollution and its influence on human health and welfare: an overview. Environ Sci Pollut Res 27, 24815–24830. https://doi.org/10.1007/s11356-020-09042-2.

Amin, Z., Rahmawati, F.N., 2017. Recent insight into potential acute respiratory distress syndrome. SMJ 38, 344–349. https://doi.org/10.15537/smj.2017.4.15843.

An, Z., Jin, Y., Li, J., Li, W., Wu, W., 2018. Impact of Particulate Air Pollution on Cardiovascular Health. Curr Allergy Asthma Rep 18, 15. https://doi.org/10.1007/s11882-018-0768-8.

Aufderheide, M., 2005. Direct exposure methods for testing native atmospheres. Experimental and Toxicologic Pathology 57, 213–226. https://doi.org/10.1016/j.etp.2005.05.019.

Aufderheide, M., Knebel, J.W., Ritter, D., 2002. A Method for the *In Vitro* Exposure of Human Cells to Environmental and Complex Gaseous Mixtures: Application to

Various Types of Atmosphere. Altern Lab Anim 30, 433–441. https://doi.org/10.1177/026119290203000406.

Barraud, C., Corbière, C., Pottier, I., Estace, E., Blanchard, K., Logie, C., Lagadu, S., Kéravec, V., Pottier, D., Dionnet, F., Morin, J.P., Préterre, D., André, V., Monteil, C., Sichel, F., 2017. Impact of after-treatment devices and biofuels on diesel exhausts genotoxicity in A549 cells exposed at air-liquid interface. Toxicology in Vitro 45, 426–433. https://doi.org/10.1016/j.tiv.2017.04.025.

Bisig, C., Comte, P., Güdel, M., Czerwinski, J., Mayer, A., Müller, L., Petri-Fink, A., Rothen-Rutishauser, B., 2018. Assessment of lung cell toxicity of various gasoline engine exhausts using a versatile in vitro exposure system. Environmental Pollution 235, 263–271. https://doi.org/10.1016/j.envpol.2017.12.061.

Churg, A., Brauer, M., 2000. Ambient Atmospheric Particles in the Airways of Human Lungs. Ultrastructural Pathology 24, 353–361. https://doi.org/10.1080/019131200750060014.

Cao, X., Coyle, J.P., Xiong, R., Wang, Y., Heflich, R.H., Ren, B., Gwinn, W.M., Hayden, P., Rojanasakul, L., 2021. Invited review: human air-liquid-interface organotypic airway tissue models derived from primary tracheobronchial epithelial cells—overview and perspectives. In Vitro Cell.Dev.Biol.-Animal 57, 104–132. https://doi.org/10.1007/s11626-020-00517-7.

Cervena, T., Vojtisek-Lom, M., Vrbova, K., Ambroz, A., Novakova, Z., Elzeinova, F., Sima, M., Beranek, V., Pechout, M., Macoun, D., Klema, J., Rossnerova, A., Ciganek, M., Topinka, J., Rossner, P., 2020. Ordinary Gasoline Emissions Induce a Toxic Response in Bronchial Cells Grown at Air-Liquid Interface. IJMS 22, 79. https://doi.org/10.3390/ijms22010079.

Cheng, M.-D., Malone, B., Storey, J.M.E., 2003. Monitoring cellular responses of engine-emitted particles by using a direct air–cell interface deposition technique. Chemosphere 53, 237–243. https://doi.org/10.1016/S0045-6535(03)00555-1.

Combes, A., Franchineau, G., 2019. Fine particle environmental pollution and cardiovascular diseases. Metabolism 100, 153944. https://doi.org/10.1016/j.metabol.2019.07.008.

de Bruijne, K., Ebersviller, S., Sexton, K.G., Lake, S., Leith, D., Goodman, R., Jetters, J., Walters, G.W., Doyle-Eisele, M., Woodside, R., Jeffries, H.E., Jaspers, I., 2009. Design and testing of Electrostatic Aerosol in Vitro Exposure System (EAVES): an alternative exposure system for particles. Inhal Toxicol 21, 91–101. https://doi.org/10.1080/08958370802166035.

Hamanaka, R.B., Mutlu, G.M., 2018. Particulate Matter Air Pollution: Effects on the Cardiovascular System. Front. Endocrinol. 9, 680. https://doi.org/10.3389/fendo.2018.00680.

Hayes, A., Bakand, S., 2010. Inhalation toxicology. EXS 100, 461–488. https://doi.org/10.1007/978-3-7643-8338-1_13.

Holder, A.L., Lucas, D., Goth-Goldstein, R., Koshland, C.P., 2008. Cellular response to diesel exhaust particles strongly depends on the exposure method. Toxicol Sci 103, 108–115. https://doi.org/10.1093/toxsci/kfn014.

Holder, A.L., Lucas, D., Goth-Goldstein, R., Koshland, C.P., 2007. Inflammatory response of lung cells exposed to whole, filtered, and hydrocarbon denuded diesel exhaust. Chemosphere 70, 13–19. https://doi.org/10.1016/j.chemosphere.2007.07.036.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer, 2015. Outdoor air pollution.

Joeng, L., Hayes, A., Bakand, S., 2013. Validation of the dynamic direct exposure method for toxicity testing of diesel exhaust in vitro. ISRN Toxicol 2013, 139512. https://doi.org/10.1155/2013/139512.

Killin, L.O.J., Starr, J.M., Shiue, I.J., Russ, T.C., 2016. Environmental risk factors for dementia: a systematic review. BMC Geriatr 16, 175. https://doi.org/10.1186/s12877-016-0342-y.

Klaunig, J.E., 2019. Oxidative Stress and Cancer. CPD 24, 4771–4778. https://doi.org/10.2174/1381612825666190215121712.

Knebel, J.W., Ritter, D., Aufderheide, M., 2002. Exposure of human lung cells to native diesel motor exhaust— development of an optimized in vitro test strategy. Toxicology in Vitro 16, 185–192. https://doi.org/10.1016/S0887-2333(01)00110-2.

Lacroix, G., Koch, W., Ritter, D., Gutleb, A.C., Larsen, S.T., Loret, T., Zanetti, F., Constant, S., Chortarea, S., Rothen-Rutishauser, B., Hiemstra, P.S., Frejafon, E., Hubert, P., Gribaldo, L., Kearns, P., Aublant, J.-M., Diabaté, S., Weiss, C., de Groot, A., Kooter, I., 2018. Air–Liquid Interface *In Vitro* Models for Respiratory Toxicology Research: Consensus Workshop and Recommendations. Applied In Vitro Toxicology 4, 91–106. https://doi.org/10.1089/aivt.2017.0034.

Le Prieur, E., Vaz, E., Bion, A., Dionnet, F., Morin, J.-P., 2000. Toxicity of diesel engine exhausts in an in vitro model of lung slices in biphasic organotypic culture: induction

of a proinflammatory and apoptotic response. Archives of Toxicology 74, 460–466. https://doi.org/10.1007/s002040000138.

Lichtveld, K.M., Ebersviller, S.M., Sexton, K.G., Vizuete, W., Jaspers, I., Jeffries, H.E., 2012. In vitro exposures in diesel exhaust atmospheres: resuspension of PM from filters versus direct deposition of PM from air. Environ Sci Technol 46, 9062–9070. https://doi.org/10.1021/es301431s.

Liu, J., Ye, X., Ji, D., Zhou, X., Qiu, C., Liu, W., Yu, L., 2018. Diesel exhaust inhalation exposure induces pulmonary arterial hypertension in mice. Environmental Pollution 237, 747–755. https://doi.org/10.1016/j.envpol.2017.10.121.

Maikawa, C.L., Zimmerman, N., Rais, K., Shah, M., Hawley, B., Pant, P., Jeong, C.-H., Delgado-Saborit, J.M., Volckens, J., Evans, G., Wallace, J.S., Godri Pollitt, K.J., 2016. Murine precision-cut lung slices exhibit acute responses following exposure to gasoline direct injection engine emissions. Sci Total Environ 568, 1102–1109. https://doi.org/10.1016/j.scitotenv.2016.06.173.

Manigrasso, M., Vernale, C., Avino, P., 2017. Traffic aerosol lobar doses deposited in the human respiratory system. Environ Sci Pollut Res 24, 13866–13873. https://doi.org/10.1007/s11356-015-5666-1.

Moller, P., Danielsen, P.H., Karottki, D.G., Jantzen, K., Roursgaard, M., Klingberg, H., Jensen, D.M., Christophersen, D.V., Hemmingsen, J.G., Cao, Y., Loft, S., 2014. Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles. Mutat Res Rev Mutat Res 762, 133–166. https://doi.org/10.1016/j.mrrev.2014.09.001.

Moorthy, B., Chu, C., Carlin, D.J., 2015. Polycyclic Aromatic Hydrocarbons: From Metabolism to Lung Cancer. Toxicol. Sci. 145, 5–15. https://doi.org/10.1093/toxsci/kfv040.

Morin, J.P., Fouquet, F., Monteil, C., Le Prieur, E., Vaz, E., Dionnet, F., 1999a. Development of a new in vitro system for continuous in vitro exposure of lung tissue to complex atmospheres: application to diesel exhaust toxicology. Cell Biol. Toxicol. 15, 143–152.

Morin, Jean Paul, Leprieur, E., Dionnet, F., Robin, L., 1999b. The Influence of a Particulate Trap on the in Vitro Lung Toxicity Response to Continuous Exposure to Diesel Exhaust Emissions. Presented at the 34th Intersociety Energy Conversion Engineering Conference 1999-01–2710. https://doi.org/10.4271/1999-01-2710.

Mülhopt, S., Dilger, M., Diabaté, S., Schlager, C., Krebs, T., Zimmermann, R., Buters, J., Oeder, S., Wäscher, T., Weiss, C., Paur, H.-R., 2016. Toxicity testing of combustion aerosols at the air–liquid interface with a self-contained and easy-to-use exposure system. Journal of Aerosol Science 96, 38–55. https://doi.org/10.1016/j.jaerosci.2016.02.005.

Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A.C.R., Gehr, P., Burtscher, H., Morin, J.-P., Konstandopoulos, A., Rothen-Rutishauser, B., 2010. New exposure system to evaluate the toxicity of (scooter) exhaust emissions in lung cells in vitro. Environ. Sci. Technol. 44, 2632–2638. https://doi.org/10.1021/es903146g.

Müller, L., Comte, P., Czerwinski, J., Kasper, M., Mayer, A.C.R., Schmid, A., Rosinus, L., Clift, M.J.D., Steiner, S., Gehr, P., Rothen-Rutishauser, B., 2012. Investigating the potential for different scooter and car exhaust emissions to cause cytotoxic and (pro-)inflammatory responses to a 3D in vitro model of the human epithelial airway. Toxicological & Environmental Chemistry 94, 164–180. https://doi.org/10.1080/02772248.2011.632509.

Oeder, S., Kanashova, T., Sippula, O., Sapcariu, S.C., Streibel, T., Arteaga-Salas, J.M., Passig, J., Dilger, M., Paur, H.-R., Schlager, C., Mülhopt, S., Diabaté, S., Weiss, C., Stengel, B., Rabe, R., Harndorf, H., Torvela, T., Jokiniemi, J.K., Hirvonen, M.-R., Schmidt-Weber, C., Traidl-Hoffmann, C., BéruBé, K.A., Wlodarczyk, A.J., Prytherch, Z., Michalke, B., Krebs, T., Prévôt, A.S.H., Kelbg, M., Tiggesbäumker, J., Karg, E., Jakobi, G., Scholtes, S., Schnelle-Kreis, J., Lintelmann, J., Matuschek, G., Sklorz, M., Klingbeil, S., Orasche, J., Richthammer, P., Müller, L., Elsasser, M., Reda, A., Gröger, T., Weggler, B., Schwemer, T., Czech, H., Rüger, C.P., Abbaszade, G., Radischat, C., Hiller, K., Buters, J.T.M., Dittmar, G., Zimmermann, R., 2015. Particulate matter from both heavy fuel oil and diesel fuel shipping emissions show strong biological effects on human lung cells at realistic and comparable in vitro exposure conditions. PLoS ONE 10, e0126536. https://doi.org/10.1371/journal.pone.0126536.

Oldham, M.J., Castro, N., Zhang, J., Rostami, A., Lucci, F., Pithawalla, Y., Kuczaj, A.K., Gilman, I.G., Kosachevsky, P., Hoeng, J., Lee, K.M., 2020. Deposition efficiency and uniformity of monodisperse solid particle deposition in the Vitrocell® 24/48 Air–Liquid-Interface in vitro exposure system. Aerosol Science and Technology 54, 52–65. https://doi.org/10.1080/02786826.2019.1676877.

Oldham, M.J., Robinson, R.J., 2006. Calculated Deposition in Growing Tracheobronchial Airways: Effect of Growth-Rate Assumptions. Inhalation Toxicology 18, 803–808. https://doi.org/10.1080/08958370600748349.

Oostingh, G.J., Papaioannou, E., Chasapidis, L., Akritidis, T., Konstandopoulos, A.G., Duschl, A., 2013. Development of an on-line exposure system to determine freshly produced diesel engine emission-induced cellular effects. Toxicol In Vitro 27, 1746–1752. https://doi.org/10.1016/j.tiv.2013.04.016.

Rehberger, K., Kropf, C., Segner, H., 2018. In vitro or not in vitro: a short journey through a long history. Environ Sci Eur 30, 23. https://doi.org/10.1186/s12302-018-0151-3.

Rissler, J., Gudmundsson, A., Nicklasson, H., Swietlicki, E., Wollmer, P., Löndahl, J., 2017. Deposition efficiency of inhaled particles (15-5000 nm) related to breathing pattern and lung function: an experimental study in healthy children and adults. Part Fibre Toxicol 14, 10. https://doi.org/10.1186/s12989-017-0190-8.

Romeo, D., Salieri, B., Hischier, R., Nowack, B., Wick, D., 2020. An integrated pathway based on in vitro data for the human hazard assessment of nanomaterials. Environment International 137, 105505. https://doi.org/10.1016/j.envint.2020.105505.

Rossner, P., Cervena, T., Vojtisek-Lom, M., Neca, J., Ciganek, M., Vrbova, K., Ambroz, A., Novakova, Z., Elzeinova, F., Sima, M., Simova, Z., Holan, V., Beranek, V.,

Pechout, M., Macoun, D., Rossnerova, A., Topinka, J., 2021. Markers of lipid oxidation and inflammation in bronchial cells exposed to complete gasoline emissions and their organic extracts. Chemosphere, 130833. https://doi.org/10.1016/j.chemosphere.2021.130833.

Rossner, P., Cervena, T., Vojtisek-Lom, M., Vrbova, K., Ambroz, A., Novakova, Z., Elzeinova, F., Margaryan, H., Beranek, V., Pechout, M., Macoun, D., Klema, J., Rossnerova, A., Ciganek, M., Topinka, J., 2019. The Biological Effects of Complete Gasoline Engine Emissions Exposure in a 3D Human Airway Model (MucilAirTM) and in Human Bronchial Epithelial Cells (BEAS-2B). IJMS 20, 5710. https://doi.org/10.3390/ijms20225710.

Roth, M., Usemann, J., Bisig, C., Comte, P., Czerwinski, J., Mayer, A.C.R., Beier, K., Rothen-Rutishauser, B., Latzin, P., Müller, L., 2017. Effects of gasoline and ethanol-gasoline exhaust exposure on human bronchial epithelial and natural killer cells in vitro. Toxicology in Vitro 45, 101–110. https://doi.org/10.1016/j.tiv.2017.08.016.

Seagrave, J., Dunaway, S., McDonald, J.D., Mauderly, J.L., Hayden, P., Stidley, C., 2007. Responses of differentiated primary human lung epithelial cells to exposure to diesel exhaust at an air-liquid interface. Experimental Lung Research 33, 27–51. https://doi.org/10.1080/01902140601113088.

Shvedova, A.A., Yanamala, N., Murray, A.R., Kisin, E.R., Khaliullin, T., Hatfield, M.K., Tkach, A.V., Krantz, Q.T., Nash, D., King, C., Ian Gilmour, M., Gavett, S.H., 2013. Oxidative Stress, Inflammatory Biomarkers, and Toxity in Mouse Lung and Liver after Inhalation Exposure to 100% Biodiesel or Petroleum Diesel Emissions. Journal of Toxicology and Environmental Health, Part A 76, 907–921. https://doi.org/10.1080/15287394.2013.825217.

Sies, H., Jones, D.P., 2020. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat Rev Mol Cell Biol 21, 363–383. https://doi.org/10.1038/s41580-020-0230-3.

Steiner, S., Czerwinski, J., Comte, P., Müller, L.L., Heeb, N.V., Mayer, A., Petri-Fink, A., Rothen-Rutishauser, B., 2013a. Reduction in (pro-)inflammatory responses of lung cells exposed in vitro to diesel exhaust treated with a non-catalyzed diesel particle filter. Atmospheric Environment 81, 117–124. https://doi.org/10.1016/j.atmosenv.2013.08.029.

Steiner, S., Czerwinski, J., Comte, P., Popovicheva, O., Kireeva, E., Müller, L., Heeb, N., Mayer, A., Fink, A., Rothen-Rutishauser, B., 2013b. Comparison of the toxicity of diesel exhaust produced by bio- and fossil diesel combustion in human lung cells in vitro. Atmospheric Environment 81, 380–388. https://doi.org/10.1016/j.atmosenv.2013.08.059.

Steiner, S., Mueller, L., Popovicheva, O.B., Raemy, D.O., Czerwinski, J., Comte, P., Mayer, A., Gehr, P., Rothen-Rutishauser, B., Clift, M.J.D., 2012. Cerium dioxide nanoparticles can interfere with the associated cellular mechanistic response to diesel exhaust exposure. Toxicol. Lett. 214, 218–225. https://doi.org/10.1016/j.toxlet.2012.08.026.

Tsukue, N., Okumura, H., Ito, T., Sugiyama, G., Nakajima, T., 2010. Toxicological evaluation of diesel emissions on A549 cells. Toxicology in Vitro 24, 363–369. https://doi.org/10.1016/j.tiv.2009.11.004.

Upadhyay, S., Palmberg, L., 2018. Air-Liquid Interface: Relevant In Vitro Models for Investigating Air Pollutant-Induced Pulmonary Toxicity. Toxicological Sciences 164, 21–30. https://doi.org/10.1093/toxsci/kfy053.

Usemann, J., Roth, M., Bisig, C., Comte, P., Czerwinski, J., Mayer, A.C.R., Latzin, P., Müller, L., 2018. Gasoline particle filter reduces oxidative DNA damage in bronchial epithelial cells after whole gasoline exhaust exposure in vitro. Sci Rep 8, 2297. https://doi.org/10.1038/s41598-018-20736-z.

Vaughan, A., Stevanovic, S., Banks, A.P.W., Zare, A., Rahman, M.M., Bowman, R.V., Fong, K.M., Ristovski, Z.D., Yang, I.A., 2019. The cytotoxic, inflammatory and oxidative potential of coconut oil-substituted diesel emissions on bronchial epithelial cells at an air-liquid interface. Environ Sci Pollut Res 26, 27783–27791. https://doi.org/10.1007/s11356-019-05959-5.

Vojtisek-Lom, M., Pechout, M., Macoun, D., Rameswaran, R., Kumar Praharaj, K., Cervena, T., Topinka, J., Rossner, P., 2019. Assessing Exhaust Toxicity with Biological Detector: Configuration of Portable Air-Liquid Interface Human Lung Cell Model Exposure System, Sampling Train and Test Conditions. SAE Technical Paper 2019-24-0050.

Yu, T., Zhang, X., Zhong, L., Cui, Q., Hu, X., Li, B., Wang, Z., Dai, Y., Zheng, Y., Bin, P., 2017. The use of a 0.20 μm particulate matter filter decreases cytotoxicity in lung epithelial cells following air-liquid interface exposure to motorcycle exhaust. Environmental Pollution 227, 287–295. https://doi.org/10.1016/j.envpol.2017.04.080.

Yu, T., Zhang, X.Y., Li, S.F., Zhou, Y.M., Li, B., Wang, Z.X., Dai, Y.F., Adamson, S.X.-F., Zheng, Y.X., Bin, P., 2021. Assessment of Benchmark Dose in BEAS-2B Cells by Evaluating the Cell Relative Viability with Particulates in Motorcycle Exhaust via the Air-liquid Interface Exposure. Biomed Environ Sci 34, 272–281. https://doi.org/10.3967/bes2021.036.

Zarcone, M.C., Duistermaat, E., Alblas, M.J., van Schadewijk, A., Ninaber, D.K., Clarijs, V., Moerman, M.M., Vaessen, D., Hiemstra, P.S., Kooter, I.M., 2018. Effect of diesel exhaust generated by a city bus engine on stress responses and innate immunity in primary bronchial epithelial cell cultures. Toxicology in Vitro 48, 221–231. https://doi.org/10.1016/j.tiv.2018.01.024.

Zarcone, M.C., Duistermaat, E., van Schadewijk, A., Jedynska, A., Hiemstra, P.S., Kooter, I.M., 2016. Cellular response of mucociliary differentiated primary bronchial epithelial cells to diesel exhaust. Am J Physiol Lung Cell Mol Physiol 311, L111–123. https://doi.org/10.1152/ajplung.00064.2016.

Zavala, J., Freedman, A.N., Szilagyi, J.T., Jaspers, I., Wambaugh, J.F., Higuchi, M., Rager, J.E., 2020. New Approach Methods to Evaluate Health Risks of Air Pollutants: Critical Design Considerations for In Vitro Exposure Testing. IJERPH 17, 2124. https://doi.org/10.3390/ijerph17062124.