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Title: Exposure to naphthalene and beta-pinene-derived secondary organic aerosol induced divergent changes in transcript levels of BEAS-2B cells

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Related publications

Pantzke J, **Offer S**, Zimmermann E J, Kuhn E, Streibel T, Oeder S, Di Bucchianico S, Zimmermann R. **An alternative in vitro model considering cell-cell interactions in fiber-induced pulmonary fibrosis**. *Toxico. Mech. Method.* 2022. 10.1080/15376516.2022.2156008.

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Jeong S, Pantzke J, **Offer S**, Käfer U, Bendl J, Huber A, Michalke B, Etzien U, Jakobi G, Orasche J, Schnelle-Kreis J, Streibel T, Bucholz B, Adam T, Sklorz M, Di Bucchianico S, Zimmermann R. **In vitro mutagenic and genotoxic potential of combustion particles from marine fuels with different sulfur contents.** *Journal of Hazardous Materials.* Submitted.

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Zusammenfassung

Feinstaub ($PM_{2.5}$) ist einer der Luftschadstoffe, von denen bekannt ist, dass er sich negativ auf die Gesundheit auswirkt und zu einer der weltweit führenden Ursachen für vorzeitige Todesfälle zählt. Während sich viele toxikologische Studien auf die gesundheitlichen Folgen von $PM_{2.5}$ fokusieren, sind die Schadwirkungen von sekundäre organischen Aerosolen (SOA) eher unerforscht. Jedoch tragen SOA, die aus der atmosphärischen Alterung von flüchtigen organischen Verbindungen (VOC) stammen, wesentlich zur Belastung der Luftqualität durch $PM_{2.5}$ bei.

Diese Dissertation beschäftigt sich mit der Fragen nach den toxikologischen Effekten von atmosphärisch gealterten biogenen und anthropogenen Emissionen. In Simulationsversuchen wurde ein anthropogenes (Naphthalin) oder ein biogenes (β-Pinen) VOC zusammen mit einem primären Aerosol [d.h. Rußpartikel (SP)] gealtert und umfassend physikalisch und chemisch charakterisiert. Anhand von verschiedenen *in vitro* Atemwegsmodellsystemen [Bronchial- (BEAS-2B), Alveolar- (A549) und eine Kokultur aus A549- und Endothelzellen (EA.hy926)] wurden die genomischen, metabolomischen und funktionalen Effekte der Aerosole an der Luft-Flüssigkeitsgrenze (ALI) in einem automatisierten Zellexpositionssystem getestet.

Die Alterung von Naphthalin auf SP führte zu einem höher oxidierten und aromatischen SOA mit höherem oxidativen Potenzial, welches in allen Zellmodellen Zelltod, oxidative Stressreaktionen, Entzündungen und Genotoxizität induzierte. Verglichen mit der Alterung von β-Pinen, welches ein weniger oxidiertes aliphatische SOA mit geringerem oxidativen Potenzial bildet, wurden für SOA aus Naphthalin stärkere Effekte beobachtet. Darüber hinaus unterstützten die beobachteten toxikologischen Effekte in den Zellmodellen, die gealterten SP im Vergleich zu frischen SP ausgesetzt waren, die Annahme verstärkter negativer zellulärer Effekte, welche durch atmosphärische Transformationen asugelöst werden. Außerdem zeigten systembiologische Ansätze unter Verwendung des Kokulturmodel stressbedingte Umbauprozesse der Atemwege in direkt exponierten Alveolarzellen (A549) und eine entscheidende Rolle von nicht direkt exponierten Endothelzellen (EA.hy926) auf. Transkriptom- und Metabolomanalysen in den EA.hy926 Zellen ergaben Hinweise auf induzierte Effekte wie oxidativem Stress, DNA-Schäden (sekundäre Genotoxizität) und Entzündungen, die vor allem in Richtung kardiovaskuläre Erkrankungen deuteten. Diese Wirkungen waren nur im geringen Maße durch die Alterung von Naphthalin stärker ausgeprägt, welches möglicherweise eine allgemeine systemische Auswirkung, die durch die Exposition von Aerosolen hervorgerufen wird, unterstreicht.

Zusammenfassend betont diese Dissertation die zentrale Rolle der chemischen Identität des Aerosols sowie der atmosphärischen Alterung für die toxikologischen Ergebnisse in bronchialen

und alveolären Epithelzellmodellen und die Bedeutung der Verwendung von Kokultursystemen, um Wirkungen über die Lunge hinaus aufzudecken.

Abstract

Fine particulate matter ($PM_{2.5}$) is one of the air pollutants known to have adverse health effects and is one of the leading cause of premature deaths worldwide. While many studies focus on the toxicological effects of $PM_{2.5}$, the harmful impacts of secondary organic aerosols (SOA) are rather unexplored. However, SOA that is originating from the atmospheric aging of volatile organic compounds (VOC) contribute significantly to the $PM_{2.5}$ pollution.

This dissertation addresses the question about the toxicological impacts of atmospherically aged biogenic and anthropogenic emissions. In simulation experiments, an anthropogenic (naphthalene) or biogenic (β-pinene) VOC precursor was aged together with a primary aerosol [i.e. soot particles (SP)] and comprehensively physical and chemical characterized. Using different *in vitro* airway model systems [bronchial (BEAS-2B), alveolar (A549) and a coculture of A549 and endothelial cells (EA.hy926)] in an automated air-liquid interphase (ALI) exposure system, the cellular effects triggered by the aerosols were tested by evaluating the genomic, metabolomic and functional changes.

The aging of naphthalene on SP resulted in a higher oxidized and aromatic SOA of higher oxidative potential, and induced to a greater extent cell death, oxidative stress responses, inflammation and genotoxicity in all cell models, compared to the aging of β-pinene forming a less oxidized aliphatic SOA of lower oxidative potential. In addition to that, the toxicological outcomes of cells exposed to aged SP compared to fresh SP supported the assumption of enhanced cellular adverse effects triggered by atmospherically transformations. Moreover, systems biology approaches using the coculture model outlined stress-related airways remodeling processes in directly exposed alveolar cells and a crucial role of non-directly exposed endothelial cells. Transcriptomic and metabolomic analysis revealed the occurrence of oxidative stress, DNA damage (secondary genotoxicity) and inflammation in endothelial cells, pointing in the direction of cardiovascular diseases. These effects were only to some minor degree more noticeable by the aging of naphthalene, therefore, emphasizing the generalized systemic effects induced by the exposure to aerosols.

In summary, this dissertation highlights the pivotal role of aerosol chemical identity, as well as atmospherically aging on the toxicological outcomes in bronchial and alveolar epithelial cell models and the importance of using coculture systems to reveal effects beyond the lung.

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Abbreviations

ALI	air-liquid interphase
AhR	aryl hydrocarbon receptor
AOP	adverse outcome pathway
ARG2	Arginase 2
AT1	alveolar type I cells
AT2	alveolar type II cells
COPD	chronic obstructive pulmonary disease
Cyp	cytochrom P450
DMEM/F12	high-glucose Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
ELISA	enzyme-based immunosorbent assay
ER	endoplasmic reticulum
ERRFI1	ERBB Receptor Feedback Inhibitor 1
FBS	fetal bovine serum
FCGB	FC gamma binding protein
FOSB	FosB Proto-Oncogene
GCLM	glutamate-cysteine ligase regulatory subunit
GSH	glutathione
HMOX1	heme oxygenase 1
IL	interleukin
INFγ	interferon γ
iNOS	inducible nitric oxide synthase
IPA	ingenuity pathway analysis
KE	key events
LDH	lactate dehydrogenase
NR4A1	Nuclear Receptor Subfamily 4 Group A Member 1
MIE	molecular initaing event
MDA	malondialdehyde
mRNA	messenger RNA
mRNA	messenger RNA

MUC5AC	mucin 5AC
MUC5B	mucin 5B
NO	nitric oxide
NO_3	nitrate
NRF2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H dehydrogenase
O_3	ozone
OA	organic aerosol
OH	hydroxyl radicals
OFR	oxidation flow reactor
OPROSI	online particle-bound ROS instrument
OS_c	oxidation state
P13/AKT	phosphatidylinositol 3-kinase/protein kinase B
PAH	polycyclic aromatic hydrocarbons
PAM	potential aerosol mass
PDGF	platelet-derived growth factor
PM	particulate matter
$PM_{2.5}$	PM with upper size limits of 2.5 μm
P/S	penicillin and streptomycin
PTGS2	prostaglandin endoperoxide synthase
ROS	reactive oxygen species
RNASeq	RNA sequencing
SOX2	sex determining region Y-box 2
SOA	secondary organic aerosols
SO_x	sulfur oxides
SP	soot particle
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor
VOC	volatile organic compounds
Wnt4	wnt family member 4

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1. Introduction: Atmospheric aerosols and their health effects

This PhD work has focused on gaining better understanding of the cellular responses after the *in vitro* exposure at the air-liquid interphase (ALI) to biogenic and anthropogenic models of atmospheric aerosols. In 2017 Cohen and colleagues published the Global Burden of Diseases, Injuries, and Risk Factors Study 2015 (GBD 2015) identifying air pollution as a substantially contributor to the global disease burden with increasing trends over the past 25 years *(Cohen et al. 2017)*. In numbers, Cohen and colleagues concluded that 3.5 million deaths were attributed to ambient fine particulate matter ($PM_{2.5}$) in 1990, whereas in 2015 already 4.2 million deaths were recorded. With this increasing tendency of mortalities and morbidities it is of major importance to investigate the toxicological impact of atmospheric aerosols in view of health effects.

1.1 Classification of atmospheric aerosols

The term aerosol comprises the condensed phase particles and the gaseous medium the particles are scattered in. The chemical composition, size and phase of atmospheric aerosols are of high variability accounting of a wide variety of natural and anthropogenic sources and numerous formation mechanisms *(Putaud et al. 2010)* (***Figure 1***). Primary aerosols are specified as aerosol particles or PM that are directly emitted to the atmosphere, e.g. from biomass burning, volcanic eruption and combustion of fossil fuels. Secondary aerosols, however, are formed and altered ("aged") by oxidative gas-to-particle conversion of low volatile compounds through condensation and nucleation processes with ozone (O_3), nitrate (NO_3) and hydroxyl radicals (OH), leading to complex chemical composition and size profiles *(Hallquist et al. 2009)*. Furthermore, PM can be characterized by their size and especially fine PM with upper size limits of 2.5µm ($PM_{2.5}$) are able to penetrate deep into the respiratory regions and induce adverse health effects *(Lelieveld et al. 2020; Manisalidis et al. 2020)*. The chemical composition of ambient aerosols can be divided into an inorganic and an organic fraction. The major inorganic compounds include sulfates and nitrates that have been formed by the oxidation of SO_2 and nitrogen oxides (NO_x) *(Ziemann and Atkinson 2012),* as well as chlorides and alkali/alkaline earth metals that are especially found in sea spray aerosols *(Huffman and Duce 1977)*. Notably, 20 to 90 % of $PM_{2.5}$ are comprised of organic aerosols (OA) throughout the continental boundary layer with a complex composition ranging from simple hydrocarbons to highly oxidized compounds and studies showed that

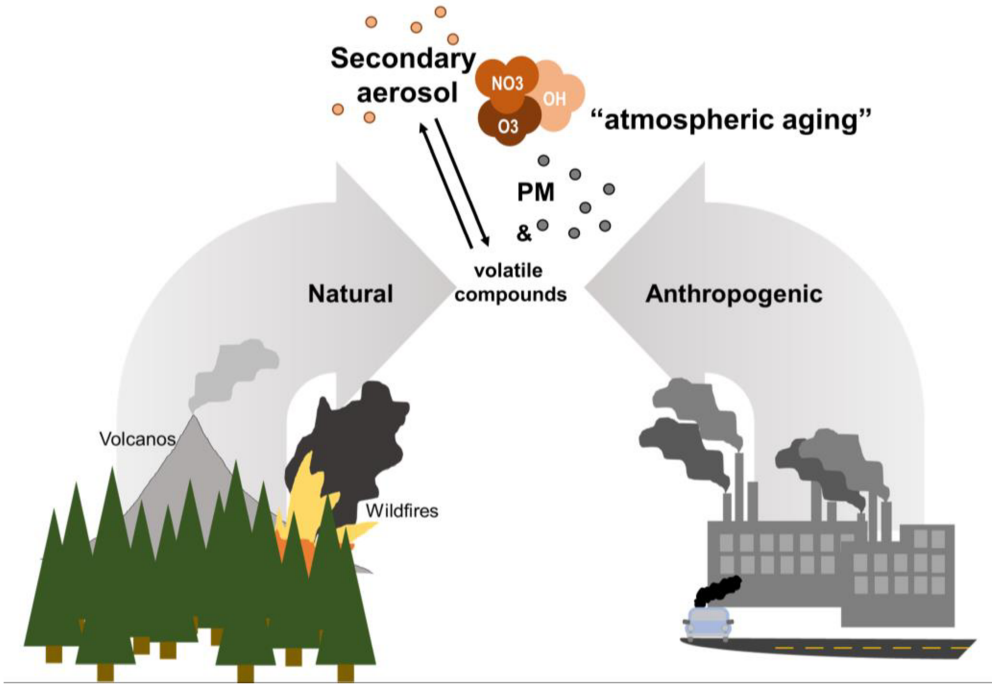


Figure 1. Overview of natural and anthropogenic sources of primary PM and volatile compounds emissions and their "aging" process.

secondary OA (SOA) account for a major fraction of the OA burden *(Jimenez et al. 2009; Zhang et al. 2007)*. Thus, this results to the hypothesis of SOA as significant contributors to adverse health outcomes and the interest of this study.

1.1.1 Formation and chemical characterization of SOA

The major classes of SOA precursors are volatile and semi-volatile alkanes, alkenes, aromatic hydrocarbons, and oxygenated compounds *(Atkinson and Arey 2003)*. The formation of SOA can be induced by biogenic volatile organic compounds (VOC) such as isoprene and monoterpenes (e.g. α- and β-pinene) deriving from terrestrial vegetation *(Guenther et al. 2012)*. In addition to that, VOC emitted from biomass burning and anthropogenic SOA precursors are contributing to the annual SOA fluxes *(Hallquist et al. 2009)*. It has to be considered that ambient measurements have shown higher concentrations of anthropogenic compared to biogenic VOC in urban and industrial environments highlighting the importance of anthropogenic SOA precursors (e.g. aromatics such as naphthalene) particularly in polluted areas *(Stone et al. 2010; Volkamer et al. 2006)*. Besides the compound class of aromatics that comprises around 20 % of anthropogenic SOA precursors, other main compounds are alkanes with around 40 % and alkenes with 10 % *(Ziemann and Atkinson 2012)*. As mentioned above, VOC emitted to the atmosphere are not stable and chemical processes known as "atmospheric aging" lead to the degradation of VOC and the formation of SOA within hours to days *(Parrish et al. 2007)*. The oxidation of VOC during daytime is mainly induced by OH radicals

that are produced from the photolysis of O_3 and subsequent reaction of the formed singlet oxygen with water vapor. Important sources of ozone are the vertical transport from the stratosphere and the photochemical degradation of VOC in presence of NO_x *(Atkinson 2000)*, which is known as ozone formation potential of VOC *(Bufalini et al. 1976)*. During nighttime in absence of sunlight, $NO_3^{\bullet}$ produced by O_3 oxidizing NO_2 is the main oxidant *(Rollins et al. 2012)*. One general pathway of VOC gas phase reaction to form oxidized products is the incorporation of functional groups such as carbonyl (C=O), hydroxyl (-OH), nitrate ($-ONO_2$) and hydroperoxy (-OOH) into the carbon backbone. This goes along with the reduction of the vapor pressure and the partition into the particle phase. In the other general pathway, the formation of smaller oxidized products results from molecular fragmentation. Hereby, the vapor pressure usually increases and the reaction product can undergo further oxidation forming second- and higher-generation reaction products *(Pankow 1994; Robinson et al. 2007)*. Therefore, the yield of SOA and their physical and chemical properties are largely depending on the reaction pathways taking place during day- or nighttime, availability of oxidants, VOC precursors and the ambient levels of NO_x species *(Hallquist et al. 2009; Lambe et al. 2015).* The complexity of the situation is compounded further by the fact that atmospheric particles can act as seed molecules providing surfaces for the vapor condensation *(Kroll and Seinfeld 2008)*. For instance, soot particles emitted by anthropogenic (e.g. traffic, industry) or naturally occurring (e.g. wildfire) combustion processes are known to be quickly aged and coated by VOC in the atmosphere *(Moore et al. 2014; Xu et al. 2020)*.

1.1.2 Simulation of atmospheric aging in laboratory studies

The simulation of atmospheric aerosol transformation in laboratory studies is mainly investigated through chamber experiments, such as the smog chamber and oxidative flow reactors *(Bruns et al. 2015)*. Smog chambers are considered as a realistic model for laboratory-aging of aerosols, but suffer from limitations in equivalent atmospheric aging times of about 1.5 days. Since the atmospheric residence time of particles may be up to two weeks, higher average carbon oxidation states (OS_C) of ambient aerosols are observed than in smog chamber experiments. More intense oxidation can be achieved in oxidation flow reactors, exposing aerosols to high concentrations of atmospheric oxidants. Although the OS_C in ambient and OFR-aged is similar, the molecular composition may be not the same *(Peng and Jimenez 2020)*. The smog chamber is dimensioned of several cubic meters in volume, made from Teflon films and equipped with UV-lamps for the simulation of sun radiation *(Zador et al. 2006)*. Oxidation flow reactors (OFR), with the potential aerosol mass (PAM) as the most popular OFR, are designed as cylinders and also equipped with UV-lamps with

intense emission peaks at 185 and 254 nm required for the production of OH radicals *(Kang et al. 2007)*. In the OFR185 mode, 185 nm radiation photolyzes H_2O and directly produce OH radicals (R1).

$$H_2O + O_2 + hv(\lambda = 185\,nm) \rightarrow OH + HO_2 \quad \text{(R1)}$$

The second pathway resembles the stratospheric OH production. First, ozone is in situ generated from the photolysis of O_2 by 185 nm radiation, generating triplet oxygen $O(^3P)$, which subsequently reacts with O_2 to O_3 (R2).

$$3\,O_2 + hv(\lambda = 185\,nm) \rightarrow 2\,O_3 \quad \text{(R2)}$$

Radiation of 254 nm photolyzes the O_3 and generates singlet oxygen $O(^1D)$ (R3), which finally reacts with water vapor to two equivalents of OH radicals (R4).

$$O_3 + hv(\lambda = 254\,nm) \rightarrow O_2 + O\,(D^1) \quad \text{(R3)}$$

$$O\,(D^1) + H_2O \rightarrow 2OH \quad \text{(R4)}$$

1.2 Effects of atmospheric aerosols on health

The exposure to ambient aerosols has been linked to local as well as systemic effects, which can induce pulmonary diseases (e.g. chronic obstructive pulmonary disease (COPD) or lung cancer) and cardiovascular diseases (e.g. vascular dysfunction, myocardial infarction and atherosclerosis) *(Hamanaka and Mutlu 2018; Konduracka and Rostoff 2022; Niemann et al. 2017; Pun et al. 2017)*. Compared to larger particles, which can be directly removed by mucociliary clearance, $PM_{2.5}$ are effectively retaining in the lungs and are accounting for a significant part of total particles accumulated in the pulmonary parenchyma *(Churg and Brauer 1997)*. Moreover, those particles are not exclusively remaining in the bronchioles or on the surface of alveoli, can also cross the blood-gas barrier and deposit in extrapulmonary organs, such as the liver and the kidney, as shown *in vivo* for polystyrene particles with a diameter between 0.2 and 2 µm inhaled by mice *(Li et al. 2019)* (***Figure 2***). Besides the size, it is presumed that also aerosol formation mechanisms and sources play important impacts on

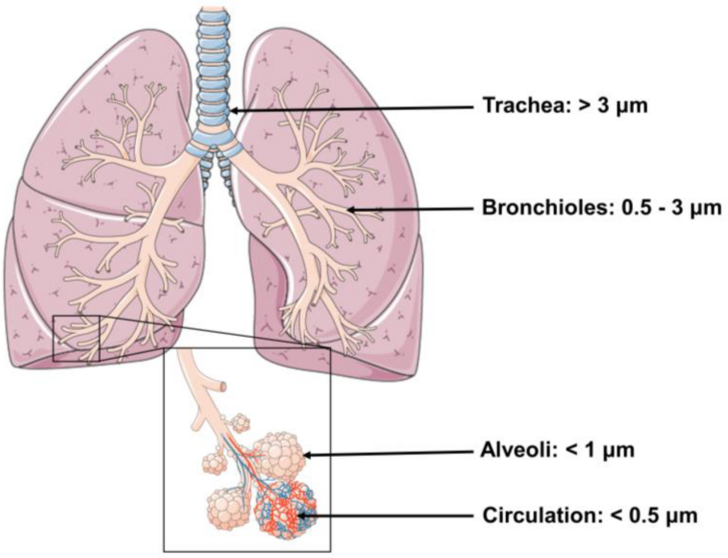


Figure 2. Pulmonary deposition of inhaled particles in different regions of the lung. Illustrated by using figures provided by smart.servier.com

the resulting biological responses beyond the lung. However, how exactly the mass, shape, surface composition or chemical properties of the PM and the gas phase is decisive remains largely unknown *(Park et al. 2018; Wyzga and Rohr 2015)*.

1.2.1 Airway damage

As mentioned above, to study effects of aerosols in the lungs, especially the bronchioles and alveoli are of interest. The bronchioles are lined by respiratory epithelial cells that can be classified into three main categories – secretory cells, cilia cells and basal cells *(Ganesan et al. 2013)*. The secretory cells produce and secrete mucus and are capable to self-renew and differentiate into ciliated cells. The cilia cells account for more than 50 % of all epithelial cell types and facilitate the transport of mucus. Basal cells are able to self-renew and to differentiate to other cell types upon injury. Therefore, the bronchial epithelial cells are extremely important for maintaining airway functions and their deregulation may contribute to several diseases such as COPD or chronic lung inflammation *(Gao et al. 2015)*. The alveolar epithelium is a mosaic of two cell types named alveolar type I cells (AT1) and alveolar type II cells (AT2) *(Crapo et al. 1982; Weibel 2009)*. More than 95 % of the epithelial lining is covered by AT1 cells that are having a thin and flat morphology. Due to this extended surface area, the AT1 cells are in close relationship to the beneath laying endothelial cells and provide the interface for an effective blood-gas exchange. In contrast to that, AT2 cells comprise less than 5 % of the alveolar epithelium, are formed in a cuboidal shape and are responsible for the secretion of surfactant and for the maintaining of the lung homeostasis. Moreover, AT2 cells play an essential role in cell-mediated lung repair with the ability to

self-renew and to transdifferentiate into oxygen-exchanging AT1 cells, which are prone to be more sensitive to injury. Therefore, AT2 cells are also referred as alveolar progenitor cells *(Barkauskas et al. 2013; Liu et al. 2015)*. Recently, a study focused on the influence of long term exposures to biomass fuel $PM_{2.5}$ on alveolar response mechanism. *Yu et al. (2022)* found reduced transition of AT2 to AT1 cells that went along with altered gene expression in several signaling pathways involved in cell differentiation and the destruction of alveoli in mice. In general, the deposition of $PM_{2.5}$ in lung alveoli can decline lung function *(Rice et al. 2015)*, trigger asthma *(Guarnieri and Balmes 2014)*, exacerbate COPD *(Huh et al. 2021)* or provoke the development of lung cancer *(Laden et al. 2006)*.

1.2.2 Cardiovascular impairments

The fact that $PM_{2.5}$ after inhalation can cross the blood-gas barrier or chemical components of the aerosol can translocate and deposit in extrapulmonary organs is accompanied by an increased risk to induce cardiovascular injuries (***Figure 3***). In addition to that, also the release of inflammatory and oxidative mediators as a result of the inhalation of aerosols to the respiratory system can remote cardiovascular effects. A cohort of healthy individuals highlights the importance of endothelial cell injury and systemic inflammation upon exposure

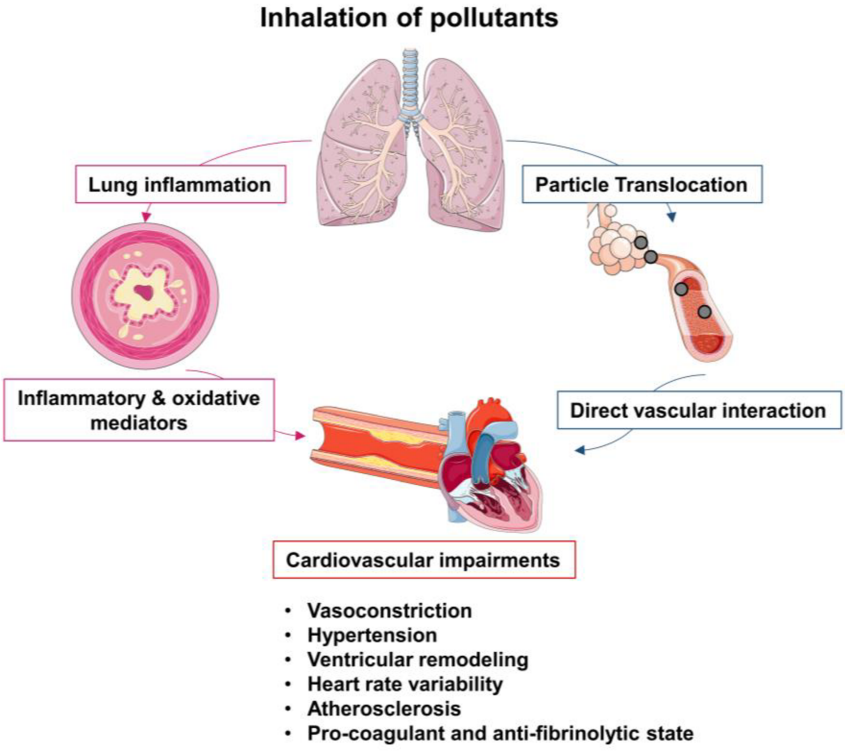


Figure 3. Overview of the main two hypotheses on cardiovascular effects caused by inhaled pollutants. Adapted from Niemann et al. (2017) by using smart.servier.com

to $PM_{2.5}$ levels *(Pope et al. 2016)*. Notably, the impairment of the vascular homeostasis through endothelial dysfunctions can lead to vasoconstriction followed by hypertension and/or the development of atherosclerosis *(Adar et al. 2013; Ying et al. 2014)*. Also the shift of the homeostatic balance towards a pro-coagulant and anti-fibrinolytic state *(Brook et al. 2010)* and a reduction in the heart rate variability has been observed *(He et al. 2011)*. Furthermore, an *in vivo* study in mice revealed the deposition of $PM_{2.5}$ in the cardiac tissue, which led to the induction of structural changes and ventricular remodeling *(Wold et al. 2012)*.

1.2.3 Potential mechanisms

Important molecular mechanism of aerosol-mediated toxicity inducing airways damage and cardiovascular impairments include the altering of the cellular metabolic activity, the induction of oxidative stress and genotoxicity as well as the causing of inflammatory responses (***Figure 4).*** Besides alveolar resident macrophages, epithelia cells are counted as the first line of defense after particle inhalation and the cellular uptake of $PM_{2.5}$ often results in particles accumulation in intracellular membrane-like structures, e.g. phagolysosomes *(Okada et al. 2021)*. Receptors of xenobiotic pathways, such as the aryl hydrocarbon receptor (AhR), can be activated and increase the expression of genes that are involved e.g. phase I or II metabolism through the release of organic compounds bound to particles *(Al Zallouha et al. 2017; Ren et al. 2020)*. Especially polycyclic aromatic hydrocarbons (PAH), together with the activation of the AhR, induce a wide range of downstream effects in which the levels of intracellular reactive oxygen species (ROS) exceed the detoxification capacity of antioxidant defenses *(Grishanova and Perepechaeva 2022)*. This imbalance defines the term “oxidative stress”. Oxidative stress and endoplasmic reticulum (ER) stress is known to be an important mechanism of carcinogenesis by causing of nuclear and mitochondrial DNA strand breaks and impairing DNA repair mechanism with biological consequences, such as mutation, base modifications, and chromosomal aberrations *(Klaunig et al. 2010; Valko et al. 2006)*. This can lead to severe cell cycle alterations with cells having genomic unbalance not often resulting in cell apoptosis *(Longhin et al. 2013)*. Besides attacking DNA, oxidative and ER stress can interact and damage additional biomolecules (proteins, lipids and carbohydrates) and can trigger a cascade of events associated with cell autophagy, impaired mitochondrial activity and inflammation *(Thimmulappa et al. 2019)*. It is believed that especially the interplay between oxidative stress and inflammation plays a key role in the adverse health effects induced by the inhalation of airborne $PM_{2.5}$. Experimental studies showed a strong correlation between intracellular ROS and the activation of redox-sensitive transcriptional

factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB)* and nuclear factor erythroid 2-related factor 2 (*NRF2*), known to regulate the expression of a wide range of pro-inflammatory cytokines and chemokines, acute phase proteins and immunoreceptors that play an important role in the recruitment of immune and other non-epithelial cells *(Christman et al. 2000; Lingappan 2018)*. For example, the secretion of interleukin 1β (IL-1β) and IL-12 is orchestrating acute and chronic lung inflammation by activating group 2 innate lymphoid cells *(Bal et al. 2016)*, while IL-8 is a prominent neutrophil chemoattractant and is found in several lung pathologies such as asthma and cancer *(Al-Alwan et al. 2013; Mukaida 2003; Shieh et al. 2014)*. Moreover, tumor necrosis factor α (TNFα), IL-6 as well as IL-1β can polarize macrophages into a M1 pro-inflammatory way *(Lee et al. 2021)*.

As described above, $PM_{2.5}$ might translocated to the endothelial cells inducing direct effects, but also the release of inflammatory and oxidative mediators by e.g. lung epithelial cells can trigger a cascade of secondary or indirect effects. Also here the concept of oxidative and ER stress with the initiated downstream effects of DNA damage, cell apoptosis, autophagy and/or increased membrane permeability is paramount *(Miao et al. 2019; Wang and Tang 2020)*. Moreover, the endothelial homeostasis can be affected by impaired generation of the vasoprotective molecule nitric oxide (NO) that is closely related to the excess of ROS and vice versa *(Wauters et al. 2013)*. Activated endothelial cells are prone to release inflammatory mediators, such as prostaglandin endoperoxide synthase (PTGS2), IL-8, IL-6 and TNFα, which are known to play a crucial role in inducing angiogenesis *(Bengalli et al. 2017)* and

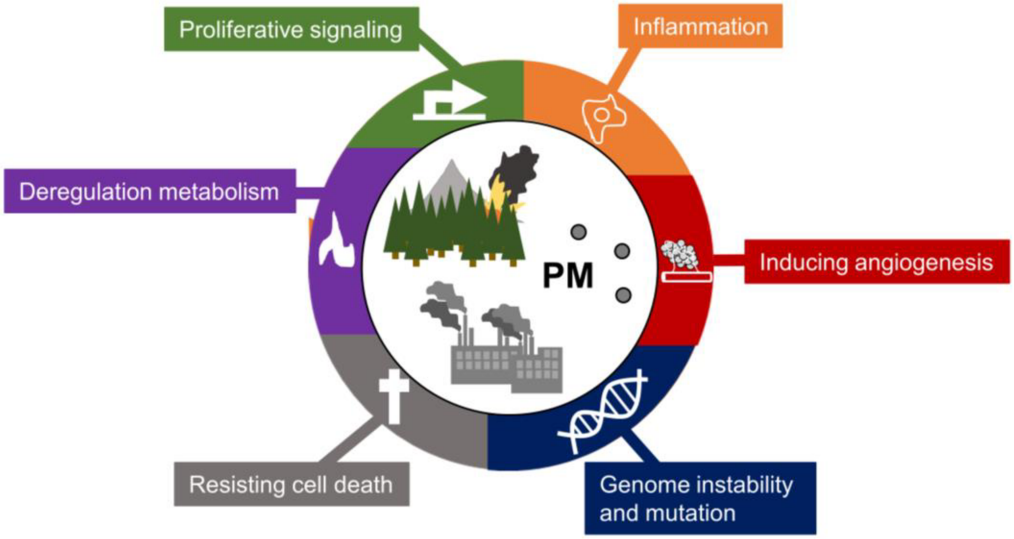


Figure 4. Aerosols and their role in six hallmarks of environmental insults adapted from Peters et al. (2021).

atherosclerosis *(Zhang et al. 2020)*. Besides initiating cardiovascular diseases, those pro-inflammatory mediators can worsening existing conditions by destabilization of atherosclerotic plaques and thus, increasing the chance for thrombotic outcomes *(Pope et al. 2016)*.

However, it is still largely unknown which aerosol properties, such as size, mass or chemical properties, induce those cellular effects. Moreover, VOC precursors and atmospheric aging recently gained more attention as a pivotal player in the detrimental effects of air pollution, but are widely unexplored. Altogether, this highlights the importance of studying the effects of primary combustion emission (e.g. SP) and atmospheric aging of a typical anthropogenic (e.g. naphthalene) or biogenic (e.g. β-pinene) on different types of lung cells and endothelial cells and was the focus of this PhD thesis. Furthermore, our interest was on cellular and molecular effects within the categories of proliferative signaling, inflammation, angiogenesis, genome instability and mutation, resisting cell death and deregulation of metabolism, which resemble some of the hallmarks of environmental insults and enables to understand the interactions of ambient aerosols on a cellular and molecular level and addresses the complexity of the issue.

2. The scope of the work

This PhD thesis is targeted to reveal the unresolved question of the toxicological effects of anthropogenic versus biogenic aerosols. Therefore, the aim of this PhD thesis was to gain a better understanding of cellular and molecular responses to atmospheric aged biogenic and anthropogenic SOA. A focus was put in elucidating the importance of chemical compositions of the generated aerosols in driving adverse outcome effects in different *in vitro* model systems, including mono- and cocultures consisting of lung epithelial cell lines and endothelial cells. The SOA were generated from a typical anthropogenic (naphthalene) and biogenic (β-pinene) VOC, which were atmospherically processed in an OFR in the presence of freshly formed combustion soot particles (SP). This resulted in the coating of SP with condensed organic matter form naphthalene (SOA_{NAP}-SP) and β-pinene ($SOA_{\beta PIN}$-SP) with an averaged particle diameter of ~ 115 nm, defining them as quasi ultrafine. Moreover, fresh and aged SP were used as reference and additional aerosols in order to better understand the atmospherically aging on adverse outcomes in our model systems. The specific aims of this thesis included a comprehensive physical and chemical characterization of the resulting SOA products as well as fresh (primary) and aged SP, especially with the correlation to the toxicological outcomes. Moreover, the importance of using monocultures with lung epithelial cells (A549) or lung bronchial epithelial cells (BEAS-2B), as well as, a coculture system consisting of A549 epithelial cells and EA.hy926 endothelial cells was laid down.

2.1 The specific research questions of the thesis

- Is an anthropogenic SOA more toxic than a biogenic SOA?
- Is the particle concentration, the mass concentration and/or the chemical composition of the aerosol inducing cellular adverse effects?
- Can we correlate specific compounds to the observed biological outcomes?
- What is the importance of atmospheric aging on SP toxicity?
- Are we observing local and/or systemic outcomes by comparing the toxicological effects in two monocultures and one coculture system?

3. Methods

This section gives a more detailed overview of the methodologies used in this thesis. Additional details can be found in the materials and methods sections in the publications of Offer et al. (2022), Pardo et al. (2022), Pardo et al. (2023) and Offer et al. (2023).

3.1 Cell culture

In order to balance between cost, complexity and physiological relevance, our *in vitro* lung cell culture models were grown on transferrable 24-mm Transwell inserts with a polyester membrane (0.4 μm pore-size; type #3450; Corning) at the air-liquid interphase (ALI). Culturing cells on Transwell inserts and at ALI enables the generation of a 3D airways cell model that closely mimics the respiratory tract epithelia. Bronchial and alveolar epithelial cells are playing a pivotal role in the maintenance of airways function and therefore, both cell types were used in two monoculture systems in our study (***Figure 5***). One monoculture system was consisting of AT2-secretory like epithelial cells, namely the A549 cell line, which is widely investigated, especially in toxicological studies *(Barosova et al. 2021*), and known to partially mimic an alveolar epithelium *(J Wu et al. 2017)*. However, drawbacks include their carcinogenic origin and the low expression of tight junctions or adherence proteins *(Papazian et al. 2016)*. Therefore, our second monoculture system was consisting of a human non-tumorigenic cell line, namely the BEAS-2B cell line, as an additional model system. BEAS-2B cells originated from the autopsy of a normal bronchial epithelium and have been infected with a replication-defective SV40/adenovirus 12 hybrid in order to extend the culture lifespan *(Reddel et al. 1988)*. Besides epithelial cells, alveolar macrophages are known to maintain the homeostasis of the lung in normal and in particular in diseased conditions (***Figure 5***). Pantzke et al. (2022) highlighted the importance of using an advanced cell culture model system with immunocompetent cells, such as macrophages, to study the fibrotic effects of fibers. Due to the complexity of the experimental set-up, we were not able to include macrophages in our cell model systems, however we focused on the intercellular crosstalk of epithelial and endothelial cells at the gas-blood barrier by culturing A549 cells on the upper side of the Transwell insert membrane and EA.hy926 endothelial cells on the basolateral side of the membrane (***Figure 5***).

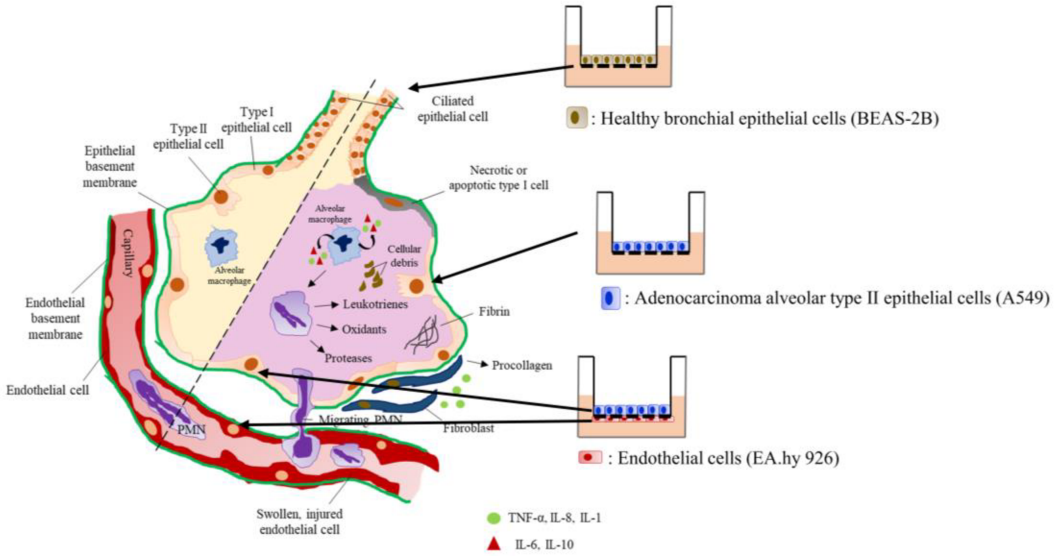


Figure 5. Simplified overview of an alveoli in normal and diseased condition, as well as the used cell culture model systems.

3.1.1 Culture Conditions

In order to set-up a co-culture model system, A549 and EA.hy926 cells were routinely cultured in the same media, namely the high-glucose Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific; 31331-028) supplemented with 5 % (vol/vol) fetal bovine serum (FBS) (ThermoFisher Scientific; 10500-064), and 100 U/mL penicillin and 100 µg/mL streptomycin (P/S; Sigma-Aldrich; P4333) and cells were used between passage 5 to 15 in all experiments. BEAS-2B cells were cultured accordingly to ATCC recommendations in BEBM™ Bronchial Epithelial Cell Growth Basal Medium (Lonza; CC3171) supplemented with the recommended BEGM™ Bronchial Epithelial SingleQuots™ Kit (Lonza; CC4175) except GA-1000 (gentamycin-amphotericin B mix), which was replaced with P/S (100 U/mL penicillin and 100 µg/mL streptomycin) and used between passage 3 to 10 in all experiments. All cells were maintained in a humidified incubator at 37 °C and 5% carbon dioxide (CO_2).

3.1.2 Seeding scheme of the cell culture models

For the first mono- and coculture model system, A549 cells were seeded 96 h before the aerosol exposure at a density of 1.8×10^5 cells/mL (3.8×10^4 cells/cm^2 growth area) per Transwell insert. After 48 h, ALI conditions were generated by removing the media from the apical side of all culture systems and fresh medium (1.5 mL) was supplied in the basolateral compartment. In general, this adaptation process to the ALI several days prior to the experiment has been shown to better result in a clear and tight epithelial phenotype *(Rothen-Rutishauser et al. 2008)*. Further 24 h later, the inserts for the coculture were inverted and 1×10^5 cells/mL (0.21×10^4 cells/cm^2 growth area) EA.hy926 cells per insert let be attached for 1 h. Then the insert was turned back and both culture systems were provided with new media (1.5 mL). On day 5, the model systems were ready for the aerosol exposures (***Figure 6***).

For the second monoculture, 72 h before the exposure experiments BEAS-2B cells were seeded at a density of 2.5 $\times10^5$ cells (5.4 $\times10^4$ cells/cm^2 growth area) on precoated Transwell insert membranes with 0.03 mg ml^{-1} bovine collagen Type 1 (Gibco, A1064401) and 0.01 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, 9048-46-8). After 24 hours, the culture medium on the apical side was removed to establish ALI conditions and further 48 h later, on day 4, the cell culture model system was ready to be used in the aerosol exposure studies.

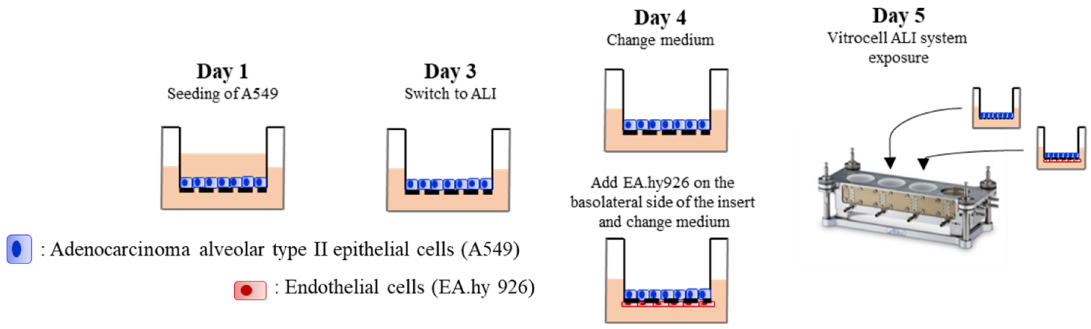


Figure 6. Seeding scheme for the mono- and coculture systems consisting of either only A549 cells or in combination with EA.hy926 cells.

3.2 Methods to mimic ambient aerosol exposure

A schematic overview of the aerosol generation, sampling and cell exposure is shown in ***Figure 7*** and was the fundamental of the conducted experiments.

3.2.1 Aerosol generation and characterization

In order to recreate a realistic exposure scenario, secondary organic aerosols (SOA) of either naphthalene (Sigma-Aldrich, 147141-25G, 99 %) or β-pinene (Sigma-Aldrich, 402753-10G, ≥ 99 %) were produced by mixing their pure vapor with soot particles (SP) and atmospherically photooxided (aging) by OH radicals in an OFR (potential aerosol mass reactor, PAM). In addition, SP were fed into the PAM with and without aging and used as additional aerosols. Several physical and chemical analysis were conducted to comprehensively characterize the generated aerosols. Online measurements were run for particle number, particle size distribution, equivalents of black carbon and brown carbon, the photochemical age of both SOA products, the elemental ratios and reactive oxygen species (ROS). Filter samples were taken and evaluated for elemental and organic carbon, analyzed by transmission electron microscopy (TEM) and in-depth characterized for the chemical composition of SOA products by GCxGC-TOFMS. In addition to that, volatile and intermediate-volatile organic compounds were quantified by chromatography time-of-flight mass spectrometry with electron ionization.

3.2.2 Cell exposure in an automated ALI exposure system

To be able to closer mimic the human inhalation of aerosols, we used two automated exposure systems (Vitrocell Systems), which enabled us to expose our cell models at the ALI. In general, exposures at the ALI allows the preservation of the physicochemical characteristics of PM on the one side and the particle deposition is controlled only by diffusion on the other side, which is of main importance in toxicological studies *(Hilton et al. 2019; Loret et al. 2016)*. The duration of exposure was 4 h and the different aerosols (SP, aged SP, $SOA_{\beta PIN}$-SP and SOA_{NAP}-SP) were tested in several concentrations [undiluted (1:1), 1:3, 1:10 and 1:30] to be able to cover realistic ambient exposure to mild occupational exposure conditions *(Paur et al. 2011)*. The ALI exposure systems were similarly operated as in previous publications *(Mülhopt et al. 2016; Oeder et al. 2015)*. In addition to the aerosol exposures, clean air (CA; purified and compressed air) was used as internal controls and incubator controls for the impact of air flow over the cells. All aerosol types, as well as, concentrations were tested in at least three independent experiments and the aerosol exposures were conducted in serum-free exposure media since FBS is known to interfere with several biological assays, such as RNASeq analysis *(Wei et al. 2016)* or LDH assay *(Thomas et al. 2015)*.

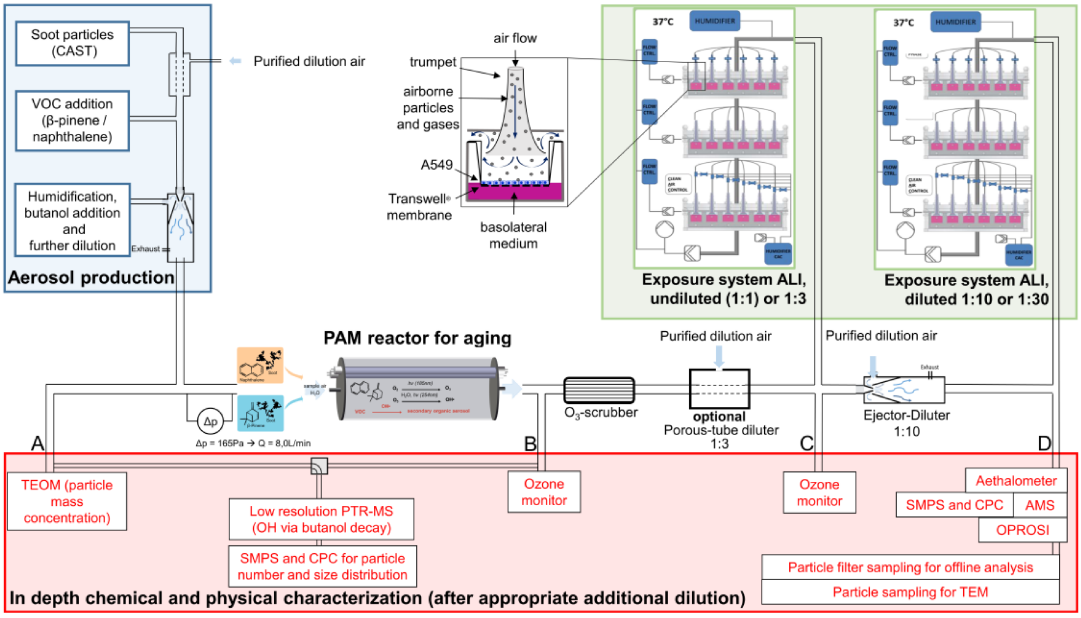


Figure 7. Schematic overview of the experimental set-up. Aerosol generation and the different sampling methods, as well as, illustrations of the two used automated ALI exposure system and cell exposure (here the A549 monoculture exposure is depicted).

3.3 Toxicological assays to determine aerosol-induced effects

Several different toxicological assays were used to determine specific aerosol-induced effects and a detailed description.

3.3.1 Cell viability assay

The resazurin assay (Presto Blue HS Cell Viability Reagent; ThermoFisher Scientific; A13262) was used to determine the metabolic activity of the monocultures A549 and BEAS-2B directly after the aerosol exposures. In live cells, the non-fluorescent blue resazurin is reduced to a red fluorescent dye (resorufin) by the active mitochondrial respiratory chain and can be then detected via spectrometer. Therefore, the metabolic activity of the exposed cells was measured after a 45 min incubation period in serum-free exposure media supplemented with 10 % PrestoBlue solution at 37 °C by determining the fluorescence at 570 nm with a spectrophotometer (MULTISKAN SKY Microplate Spectrophotometer; ThermoFischer Scientific). The results are presented as cell viability in % compared to the CA control.

3.3.2 Live cell imaging

Live cell staining enables a fast indication on the toxicity of specific compounds. Therefore, we used a staining solution with Hoechst 33342 (Sigma-Aldrich; B2261) that is able to bind to the

nuclei of living cells and propidium iodide (Sigma-Aldrich; P41770) that can only bind DNA in dead cells to determine the relation of live and dead cells after the aerosol exposure. The ability of the used microscope (Lionheart FX automated microscope) to capture a whole insert membrane allowed us to gain a good overview of the exposed regions on the membrane after a 45 min incubation at 37 °C with the above mentioned staining solution.

3.3.3 LDH assay

The lactate dehydrogenase (LDH) assay was conducted with the cell culture media collected after the aerosol exposures to determine the amount of LDH released into the cell culture media from all cell culture models. This is a good indicator of cell membrane damage upon necrosis or apoptosis *(FK Chan et al. 2013)*. The Cytotoxicity Detection KitPlus (l-LDH; Roche; 11644793001) was used in combination with a LDH standard curve ranging from 4 to 250 mU/mL (l-LDH; Roche; 10127230001). The quantification of LDH was measured by the absorbance 493 nm after 2 min of reaction time with a spectrophotometer (MULTISKAN SKY Microplate Spectrophotometer; ThermoFischer Scientific) and the respective mU/mL was calculated by using the standard curve.

3.3.4 MDA analysis via LC-MS/MS

Malondialdehyde (MDA), a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis, is generated through the reaction of ROS with cellular membrane phospholipids. It is a mutagenic and carcinogenic compound and is used as a biomarker for oxidative stress *(Del Rio et al. 2005)*. The abundance of MDA in the cell culture media was analyzed by liquid chromatographic (LC) tandem mass spectroscopy MS/MS (API 4000 Triple Quadrupole system, AB Sciex in positive MRM mode). The strength of this technique is the rapid, specific and sensitive quantification of compounds and has been successfully proven for MDA detection *(X Wu et al. 2017)*.

3.3.5 Comet assay

Studies by *Moller et al. (2014)*, revealed oxidative stress as a main player in inducing DNA damage. Therefore, we investigated the amount of DNA breaks (single- and double-strand breaks) in the cells of the mono- (A549, BEAS-2B) and coculture (A549/EA.hy926) systems after the exposure to the different aerosols. The alkaline version of the comet assay is a sensitive technique for the detection of DNA damage with the advantage of needing only a low amount of starting material and the analysis in individual cells *(Di Bucchianico et al. 2017; Singh et al. 1988)*. We used cells from each cell type exposed to 30 µM hydrogen peroxide for 5 min on ice as positive controls and prepared on each slide eight mini-gels, always including a positive and negative

control and exposed cells. Slides were stained with a 1:10.000 dilution SYBR Green (ThermoFisher Scientific; S7563), a nucleic acid stain, and images were captured by a Lionheart FX automated microscope at 20x magnification. DNA damage in nucleoids was quantified by using CometScore software (version 2.0; TriTek Corp.) and results are presented as % DNA in tail.

3.3.6 Enzyme-based immunosorbent assay (ELISA) to detect IL8 release

We used ELISA specific for the cytokine IL-8 to understand the inflammatory response in A549 cells, but also the impact of EA.hy926 cells in the response to the aerosol exposure. It is well-known that endothelial cells are main producers of IL-8 *(Nijhuis et al. 2003)* and that besides being an inflammatory cytokine, IL-8 also plays an important role in cell survival, proliferation and angiogenesis *(Li et al. 2003)*. Aliquots of the exposure media were thawed and analyzed in a 96-well plate using the ELISA for the cytokine IL-8 (ELISA; R&D Systems; DY208). Thawed samples were diluted 1:2 for the monoculture and 1:3 for the coculture in reagent diluent and 100 µL of each sample and the IL8 standard samples (31-2000 pg/mL) were pipetted in a 96-well plate and proceeded according to manufacturer's instructions. The fluorescence was measured at 450 nm and 540 nm in a microplate reader (Varioskan Lux multimode microplate reader, Thermo Scientific).

3.3.7 Angiogenesis assay

The ability of endothelial cells to form tubes is given by letting them grow on matrigel. We used this approach to characterize the impact of aerosol exposure to induce angiogenic effects in endothelial cells after a 24 h incubation with the undiluted exposure media from all aerosol exposures (SP, $SOA_{\beta PIN}$-SP and SOA_{NAP}-SP) or the CA control and both cell cultures. Therefore, EA.hy926 cells were treated with the respective sampled media, trypsinized and re-seeded in fresh cell culture media in 96-well plates, which were precoated with growth factor-reduced BME Matrigel (Cultrex *in vitro* angiogenesis assay; R&D Systems; 3470-096-K). The tube formation was captured 24 h after seeding by a Lionheart FX automated microscope at 10x magnification and the angiogenic potential was calculated by using the formula established by *Aranda and Owen (2009)*.

3.4 Identification of genomic and metabolic changes

We used RNA Sequencing (RNASeq) to determine underlying transcriptional changes and combined those with the detection of specific cytokines. Moreover, our focus was additional on the circulating metabolites found in the cell culture media after the aerosol exposures and on the integration of genomic and metabolomics results.

3.4.1 RNASeq analysis

Since the discovery of the RNA as being essential in various biological processes, such as (de)coding, regulation and gene expression, its analysis became a power fool tool in the molecular biology. The messenger RNA (mRNA) is made through the transcription of DNA, the so-called blueprint of life, can leave the nucleus and has the instructions to synthesis proteins. In 2008, the introduction of RNASeq analysis *(Lister et al. 2008)* as a new method revolutionized the conventional microarrays and let to a fast growing popularity. One of the advantages of RNASeq is the combination of new transcript discovery and gene expression quantification in a single high-throughput sequencing with comparable low costs. The protocol of RNASeq analysis starts with the conversion of enriched mRNA or total RNA into cDNA, processing steps and the subsequent sequencing or "reading" using a high-throughput platform. The raw read data are then mapped to genes as raw counts and can be processed by R analysis *(Koch et al. 2018)*. After the exposure to the 1:3 and 1:30 dilution of either SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP, fresh SP and aged SP, as well as, CA (four independent replicates, respectively for all conditions), RNAprotect solution was added and the cells (A549, BEAS-2B or EA.hy926) scrapped, collected and frozen at -20 °C until the RNA extraction. Then, samples were thawed and RNA were isolated from the cell pellet by using the RNA Plus mini kit (Qiagen, Hilden, GE) according to manufacturer's instruction. To ensure high RNA integrity, the quality of the RNA was measured by a Nanodrop and TapeStation (Agilent Technologies, CA, USA) and only samples with a RIN number above 7.9 were continued to use for RNASeq analysis. The data analysis was performed in R studio and the focus was set on genes significantly differentially expressed ($log_2FC > 0.5$ or $log_2FC < -0.5$; p-value < 0.05) compared to the CA control. Principal component analysis (PCA) is a statistical technique and was used to identify clusters of closely related data points in respect to aerosol and CA exposures. Moreover, volcano plot (scatter plot) and heatmaps helped to visualize significant up- and down-regulated genes.

3.4.2 qPCR

For quantification of mRNA expression and to confirm the results gained from the RNASeq analysis, real-time PCR was performed using the Fast SYBR Green PCR mix (Applied Biosystems, Foster City, CA, USA) in StepOnePlus Reverse Transcription (RT) PCR instrument. The reverse transcriptase creates complementary DNA (cDNA) copies of RNA, which are then amplified in several thermal cycles. In our experiment, we applied 40 cycles with different temperatures starting at 95 °C for 30 s, which allows the separation of the

nucleic acids double chain. This is followed by 60 ° C for 30 s to allow the binding of the primers to the DNA template and 72 °C for 30 s to facilitate the polymerization carried out by the DNA polymerase. Additionally to the tested genes (e.g. *HMOX-1*, *NRF2, CYP1A1, CYP1B1*) endogenous genes (β-actin and 18S) were used as controls.

3.4.3 Cytokine analysis

In addition to the measurements of IL8 by ELISA, several more cytokines (i.e. CXCL11/I-TAC, IFNγ, IL-12p70, IL-1β, IL-23, IL-6, IL-8 and TNF-α) that first have been chosen based on the RNA-seq results and second have already been correlated to PM-induced cell responses *(Pardo et al. 2022; Wang et al. 2017)* were analyzed from the frozen collected sample media by using the MILLIPLEX MAP Human High Sensitivity T Cell Panel-Immunology Multiplex Assay with the Milliplex Magpix instrument (Merck KGaA, Darmstadt, GE).

3.4.4 Metabolome analysis

Targeted and untargeted metabolome analysis reveal invaluable information to mechanistically understand the phenotype of the cell. Metabolites are small molecules that are products of biochemical reactions, have an important role in operating the living cell and are good indicators to study organisms under different conditions since metabolites can be seen as the final products of cellular regulatory pathways *(Zhang et al. 2013)*. Here, an untargeted GC/MS approach was used to identify metabolites that were altered in the cell culture media after the exposure to the different aerosols compared to the CA control (three independent experiments for all conditions). PCA and volcano plots were used to identify differences between aerosol exposures and CA controls and the significant altered metabolites were analyzed by the software MetaboAnalyst 5.0 *(Pang et al. 2022)* in order to identify altered pathways.

3.4.5 Integrated genome and metabolome analysis

The integrating of the obtained genomic and metabolic information reveals the interplay between gene expression and phenotypic shaping. Here, overlapping significant genes of A549 and EA.hy926 cells, respectively, and significant altered metabolites in the cell culture media ($log_2FC > 0.5$ or $log_2FC < -0.5$; p-value < 0.05) were evaluated for canonical pathways and network analysis with the ingenuity pathway analysis (IPA) software (QIAGEN Inc).

4. Results and discussion

The results provide further evidence on the role of chemical composition of photochemically aged SOA in inducing toxicological outcomes and emphasize the importance of using different cell culture model systems (mono- and coculture) at the air-liquid interphase (ALI) for understanding the aerosol-induced cellular effects. Previous studies described the pivotal role of ALI aerosol exposure in modelling realistic exposure scenarios through the preservation of the physicochemical characteristics of airborne PM *(Loret et al. 2016)* and the close approach (less than a factor of two) of the particle size distribution compared to the deposition in lung tissue *(Karg et al. 2020)*. Moreover, the cultivation of cells at the ALI results in a tighter epithelial phenotype that resembles to a greater extent the physiological characterizations of an alveoli *(Rothen-Rutishauser et al. 2008)*.

4.1 Physiochemical characterization of the generated aerosols

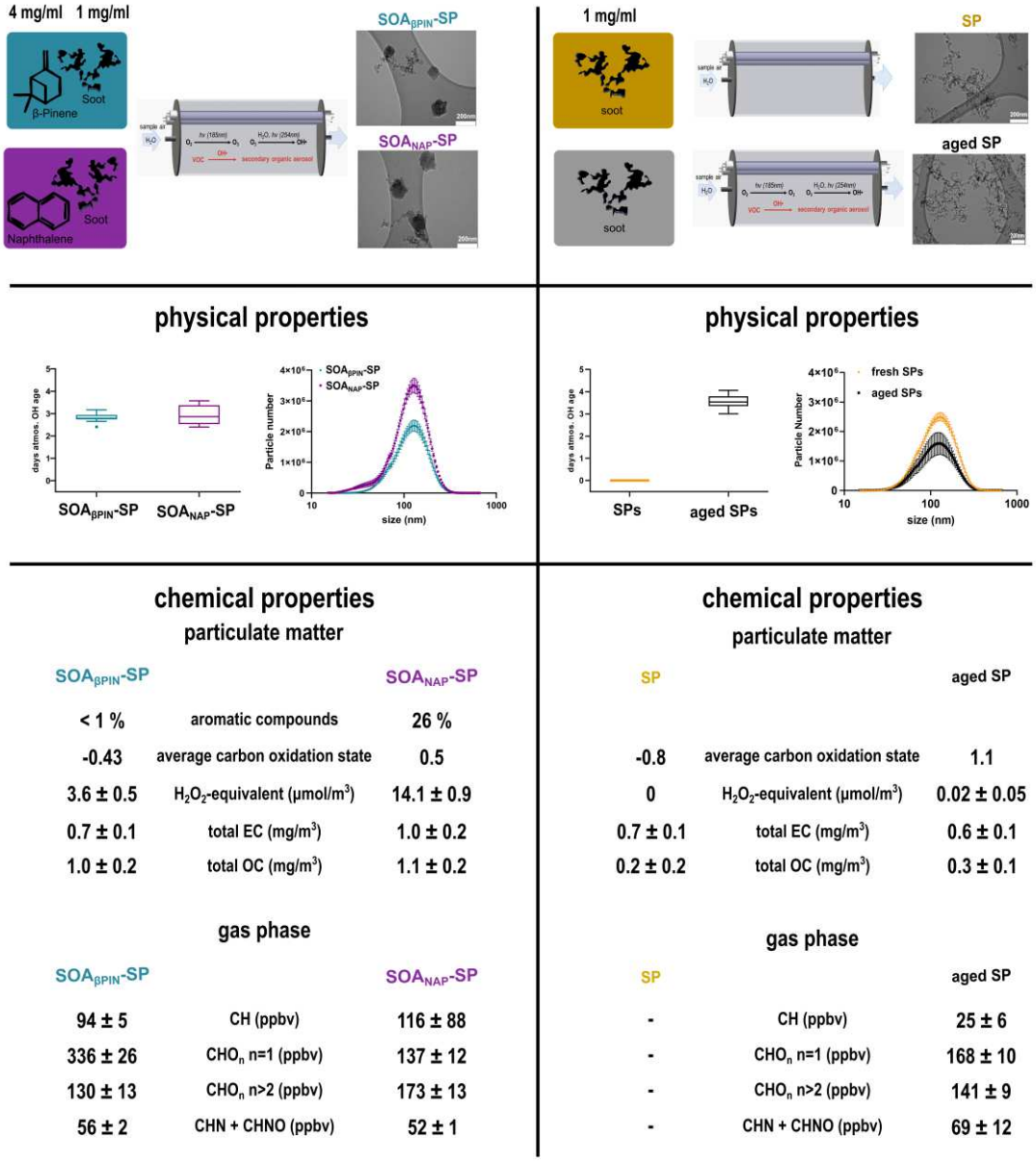
To study the impact of chemical composition on the toxicological outcomes, we generated two SOA with similar physical properties, but distinct chemical identities. Soot particles (SP) were used as seed molecules providing surface for the condensation of photochemical products of the VOC and as reference aerosol. A summary of the physical and chemical properties of SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP, SP and aged SP is depicted in ***Figure 8***.

On the one hand, we used naphthalene representing an anthropogenic and aromatic VOC, and photochemically aged it together with SP to form SOA_{NAP}-SP. Naphthalene is a semi-volatile aromatic hydrocarbon, which consists of two fused benzene rings and is classified as the smallest and most volatile polycyclic aromatic hydrocarbon (PAH) with probably carcinogenic impacts on humans *(IARC 2010)*. Due to its high vapor pressure, naphthalene is almost completely existing in the gas phase and atmospherically aging is triggering its conversion into the particle phase and the formation of SOA *(Williams et al. 2010)*. The main production sites for naphthalene are the chemical and the primary metal industries as well as gasoline and oil combustion, biomass burning or tobacco smoke *(Jia and Batterman 2010)*.

On the other hand, we aged β-pinene, a biogenic and aliphatic VOC, together with SP to generate $SOA_{\beta PIN}$-SP. β-pinene is released in large extents by the vegetation and its oxidation leads to highly oxidized extremely low volatile organic compound with enhanced toxicological effects *(Ehn et al. 2014)*. The photochemically aging of both SOA precursors resulted in equivalent days atmospheric OH age (~ 2.8 days) for both SOA products, as well as, similar particle size distribution, particle diameter (~ 115 nm), particle number and total

OC concentrations. Moreover, TEM images revealed the chains-like structure SP agglomerates coated by organic matter from condensation of secondary organic material. Analysis of SOA_{NAP}-SP revealed typical one-ring and two-ring-retaining first-generation products of naphthalene, such as 1- or 2-naphthol, naphthoquionones or 2-formylcinnamaldehyde *(Keyte et al. 2013)*. Gas-phase measurements by PTR-TOFMS showed higher oxygen content in small molecules with carbon numbers from one to ten for individual VOC in SOA_{NAP}-SP (CHO_1: 137 ± 12 ppb; $CHO_{n>1}$: 173 ± 13 ppb) than $SOA_{\beta PIN}$-SP (CHO_1: 336 ± 26 ppb; $CHO_{n>1}$: 130 ± 13 ppb). For SOA_{NAP}-SP, naphthalene was the most abundant VOC, followed by $C_5H_2O_2$, methanol (CH_4O), butene (C_4H_8) and formic acid (CH_2O_2). For $SOA_{\beta PIN}$-SP and in agreement with previous studies, we found nopinone and especially cyclic and non-cyclic compounds as the major first-generation product in the PM and small C1 and C2 species, such as acetone (C_3H_6O) or formaldehyde (CH_2O) in the gas phase *(Hohaus et al. 2015; Kaminski et al. 2017)*. It is speculated that the degree of oxidation induced by atmospheric aging has an impact on the toxicological responses *(Chowdhury et al. 2018; Han et al. 2020)*. We showed that for both SOA products the average OS_c increased at three days of atmospheric OH age, however the one for SOA_{NAP}-SP was higher than for $SOA_{\beta PIN}$-SP. This was correlating with the higher oxidation potential of SOA_{NAP}-SP measured by particle-related H_2O_2 in an OPROSI *(Zhang et al. 2022)*.

To further understand the effects of SOA precursors on cellular toxicity, we used fresh SP as a reference aerosol and SP aged with the same OH exposure as an additional aerosol. Except their particle size distribution and particle number concentration, that were similar to both SOA, fresh and aged SP retained a typical soot structure of chain-like agglomerates and a high total EC content. The aging of SP resulted in slightly higher total OC content and higher oxidation potential, however significantly lower compared to both SOA. A set of analytical techniques for organic compounds of different chemical properties gave results below the limit of detection, hence giving indication oxidation of the SP surface was increasing the content of non-refractory particle constituents, such as OC, rather than for formation of organic compounds in the narrower sense. Gas-phase measurements of individual VOC in aged SP revealed oxygen content in small molecules with carbon numbers from one to ten that was in between both SOA products.



chemical properties

particulate matter

SOA$_{βPIN}$-SP		SOA$_{NAP}$-SP
< 1 %	aromatic compounds	26 %
-0.43	average carbon oxidation state	0.5
3.6 ± 0.5	H_2O_2-equivalent (µmol/m^3)	14.1 ± 0.9
0.7 ± 0.1	total EC (mg/m^3)	1.0 ± 0.2
1.0 ± 0.2	total OC (mg/m^3)	1.1 ± 0.2

gas phase

SOA$_{βPIN}$-SP		SOA$_{NAP}$-SP
94 ± 5	CH (ppbv)	116 ± 88
336 ± 26	CHO_n n=1 (ppbv)	137 ± 12
130 ± 13	CHO_n n>2 (ppbv)	173 ± 13
56 ± 2	CHN + CHNO (ppbv)	52 ± 1

chemical properties

particulate matter

SP		aged SP
-0.8	average carbon oxidation state	1.1
0	H_2O_2-equivalent (µmol/m^3)	0.02 ± 0.05
0.7 ± 0.1	total EC (mg/m^3)	0.6 ± 0.1
0.2 ± 0.2	total OC (mg/m^3)	0.3 ± 0.1

gas phase

SP		aged SP
-	CH (ppbv)	25 ± 6
-	CHO_n n=1 (ppbv)	168 ± 10
-	CHO_n n>2 (ppbv)	141 ± 9
-	CHN + CHNO (ppbv)	69 ± 12

Figure 8. Physiochemical characterization of SOA$_{βPIN}$-SP, SOA$_{NAP}$-SP, fresh and aged SP. Schematic overview of atmospheric aging of SP together with either β-pinene (SOA$_{βPIN}$-SP) or naphthalene (SOA$_{NAP}$-SP). Moreover, fresh and aged SP were used as additional aerosol. Characterization of physical and chemical properties of the particulate matter and the gas phase.

4.2 Toxicological outcomes in different cell models

An increasing number of studies have shown the importance of atmospheric aging of SOA on the impact of human health *(Chowdhury et al. 2018; Weitekamp et al. 2020)*. However, the specific SOA-induced cellular effects in the different regions of the lung remains largely unknown. First, we addressed this uncertainty by using two monocultures consisting either of a secretory-type bronchial epithelial cell line (BEAS-2B) or a more AT2 alveolar epithelial cell line (A549). It has to be noted that the BEAS-2B cell line originated from a human non-tumorigenic lung epithelium, however had to be immortalized to enable cell proliferation, whereas the A549 cell line was established from a human pulmonary carcinoma tissue. These differences in cellular origin and the resulting consequences in the toxicity screening will be discussed in the following section. In order to enhance our toxicity testing strategy, we applied the concept of "Adverse Outcome Pathway" (AOP) that is used as a tool to link a causal event with a molecular initiating event (MIE), several intermediate key events (KE) and an adverse outcome (AO) *(Halappanavar et al. 2020)*. Our developed AOP describes the interaction of stressors (e.g. aerosols) with the alveolar cell membrane leading to tumorigenesis (***Figure 9***). Inhaled stressors can react with cells via physical, chemical or receptor-mediate interaction (MIE), triggering an increased secretion of pro-inflammatory mediators (KE 1) and subsequently inducing a cascade of events associated with an increased ROS synthesis (KE 2) and a state of oxidative stress (KE 3) *(Thimmulappa et al. 2019)*. The occurrence of oxidative stress can go along with upregulation of genes associated with antioxidant response (KE 4) and increased DNA damage & mutations (KE 5) *(Klaunig et al.*

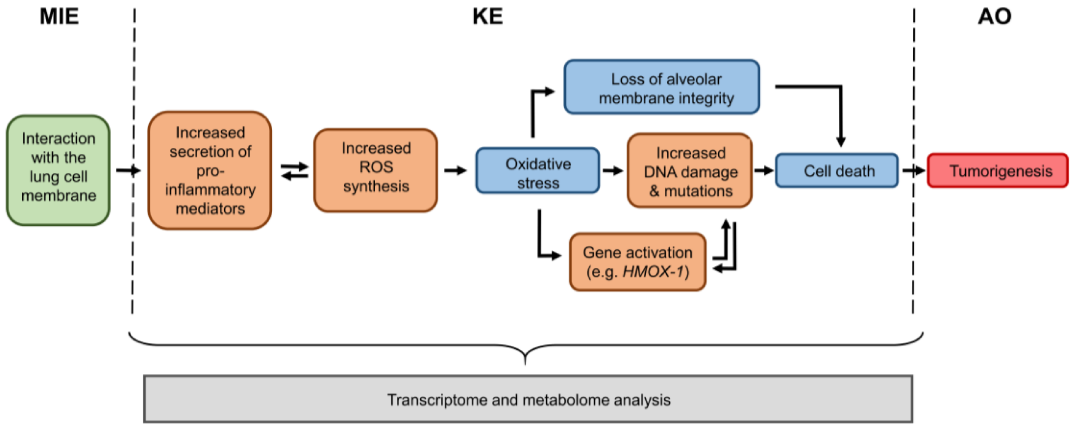


Figure 9. Adverse outcome pathways (AOP) of relevance to in vitro inhalation studies. Green: molecular initiating event (MIE); Orange: cellular level key events (KE); Blue: tissue level KE; Red: adverse outcome (AO).

2010). Besides attacking the DNA, oxidative stress can damage biomolecules, inducing the loss of alveolar membrane integrity (KE 6). All those KE events can result in cell death (KE 7) and culminating in tumorigenesis (AO). To which degree this AOP is affected by the four aerosols (SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP, SP and aged SP) and cell models is discussed in the first part of this section.

Second, we established a coculture model system focusing on the blood-gas barrier between epithelial (A549) and endothelial cells (EA.hy926), enabling an in-depth characterization of the cell-to-cell interplay after the exposure to the different aerosols. Here, we linked the results from our designed AOP with genetic and metabolic information to facilitate the inclusion of omic and functional results in order to better understand the effects of aerosol on a more systemic level. This will be discussed in the second part of this section.

4.2.1 Aerosol exposures in monocultures of A549 and BEAS-2B cells

In order to elucidate the different potentials of an anthropogenic and a biogenic SOA to locally induce toxicological effects in the lungs, we designed functional assays based on the outlined AOP approach. A summary of the observed results in A549 and BEAS-2B cells for the KE 1-7 after the exposure to SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP and additionally to fresh and aged SP is laid out in ***Figure 10***.

As KE 1, we focused on the secretion of pro-inflammatory mediators into the cell culture media after the exposure to the aerosols. We observed an increase in the release of the cytokine IL-8 after the exposure to SOA_{NAP}-SP and aged SP, however with SOA_{NAP}-SP inducing a significant higher release compared to all other tested aerosols. This is in agreement with previous studies showing an enhanced inflammatory effect of naphthalene compared to α-pinene *(Chowdhury et al. 2018)* and aged soot compared to fresh soot *(Cheng et al. 2019)*. We assumed that the inflammatory effects may be triggered by the functional groups, such as PAHs, the oxidation state and is likely due to the organic components of the particles as shown in studies with diesel engine exhaust *(Totlandsdal et al. 2012)*, SP *(Al Housseiny et al. 2020)* and $PM_{2.5}$ *(Fuentes-Mattei et al. 2010)*. This was further supported by the observation of less cytokine release in A549 and BEAS-2B cells after the exposure to SP. Xenobiotic receptors, such as the *AhR* receptor are assumed to play a pivotal role in regulating the immune system and in the uptake and metabolism of PM. Especially PAHs are known to induce its activation and notably also SP can do, however the exact interaction mechanism is not fully understood *(Niranjan et al. 2021)*.

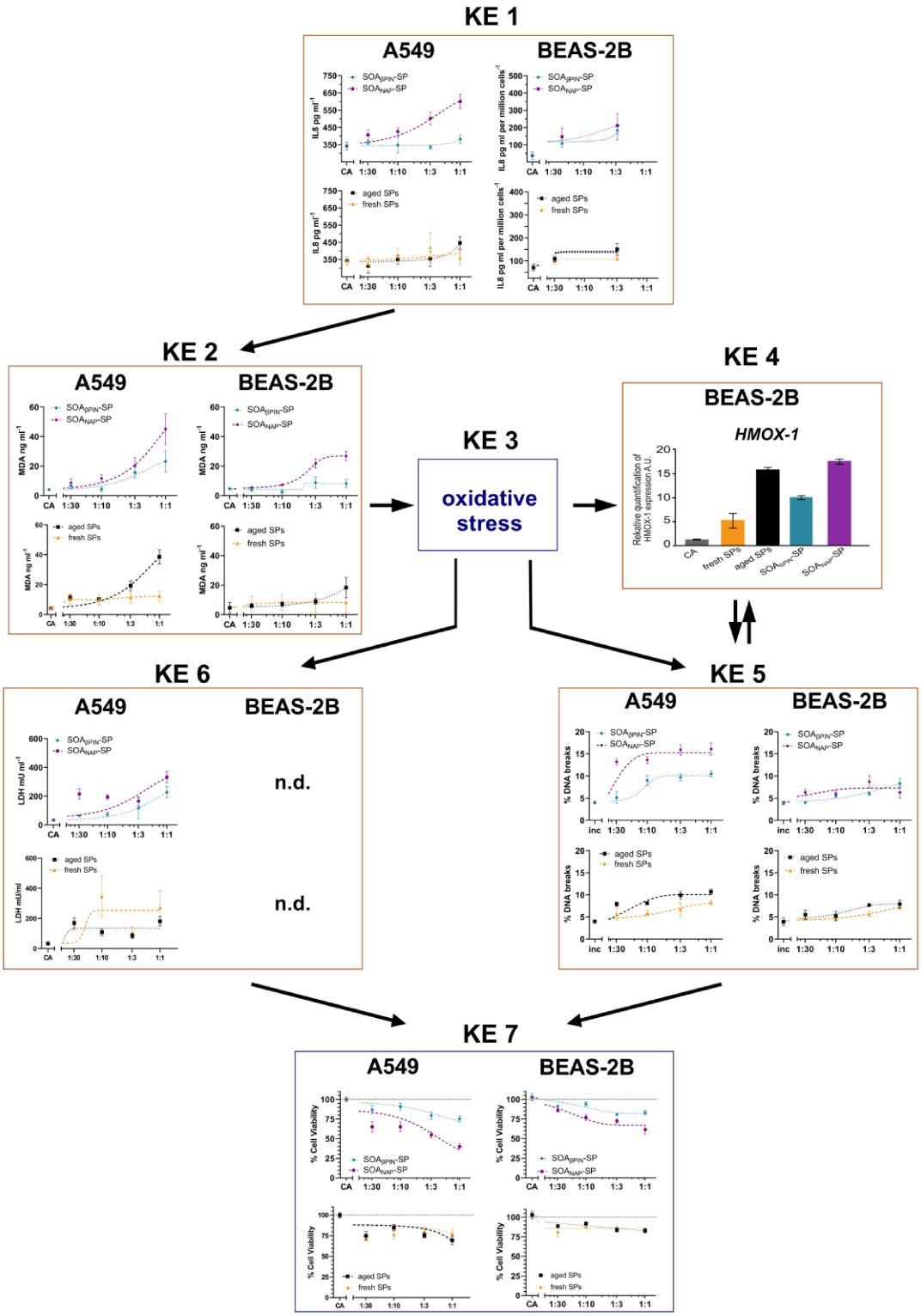


Figure 10. Adverse outcome pathway with the secretion of IL8 (KE 1) and MDA (KE 2), oxidative stress, the upregulation of antioxidant gene (KE 4), the induction of DNA breaks (KE 5), the release of LDH (KE 6) and the affected cell viability (KE 7) in A549 and BEAS-2B cells.

Here, we found an upregulation of the *AhR* gene and its downstream target gene *CYP1A1* after the exposure to fresh SP and to a greater extent after the exposure to SOA_{NAP}-SP and aged SP in BEAS-2B cells. It is speculated that atmospheric aging is enhancing the toxic potential by oxidizing PAHs and increasing the oxygen-containing moieties in the aromatic carbon rings of SP, which is in line with the results of the average carbon oxidation state being the highest in SOA_{NAP}-SP and aged SP. Interestingly, BEAS-2B cells are known to have only limited CYP gene expression activity compared to primary cells upon induction *(Garcia-Canton et al. 2013)*, demonstrating the importance of this finding. An increase in pro-inflammatory cytokines and the upregulation of xenobiotic receptors can induce downstream effects, such as the formation of ROS (KE 2) *(Grishanova and Perepechaeva 2022)*. An accumulation of ROS enhancing the antioxidant defense results in cellular oxidative stress (KE 3). Indeed, we found a dose-dependent increase in the oxidative stress marker MDA *(Del Rio et al. 2005)* in the cell culture media of A549 and BEAS-2B cells after the exposure to SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP and aged SP, however with a higher abundance after the exposure to SOA_{NAP}-SP and aged SP. This is in agreement with the assumption that intracellular ROS can be formed through the diffusion of particle-related peroxide *(Liu et al. 2020)* and the observed three times greater hydrogen H_2O_2-equivalent in SOA_{NAP}-SP compared to $SOA_{\beta PIN}$-SP, as well as, the high oxidation state detected in SOA_{NAP}-SP and aged SP. Moreover, the generation of ROS has already been observed in correlation to redox active quinones *(Gopinath et al. 2016)*. As anticipated, the particle phase of SOA_{NAP}-SP had a great abundance of several quinones, such as 1,2- and 1,4-naphthoquinone, which are early photooxidation products of naphthalene. To some extent oxidative stress can be tolerated by the cell, however overwhelming stress elicits genetic adaptation processes, genotoxicity and loss of membrane integrity up to apoptosis and cell death *(Peters et al. 2021)*. One of the crucial adaptor mechanism to oxidative stress is the upregulation of genes associated with the antioxidant response (KE 4). The *NRF2* gene is a key transcriptional factor and has been demonstrated to orchestrate cellular antioxidant responses by inducing gene expression for e.g. glutathione synthesis, iron regulation, amino acid metabolism and DNA repair *(Rojo de la Vega et al. 2018)*. In BEAS-2B cells, we observed the activation of several downstream targets of *NRF2*, e.g. heme oxygenase 1 (*HMOX-1*), NAD(P)H dehydrogenase *(NQO-1*) and glutamate-cysteine ligase regulatory subunit (*GCLM*), supporting the presumption of SOA and aged SP inducing oxidative stress. The mechanism of *NRF2* activation upon $PM_{2.5}$ exposure has been reported to involve PAH-rich particles *(JK Chan et al. 2013; Pardo et al. 2020)*, which was also consistent in our study with significant elevated gene expression level after the exposure to SOA_{NAP}-SP, a PAH-rich aerosol, compared to $SOA_{\beta PIN}$-SP.

Furthermore, the inability to clear high levels of ROS can cause irreparable damage to DNA (KE 5) resulting in programmed cell death, but also genomic instability that is a major driver in tumorigenesis *(Hanahan and Weinberg 2011)*. Notably, we already found a quite high amount of single- and double strand breaks in A549 after the exposure to both highly diluted SOA (1:30 for SOA_{NAP}-SP; 1:10 for $SOA_{\beta PIN}$-SP). For BEAS-2B cells, only the exposure to undiluted SOA_{NAP}-SP, SP and aged SP significantly induced DNA breaks. This difference between the cell lines is in line with a study by *Biola-Clier et al. (2017)* showing similar genotoxicity in both cell lines after the exposure to TiO_2-NPs, however a higher degree of repair mechanisms occurring in BEAS-2B cells compared to A549 cells. This may be due to the cancerous origin of A549 cells and an impaired upstream regulator of *NRF2* preventing DNA repair through nucleotide excision repair and base excision repair pathways *(Jugan et al. 2012)*. Moreover, oxidative stress can trigger the damage of additional biomolecules, such as proteins, lipids or carbohydrates *(Thimmulappa et al. 2019)*. To measure the loss of the alveolar membrane integrity, we conducted the LDH assay on the cell culture media collected after the aerosol exposures (KE 6). Notably, we observed a dose-dependent increase of LDH in the cell culture media of A549 cells after the exposure to both SOA, with a stronger response after SOA_{NAP}-SP. Also, the exposure to undiluted fresh and aged SP resulted in a significant increased release of LDH. Altogether, determining the cell viability as last KE (KE 7) revealed a dose-dependent decrease of cell viability that was more prone after the exposure to SOA_{NAP}-SP and in A549 cells than in BEAS-2B cells. The exposures to $SOA_{\beta PIN}$-SP, fresh and aged SP resulted in similar cell viability effects, which were again more prone in A549 than in BEAS-2B cells. This is in line with the observed effects in the KE 1-6 and also the observation of slightly different responses by diverse respiratory epithelial cells (A549 cells vs. BEAS-2B cells) has already been made elsewhere *(Biola-Clier et al. 2017; Hillyer et al. 2018)*. The heatmap in ***Figure 11*** summarizes all observed effects firstly dependent on the cellular origin and secondly on the aerosol source and dilution, highlighting the enhanced toxic effects of SOA_{NAP}-SP vs. $SOA_{\beta PIN}$-SP and fresh SP vs. aged SP, as well as, the greater response in A549 cells vs. BEAS-2B cells.

These results strengthened the hypothesis that physically similar aerosols show distinct toxic effects corresponding to their chemical composition and oxidation state in lung epithelial cells and emphasized the importance of environmental conditions like atmospheric aging and the occurrence of VOC. Amongst others, those environmental conditions are highly dependent

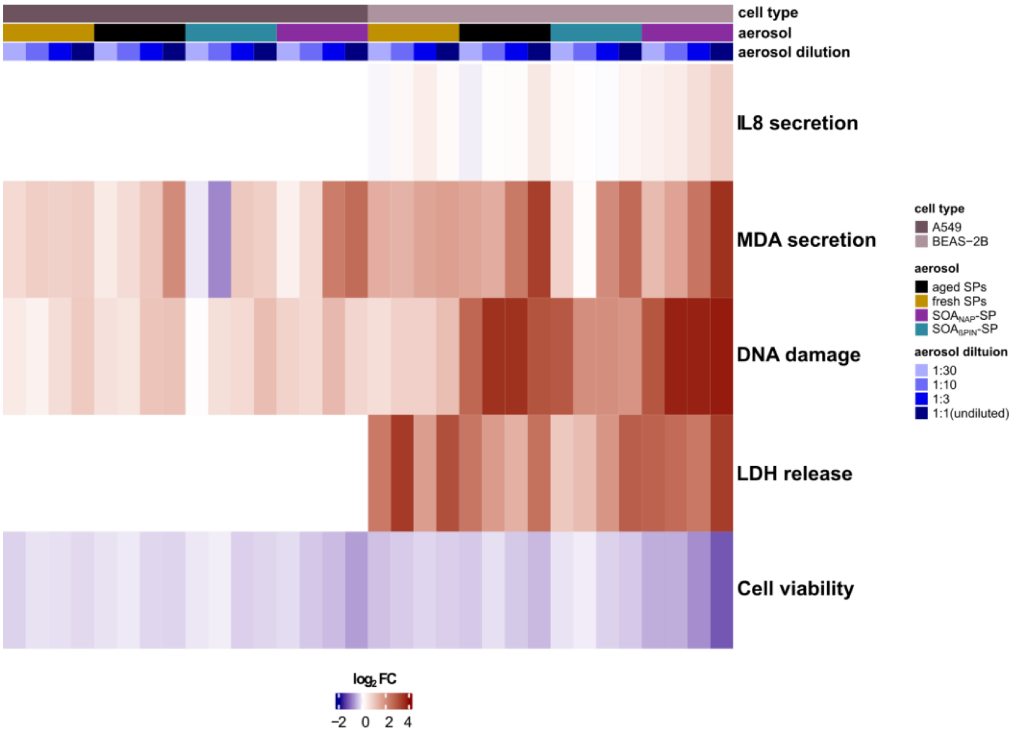


Figure 11. Summary of the toxicological effects in A549 and BEAS-2B cells after the exposure to different dilutions of SOA_{NAP}-SP, $SOA_{\beta PIN}$-SP, fresh and aged SP.

on climate change and studies predicted with an increase of temperature an upward abundance of VOC and NO_x emissions in urban areas and high ozone events *(Nussbaumer and Cohen 2020; Wu et al. 2008)*. Therefore, to understand cellular effects triggered by atmospheric aging of VOC gained more and more attention and a reduction of VOC emissions have been widely discussed and seemed to be indispensable.

A higher oxidized, aromatic SOA of higher oxidative potential induced to a greater extent a cascade of toxic events, including cell death, oxidative stress response, inflammation and genotoxicity, compared to a less oxidized, aliphatic SOA of lower oxidative potential. Importantly, fresh SP showed only minor toxic effects. Coherent with previous studies, anthropogenic SOA triggered an increased toxic response compared to a biogenic SOA. Especially ring-opening and ring-retaining compounds, such as 1-naphthol or 2-naphthol which can be further oxidized to naphthoquinones, in the particulate phase and aromatic compounds, such as naphthalene, in the gas phase have already been correlated to adverse health effects *(Jia and Batterman 2010; Wang et al. 2018)*. Thus besides the physical properties of SOA, such as particle diameter that enables a particle deposition in deep lung areas, the chemical properties of the aerosol induces specific toxicological effects in the lung cells depending on their functional groups and their oxidative potential. Recent studies evinced the importance of short-lived unsaturated carbonyls *(Han et al. 2020)*, products of

oxidized furan- *(Tabaran et al. 2019)*, as well as, aldehyde-groups *(Rebollido-Rios et al. 2020)* and epoxides *(Wang et al. 2007)* in inducing adverse cellular effects. We highlighted that the oxidation of fresh SP is enhancing the cellular toxicity compared to fresh SP, affirming the pivotal role of atmospheric aging in inducing adverse health effects. Here, we speculated that the increased occurrence of oxygen-containing moieties in the aromatic carbon rings of aged SP were responsible for the observed effects.

In addition to that, the usage of one bronchial, transformed (BEAS-2B) and one alveolar, cancer-derived epithelial (A549) cell line gave us the possibility to address the drawbacks (e.g. mRNA expression, mitochondrial activity and antioxidant capacities) of those single cell lines compared to primary cells from the upper or lower airways *(Lujan et al. 2019)* and rigidified the observed toxicological effects.

4.2.2 Aerosol exposures in the coculture of A549 and EA.hy926 cells

Our interest was caught by the observation of a significant increased secretion of IL-8 (KE 1) after both SOA exposure in the cell culture medium of the coculture compared to the monoculture. Also, we detected DNA damage in EA.hy926 cells (KE 5) after the exposure to SOA_{NAP}-SP (1:1 and 1:3 dilution) indicating an activation of the not directly exposed endothelial cells and pointing towards secondary genotoxicity (***Figure 12A***). To the best of our knowledge, this was the first study showing secondary genotoxicity in a coculture model system exposed to ALI and not in conditioned media-based experiments *(Akerlund et al. 2019; Evans et al. 2019)*. This is of major importance since inflammation-driven secondary genotoxicity is linked to the development of cancer, enhancing the need for robust testing strategies in *in vitro* settings *(Evans et al. 2017)*. An increasing number of studies indicates the importance of endothelial cell injury inducing cardiovascular diseases in response to $PM_{2.5}$ exposure *(Adar et al. 2013; Pope et al. 2016; Ying et al. 2014)* and we observed an enhanced angiogenic potential of endothelial cells (AO) when incubated with conditioned media from the exposure to SOA_{NAP}-SP. Therefore, we focused on the in-depth characterization of those KE events and the AO by analyzing gene expression and metabolite secretion and revealing GO terms, KEGG terms, genes, metabolites and pathways with the help of enrichment analysis tools (***Figure 12B***).

We observed in EA.hy926 cells a deregulation of early ER stress response genes and genes related to endothelial cell biology, which are known to be involved in atherosclerotic, angiogenic and coagulation processes. This was evident after the exposure to both SOA, however more prone after the exposure to SOA_{NAP}-SP, and suggested early genomic alterations in endothelial cells to a certain degree independently of the SOA's source. Here,

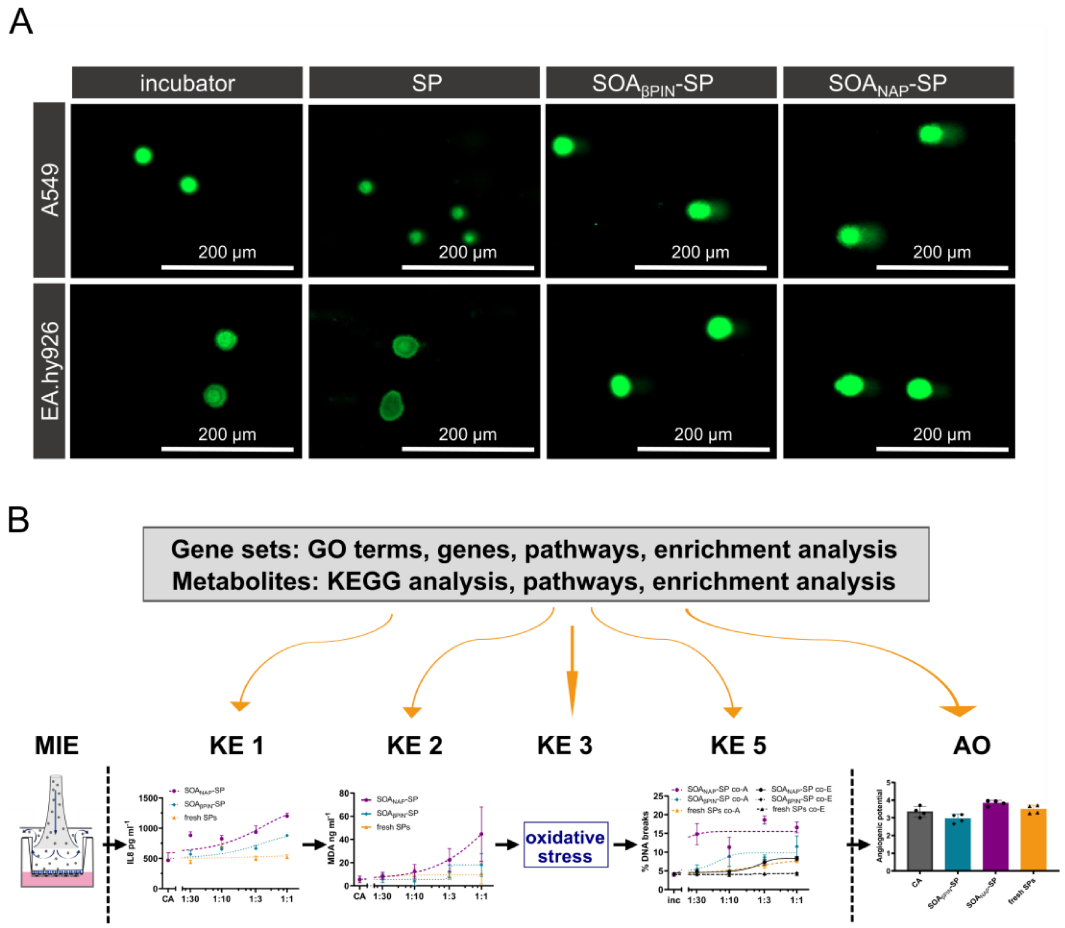


Figure 12. (A) Representative images of the analysis of DNA breaks by comet assay. Green: nucleic acid stain; SYBR gold. Scale bar: 200 µm. (B) Results and outline for the AOP in the coculture model system with results for IL8 and MDA release, as well as, the occurrence of DNA breaks and the triggered angiogenic potential.

we found a significant upregulation of the genes ERBB Receptor Feedback Inhibitor 1 (*ERRFI1*), FosB Proto-Oncogene (*FOSB*) and Nuclear Receptor Subfamily 4 Group A Member 1 (*NR4A1*) that have been grouped to immediate early genes *(Xu et al. 2006; Zhao et al. 2021)*. Especially *NR4A1* is rapidly transcribed in proliferation-inducing signals and genomic instability *(Guo et al. 2021)*, thus, affirming the secondary genotoxicity of EA.hy926 cells also on transcriptomic level. Moreover, we found a significant enrichment of genes related to angiogenesis, such as adrenomedullin (*ADM*) and B-cell translocation gene 1 (*BTG1*), which are additionally known to be involved in endothelial and epithelial barrier function *(Garcia-Ponce et al. 2016)* and in impacting the blood and lymphatic vasculature *(Karpinich et al. 2011)*. In addition to that, the secretion and gene expression of IL-6 was enhanced upon SOA exposure and has already been correlated to activation of coagulation and the reduction of the transcription of thrombosis inhibitors after PM exposure *(Mutlu et al. 2007)*. This highlights one more time the importance of studying the impact of ambient

aerosols on endothelial cells since an impaired function can lead to cardiovascular disease outcomes *(Hamanaka and Mutlu 2018; Konduracka and Rostoff 2022)*.

Furthermore, we detected a stress-related airways remodeling in A549 cells of the coculture and found supporting genomic information of the above descripted early type I immune responses especially after the exposure to SOA_{NAP}-SP. The shift from AT2-secretory like cells to more $MUC5A^{+}$ goblet-secretory like cells by the upregulation of mucin 5AC (*MUC5AC*), mucin 5B (*MUC5B*) or FC gamma binding protein (*FCGBP*) have already been observed in type 2-high asthma *(Jackson et al. 2020)* and the assumption of secretory cells playing a crucial role in the regeneration after injury has been laid out by *Basil et al. (2022)*.

Besides gene expression analysis, we uncovered the differences in the abundance of metabolites in the cell culture medium of the coculture after the SOA exposures. Only a minor number of altered metabolites were similar between the exposure to SOA_{NAP}-SP and $SOA_{\beta PIN}$-SP and some compounds seemed to origin from the aerosols, such as 3-oxy-2-naphhtoic acid (***Figure 13A***). The metabolome data showed sharp decreases in the abundance of amino acids (aa) and derivatives including arginine and norleucine after all SOA exposures and additionally glycine and pyroglutamic acid after the exposure to SOA_{NAP}-SP and SP in

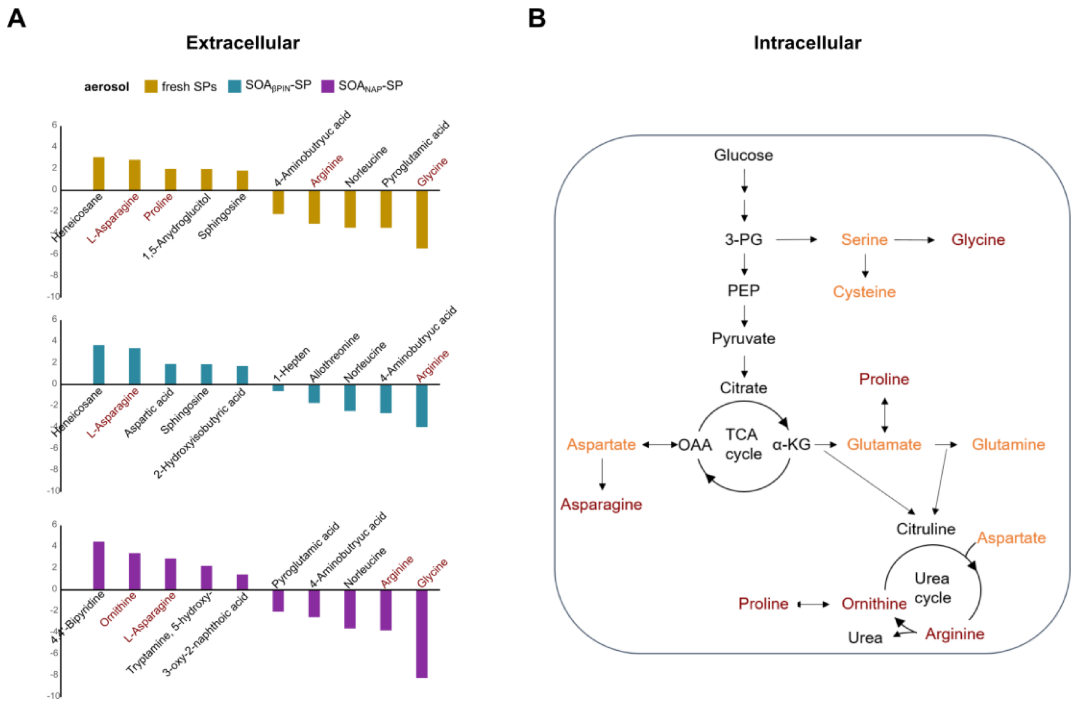


Figure 13. (A) The non-essential amino acids (neaa) that we detected by GC-MS analysis of the cell culture media after the exposure to SP and both SOA products. (B) Overview of the intracellular de novo synthesis of non-essential amino acids (labelled in orange and in red). 3-PG: 3-phosphoglyceric acid; PEP: phosphoenolpyruvate; α-KG: alpha-ketoglutarate; OAA: oxaloacetate.

the cell culture media. L-asparagine and ornithine increased especially after SOA_{NAP}-SP. This is in agreement with a study by *Breitner et al. (2016)* describing an altered glycine-ornithine-arginine metabolic axis in the blood plasma after short-term exposure to $PM_{2.5}$ in a cardiac catheterization cohort. In general, metabolic reprogramming has been widely accepted as a sign for malignancy and is included as one of the hallmarks of cancer *(Hanahan and Weinberg 2011)*. Especially, an abnormal amino acid metabolism is indispensable to balance the additional need for nutrients in order to facilitate the survival and proliferation of cancer cells. Stress factors, such as genotoxic, oxidative and nutritional challenges in the tumor microenvironment is triggering an enhanced uptake of essential aa and an increased *de novo* synthesis of non-essential amino acids (neaa) *(Wei et al. 2021)*. Those stress factors have also been observed in our study, strengthening the necessity of discussing the amino acid metabolism in the context of aerosol exposure. The aa serine and glycine can be synthesized by the conversion of glucose within the serine synthesis pathway (***Figure 13B***). Whereas, the tricarboxylic acid (TCA) cycle is not only providing substrates for forming lipids and fatty acids, but also in generating intermediates for the neaa synthesis *(Bender and Mayes 2016)*. Here, citrate is converted into α-ketoglutarate and possible in its associated aa glutamine, proline and arginine. Moreover, α-ketoglutarate is converted over several steps into oxaloacetate, which is able to form the aa aspartate and asparagine. Besides the TCA cycle, the urea cycle plays an important role in synthesizing aa *(Wei et al. 2021)*. In this cycle arginine is broken down by arginases to ornithine and urea, which is then shuttled to the mitochondria (ornithine) or excessed over the liver (urea).

Analyzing our metabolome data with the help of the IPA software revealed an affected glutathione metabolism (γ-glutamyl cycle) after the exposure to SOA_{NAP}-SP, as well as, SP exposure. Since glycine degradation is essential for the production of the antioxidant glutathione (GSH) (***Figure 14A***), a reduced abundance in the cell culture media possibly indicates an increased demand for GSH through oxidative stress *(Vivancos et al. 2010)* and higher levels of the γ-glutamyl transferases are observed in lung cancer *(Jean et al. 2003)*. Moreover, metabolome data of COPD patients revealed a correlation between reduced glycine serum levels and the occurrence of emphysema, which is beside chronic bronchitis one of the causes to airflow obstruction *(Godbole and Bowler 2022; Ubhi et al. 2012)*. In addition to that, the uptake of glycine by endothelial cells significantly promotes angiogenesis

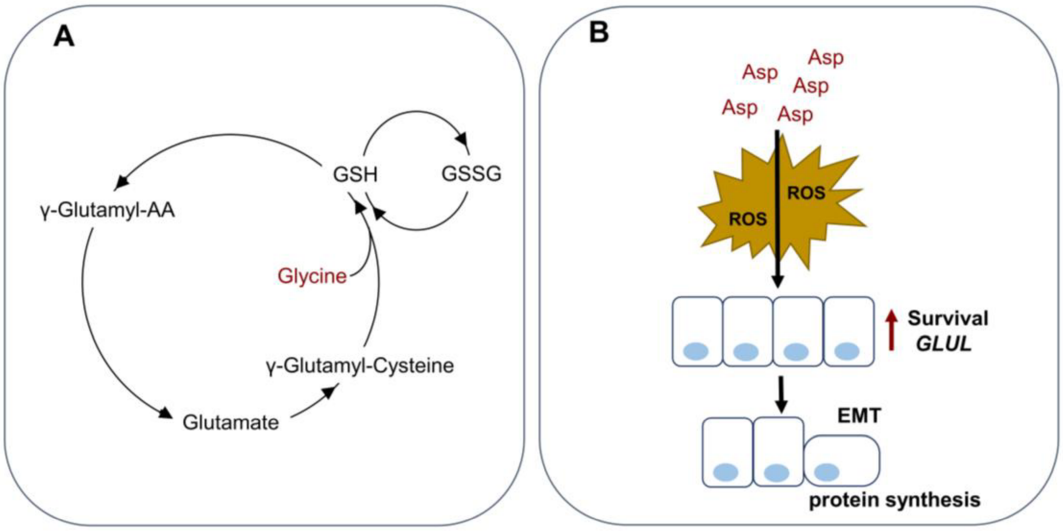


Figure 14. (A) Overview of the intracellular glutathione biosynthesis that is linked by the γ-glutamyl cycle. (B) Hypothesis on possible effects of high exogenous asparagine (Asp) level on lung epithelial cells inclusing increased cellular survival and the upregulation of GLUL, resulting in induced EMT and the maintenance of protein synthesis.

and is a key mediator in vascular endothelial growth factor (VEGF) signaling *(Guo et al. 2017)*. This is in line with the observed greater angiogenic potential in the EA.hy926 cells that have been treated with the conditioned media collected after the exposure to SOA_{NAP}-SP (***Figure 12B***) and the enhanced expression of the gene vascular endothelial growth factor A (*VEGFA*; 1.0 log_2FC after SOA_{NAP}-SP exposure).

In addition to that the observed reduced arginine availability in the cell culture media can be possibly due to a low *de novo* production and/or increased conversion to ornithine and urea by arginases (***Figure 13B***). We detected a slightly upregulation of the gene arginase 2 (*Arg2*) in the EA.hy926 cells after the exposure to SOA_{NAP}-SP (0.67 log_2FC), strengthening the hypothesis of an increased turnover. An increased turnover and a reduced *de novo* production of arginine have already been correlated to conditions of acute and chronic stress *(Luiking et al. 2012)*, as well as, to individuals with asthma that have been exposed to traffic-related pollutants *(Liang et al. 2019)*. One of the possible consequences of an impaired arginine metabolism can be a diminished NO synthesis that has been implicated in several cardiovascular diseases since NO has vasodilatory, anti-thrombotic and generally anti-atherogenic properties *(Kao et al. 2015; Ryoo et al. 2006; Tousoulis et al. 2007)*. This was also in line with the IPA analysis showing a significant correlation to the urea cycle and NO

signaling in the cardiovascular system of the metabolites altered in the cell culture medium after the exposure to SOA_{NAP}-SP.

An enhanced exogenous level of asparagine has been associated with multiple stages in the metastatic cascade of breast cancer *(Knott et al. 2018)* (***Figure 14B***). It is speculated that asparagine is regulating epithelial to mesenchymal transition (EMT) inducing tumor cell migration and invasion at the primary tumor site. Indeed, we found an upregulation of stem cell regulator genes (wnt family member 4 (*WNT4*) and sex determining region Y-box 2 (*SOX2*)) in A549 cells after the exposure to SOA_{NAP}-SP that may in turn induce the transcription of EMT-regulatory proteins. Interestingly, an addition of asparagine to the circulation positively correlated with an increased protection of cancer cells against oxidative stress and shear *(Knott et al. 2018)*. Moreover, *Pavlova et al. (2018)* observed the maintaining of protein synthesis by asparagine when glutamine is depleted and detected an upregulation of the gene Glutamate-Ammonia Ligase (*GLUL*) that is known to facilitate cell growth and survival, e.g. at distant metastatic sites. In our dataset, we found an upregulation of *GLUL* in A549 cells (1.3 log_2FC after SOA_{NAP}-SP, 0.8 log_2FC after $SOA_{\beta PIN}$-SP and SP) and EA.hy926 cells (1.4 log_2FC after SOA_{NAP}-SP; 0.9 log_2FC after $SOA_{\beta PIN}$-SP and SP), which is nicely correlating to the enhanced asparagine occurrence in the cell culture media after all aerosol exposures.

We evaluated whether our metabolome data can be further integrated with our gene expression data. The most common significant altered canonical pathways were highlighting the indirect impact of SOA on EA.hy926 cells pointing into the direction of cell activation and DNA damage. The observed altered iNOS, PDGF and apelin signaling that seem to play a crucial role in vascular endothelial cell dysfunction, including increased cell migration *(Zhu et al. 2016)*, vascular growth *(Helker et al. 2020)* and possibly orchestrating the development of respiratory diseases *(Yan et al. 2020)* or fibrosis *(Layton et al. 2022)*. Notably, the significant altered ceramide and sphingosine-1-phosphate signaling pathways coincided with the exposure to SOA_{NAP}-SP, which was also consistent with a study correlating those pathways with elevated levels of inflammatory mediators and the exposure to PAHs in asthmatic patients *(Wu et al. 2022)*. One of the most important pathways for intracellular signal transduction as a cellular response towards DNA damage and oxidative stress, as well as, typically activated for effective angiogenesis, is the PI3K/AKT-signaling pathway *(Karimian et al. 2019; Pober and Sessa 2007)*. Especially in the EA.hy926 cells an alteration of this pathway was observed after the exposure to SOA_{NAP}-SP both at transcript and

metabolite levels, which is in agreement with the observed upregulation of early stress-related genes as well as the detected secondary genotoxicity and the induced angiogenesis.

Our results further confirmed the crucial role of endothelial cells in exposure studies by showing early responses to stress events such as oxidative stress, DNA damage and a pro-inflammatory state on gene and metabolome level. Furthermore, our study suggested that endothelial cells are affected more generalized and to a greater extent independently of the aerosol source, highlighting the adverse systemic outcomes of exposures to ambient aerosols. Moreover, in-depth characterization of A549 cells revealed a stress-related airways remodeling process towards $MUC5A^+$ goblet-secretory like cells, which was more prone after the exposure to SOA_{NAP}-SP. This is possible enlisted in the injury response that is in line with showing that a higher oxidized, aromatic SOA of higher oxidative potential induced to a greater extent a cascade of toxic events in epithelial cells (A549 and BEAS-2B cells), compared to a less oxidized, aliphatic SOA of lower oxidative potential. In conclusion, we were able to show local adverse effects on epithelial cells that were depending on the chemical composition of the particles and the gas phase, whereas we observed a systemic adverse effect on endothelial cells that seemed to depend more on the cell-to-cell interplay. This is of immense importance since numerous epidemiological studies correlated air pollution not only to local effects in the lung, but also to increased occurrence of cardiovascular diseases. Therefore, the obtained results are benefitting the need to study aerosol exposure under realistic conditions with cell culture systems enabling a cell-to-cell interplay and to assess the potential of inducing genotoxicity by secondary mechanisms in *in vitro* settings.

5. Summary and outlook

Air pollution with $PM_{2.5}$ is a world leading cause for premature deaths and is expected to increase in the following years as a result of climate change. Different to sulfur oxides (SO_x) and nitrogen oxides (NO_x), the awareness of health impacts originating from VOC precursors, as well as, SOA component of $PM_{2.5}$ is just in the beginning. An increase in temperature can have serious consequences on the concentration of VOC with upward abundances in urban areas *(Nussbaumer and Cohen 2020)*. Therefore a better understanding of their toxicity can be accompanied by emission reduction scenarios, which potentially results in health benefits. The focus of this study was put on the identification of the toxicological potential of an atmospheric aged biogenic (β-pinene) or anthropogenic (naphthalene) VOC by being exposed to three different lung cell model systems. The atmospheric aging of VOC together with a primary aerosol (e.g. SP) to form SOA through condensation processes is found in the atmosphere and this experimental set-up enabled to resemble realistic exposure scenarios. The formed SOA were similar in their physical characteristics, however differed in the chemical composition with more aromatic, oxidized components for SOA_{NAP}-SP and more aliphatic, less oxidized components for $SOA_{\beta PIN}$-SP. Moreover, fresh and aged SP were used for further comparisons of the toxicological cellular effects.

The exposure to both SOA and aged SP showed in all three lung cell models a decrease in cell viability and an increase in cellular cytotoxicity, oxidative stress, genotoxicity and pro-inflammatory cytokines, whereas the exposure to fresh SP only showed minor effects. Moreover, the observed effects were more prone after the exposure to SOA_{NAP}-SP compared to $SOA_{\beta PIN}$-SP and aged SP. This is in line with the assumption that the aerosols toxicity is possibly related to the aerosols' organic components and that its chemical composition and oxidative state play major roles. The significant upregulation of the *NRF2* gene, the *AhR* gene and its downstream target gene *CYP1A1* in BEAS-2B cells was only found after the exposure to SOA_{NAP}-SP and aged SP, which pointed into the direction of aromatic ring structures, such as PAHs, as activators. In A549 cells of the coculture model, genomic information suggested a stress-related airways remodeling of AT2-secretory like cells to more $MUC5A^+$ goblet-secretory like cells. Furthermore, evidence was found that the exposure to both SOA, different to fresh SP, induced an early type I inflammatory response in all three lung cell models. Despite studying aerosol effects in monocultures of lung epithelial cells, the coculture of A549 and endothelial (EA.hy926) cells revealed a pivotal role of not directly exposed cells in the outcome of aerosol exposures. EA.hy926 cells showed indications of secondary genotoxicity with an upregulation of early stress response genes and detectable single- and

double- strand DNA breaks especially after the exposure to SOA_{NAP}-SP. This was in line with the findings of the integrated IPA analysis of gene expression and metabolite abundance highlighting the involvement of canonical pathways such as, iNOS, PDGF and P13K/AKT signaling. In addition to that, a correlation between SOA exposure and the upregulation of cardiovascular disease-related genes were observed. In defiance of the greatest effect after the exposure to SOA_{NAP}-SP, also $SOA_{\beta PIN}$-SP and fresh SP led to significant toxico-genomic effects and strengthened the hypothesis of an early involvement of cocultured endothelial cells upon aerosol exposure. This results in the hypothesis of a more systemic effect triggered by the endothelial cells, which possible leads to cardiovascular adverse health outcomes (***Figure 15***).

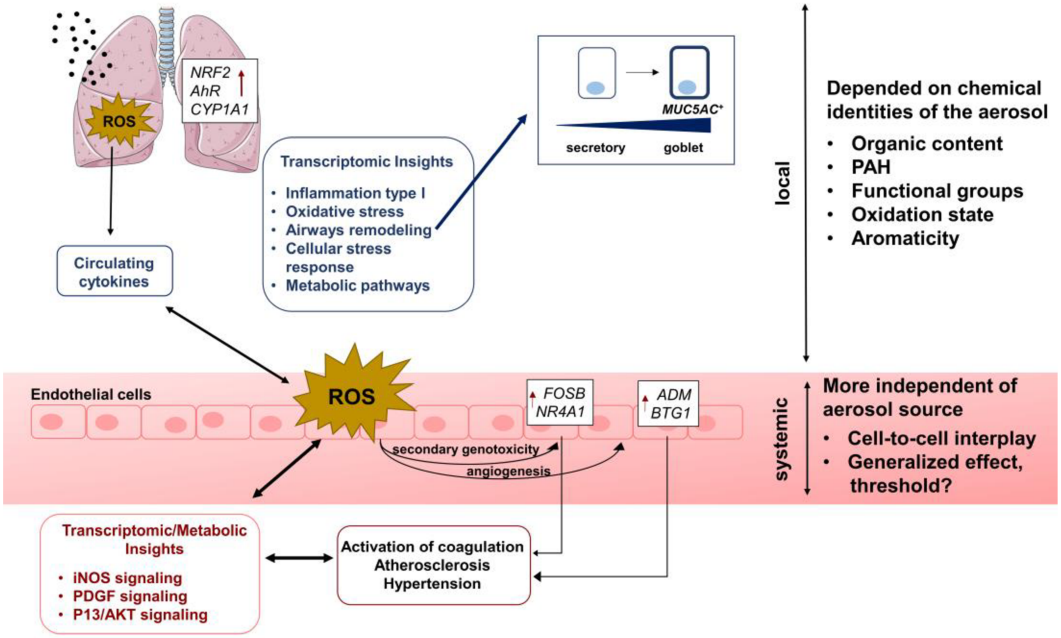


Figure 15. Proposed pathomechanisms triggered by aerosol exposure. Aerosols confer most of their effects via damage of the lung (inflammation, oxidative stress, genotoxicity and airways remodeling towards $MUC5AC^+$ goblet-secretory like cells). This seemed to be dependent on the specific aerosols chemical composition (organic content, PAHs, functional groups, oxidation state, aromaticity). The primary target organ damaged by aerosols converges at the cardiovascular level by inducing dysfunctions in endothelial cells (secondary genotoxicity), inflammation, metabolic reprogramming and angiogenesis. This possibly results in activation of coagulation, atherosclerosis and/or hypertension via iNOS, PDGF and/or P13LAKT signaling.

Altogether, this thesis contributes to the understanding of cellular and molecular toxicological impacts of physically similar, but chemically distinct SOA and highlights the importance of using more complex cell culture model systems allowing cell-to-cell interplay. The results emphasize the necessary connection between atmospheric and biological sciences to further understand the complexity of aerosol composition and the resulting cellular effects in order to establish restriction for beneficial health outcomes. Future studies are planned to overcome the simplicity of a single VOC precursor aging to the usage of more complex aerosols, such as aged car exhaust or wood combustion emissions.

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Appendix

Contribution to conferences

1. **Offer S**, Hartner E, Rudich Y, Kiendler-Scharr Y, Di Bucchianico S, Zimmermann R. (2021). Differential impact of biogenic and anthropogenic secondary organic aerosol compounds adsorbed on soot particles in lung cell models at the air-liquid interface. 24th ETH-Conference on Combustion Generated Nanoparticles. Online. 22-24 June. Conference Talk.
2. **Offer S**, Di Bucchianico S, Czech H, Pardo M, Rudich Y, Zimmermann R. (2022). Effect of Atmospheric Aging on Soot Particle Toxicity in Airway Epithelial-Endothelial Co-culture Models at the Air–Liquid Interface: Differential Toxicological Impacts of Biogenic and Anthropogenic Secondary Organic Aerosols (SOAs). Analytica Conference Munich. 21-23 June. Poster Presentation.
3. **Offer S**, Di Bucchianico S, Czech H, Pardo M, Rudich Y, Zimmermann R (2022). Effect of Atmospheric Aging on Soot Particle Toxicity in Airway Epithelial-Endothelial Co-culture Models at the Air–Liquid Interface: Differential Toxicological Impacts of Biogenic and Anthropogenic Secondary Organic Aerosols (SOAs). International Aerosol Conference in Athens. 4-9 September. Conference Talk.

Curriculum Vitae

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