Universität Rostock
HELMHOLTZ MUNICH
JOINT MASS SPECTROMETRY CENTRE

Multicellular *In Vitro* Systems Assessing Primary and Secondary Genotoxicity Involved in Particle-Driven Pro-Fibrotic Conditions

Cumulative Dissertation

submitted to
the Faculty of Mathematics and Natural Sciences of the University of Rostock
in fulfilment of the requirements for the academic degree
doctor rerum naturalium (Dr. rer. nat.)

by

Jana Pantzke
born February 19th, 1993 in Berlin

Rostock, March 2023

https://doi.org/10.18453/rosdok_id00004397

Reviewer:

Prof. Dr. Ralf Zimmermann, University of Rostock, Institute of Chemistry

Assoc. Prof. Dr. Hanna Karlsson, Karolinska Institute, Institute of Environmental Medicine

Year of submission: 2023

Year of defense: 2023

- Nicht für die Schule, sondern fürs Leben lernen wir.

List of original publications

First-authorships

The following manuscripts were created by Jana Pantzke as first author and published in peer-reviewed journals. The contribution of Jana Pantzke is given below.

Title: **An alternative *in vitro* model considering cell-cell interactions in fiber-induced pulmonary fibrosis**

Authors: **Pantzke J**, Offer S, Zimmermann E J, Kuhn E, Streibel T, Oeder S, Di Bucchianico S and Zimmermann R.

Journal: Toxicology Mechanisms and Methods

Year: 2022

DOI: 10.1080/15376516.2022.2156008

Jana Pantzke contributed to this study by developing the study design and the implementation of the biological part of the study. In short, the development and handling of the used cell model system throughout the whole study, the performance of the assays determining the cell count, LDH, colony forming efficiency and IL8 release, as well as the genotoxicity assessment and the immunostaining. Furthermore, she conducted the physical characterization of the material in suspension. Moreover, she did the evaluation of all published biological data, as well as, wrote and revised the manuscript.

Title: **Processing of carbon-reinforced construction materials releases $PM_{2.5}$ inducing inflammation and (secondary) genotoxicity in human lung epithelial cells and fibroblasts**

Authors: **Pantzke J**, Koch A, Zimmermann E J, Offer S, Rastak N, Offer S, Bisig C, Bauer S, Oeder S, Orasche J, Fiala P, Stintz M, Rüger C P, Streibel T, Di Bucchianico S and Zimmermann R.

Journal: Environmental Toxicology and Pharmacology

Year: 2023

DOI: 10.1016/j.etap.2023.104079

Jana Pantzke contributed to the investigation and analysis of all biological data, including the statistical analysis, conceptualization of the manuscript, interpretation of the data as well as the production of all illustrations. Moreover, she worked in the process of chemical and physical data integration as well as interpretation. Furthermore, she wrote and revised the manuscript.

Co-authorship

Title: **Effects of Atmospheric Aging on Soot Particle Toxicity in Lung Cell Models at the Air-Liquid Interface: Differential Toxicological Impacts of Biogenic and Anthropogenic Secondary Organic Aerosols (SOAs)**

Authors: Offer S, Hartner E, Di Bucchianico S, Bisig C, Bauer S, **Pantzke J**, Zimmermann E J, Cao X, Binder S, Kuhn E, Huber A, Jeong S, Käfer U, Martens P, Mesceriakovas A, Bendl J, Brejcha R, Buchholz A, Gat D, Hohaus T, Rastak N, Jakobi G, Kalberer M, Kanashova T, Hu Y, Ogris C, Marsico A, Theis F, Pardo M, Gröger T, Oeder S, Orasche J, Paul A, Ziehm T, Zhang Z-H, Adam T, Sippula O, Sklorz M, Schnelle-Kreis J, Czech H, Kiendler-Scharr A, Rudich Y and Zimmermann R.

Journal: Environmental Health Perspectives

Year: 2022

DOI: 10.1289/EHP9413

Jana Pantzke contributed to this study by developing the applied co-culture model and by the implementation of the genotoxicity assessment. Moreover, she was involved in the revision of the manuscript.

Related publications

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.** *Science of the Total Environment.* Submitted.

Zimmermann E J, Candeias J, Gawlitta N, Bisig C, Binder S, **Pantzke J**, Offer S, Rastak N, Bauer S, Huber A, Kuhn E, Buters J, Groeger T, Delaval M N, Oeder S, Di Bucchianico S, Zimmermann R. **Impact of sequential exposures to allergens and ultrafine particles on human bronchial epithelial BEAS-2B cells at the air liquid interface.** *J. Appl. Toxicol.* 2023. Accepted.

Pardo M, Czech H, Offer S, Sklorz M, Di Bucchianico S, Hartner E, **Pantzke J**, Kuhn E, Paul A, Ziehm T, Zhang Z-H, Jakobi G, Bauer S, Huber A, Zimmermann E J, Rastak N, Binder S, Brejcha R, Schneider E, Orasche J, Rueger C, Groeger T, Oeder S, Schnelle-Kreis J, Hohaus T, Kalberer M, Sippula O, Kiendler-Scharr A, Zimmermann R, Rudich Y. **Atmospheric aging increases the cytotoxicity of bare soot particles in BEAS-2B lung cells.** *Aerosol Science & Engineering 2023*. 10.1080/02786826.2023.2178878

Romano M, González Gómez M, Santonicola P, Aloi N, Offer S, **Pantzke J**, Raccosta S, Longo V, Surpi A, Alacqua S, Zampi G, Dediu V, Michalke B, Zimmermann R, Manno M, Piñeiro Y, Colombo P, Schiavi E D, Rivas J, Bergrese P, Di Bucchianico S. **Synthesis and characterization of a biocompatible nanoplatform based on silica-embedded SPIONs functionalized with polydopamine**. *ACS Biomater. Sci. Eng. 2023*. 10.1021/acsbiomaterials.2c00946.

Pardo M, Offer S, Hartner E, Di Bucchianico S, Bisig C, Bauer S, **Pantzke J**, Zimmermann E J, Cao X, Binder S, Kuhn E, Huber A, Jeong S, Käfer U, Schneider E, Mesceriakovas A, Bendl J, Brejcha R, Buchholz A, Gat D, Hohaus T, Rastak N, Karg E, Jakobi G, Kalberer M, Kanashova T, Hu Y, Ogris C, Marsico A, Theis F, Shalit T, Gröger T, Rüger C, Oeder S, Orasche J, Paul A, Ziehm T, Zhang Z-H, Adam T, Sippula O, Sklorz M, Schnelle-Kreis J, Czech H, Kiendler-Scharr A, Zimmermann R, Rudich Y. **Exposure to naphthalene and beta-pinene-derived secondary organic aerosol induced divergent changes in transcript levels of BEAS-2B cells.** *Environmental International 2022.* 10.1016/j.envint.2022.107366.

Acknowledgements

I would like to say a big thank to the team of the Joint Mass Spectrometry Center (JMSC), without whom I would not have been able to complete this research and who guided me through the time of my PhD.

First of all, I would like to extend my sincere thanks to **Prof. Dr. Ralf Zimmermann** for his support and supervision, lively and enthusiastic discussion during campaigns, and after all, for giving me the opportunity to pursue my doctoral research at CMA.

Furthermore, I am especially grateful for the continuing support of my supervisor **Dr. Sebastiano Di Bucchianico**, for everything I was privileged to learn from you, passing on the unbroken passion about genotoxicity and science. I would like to thank you for the encouragement during hard times, for our inspiring conversations (with or without beers), and for always supporting my ideas and goals. Thank you so much, Seba, that you always believed in me. I could not have finished this rollercoaster ride without you.

I would also like to thank my supervisor **Dr. Sebastian Öder** for the valuable guidance to realize my projects, for your scientific and personal support and your constant patience that helped me to keep a cool head. I am very grateful, that you brought me into the group and to make me work with so many wonderful people, you immediately made me feel at home at CMA.

I could not have undertaken this journey without **Svenja** and **Elena**. We have been on this adventure together, with all its ups and downs. Whether laughing, crying, agreements, disagreements, professional or emotional mountains, we have climbed them together. Thank you for your permanent support, for all the tears you dried, for putting the brakes on when my impulsivity ran out of hand. Above all, I am extremely grateful for your continuous good mood, the unbroken enthusiasm for the small things in life, the craziness after one glass of wine, our HP movie nights and singing and dancing together.

I am extremely grateful that through CMA I was able to gain a friend like **Elias**. You have always encouraged me to stand up for my principles and pursue my wishes and dreams. Countless evenings together at the Isar (under the bridge), cooking or watching cooking shows, driving home together or going for a walk, you shared my worries and happiness. You were always there when I needed you most (and even when I didn't). I look forward to sharing such moments with you for many more years!

I am very happy, that after a short time I can count **Johannes** among my friends. Even though we haven't known each other for long, sometimes it just fits like a glove. Thank you for sharing my humor, understanding my irony, countering me and sharing earrings with me. Part of the ship, part of the crew.

Furthermore, I would like to thank all the wonderful people who have sweetened my workday day in and day out. **Ramona, Gerti, Patrick, Nadine, Seongho, Lukas, Marina**, because of you there was always a good mood at the morning coffee or the lunch table, during numerous barbecue or Isar evenings, campaigns and campaign closings. I could not have wished for better colleagues and hope for more joyful evenings together.

Apart from all colleagues of the JMSC, I would like to thank all project partners as well as the funding parties for the support, sharing their expertise and the pleasant collaboration.

Above all, I would like to thank **Jürgen**, who was the biggest fan of my work, constantly supporting me and driving me to my best achievements. We have shared many moments of joy and sorrow, always contested a common path and pushed forward our happiness. Whether chocolate cream or wine testing, drifting in the Eisbach, afternoons on the Stemmerwiese, thanks to you I had the most enjoyable time during my PhD. You showed me what is important in life and that you should always follow your dreams.

I am incredibly grateful for **all my close friends from Berlin**, who made me escape my everyday life (thanks for not talking about work). You have shown me that friendship is what really matters, I can always count on you and be myself, you are a part of me.

Last but not least, I would like to thank **my family**, who accompanied me on this rocky road and always believed in me. I thank you for all your support, for helping me to keep a clear head and for all the right advices at the right moment.

Zusammenfassung

Aufgrund der wachsenden Industrialisierung und der vermehrten Produktion von Nanomaterialien ist der Mensch verstärkt den gesundheitlichen Gefahren lungengängiger Schadstoffe ausgesetzt. Um das dadurch entstehende Risiko besser einzuschätzen und die wachsenden ethischen Anforderungen an Versuchsmethoden zu erfüllen, besteht die Notwendigkeit alternative Teststrategien zum Tierversuch zu entwickeln. In Hinblick auf die Inhalationstoxikologie sollten diese nicht nur die anatomischen und physiologischen Bedingungen in der menschlichen Lunge wiederspiegeln, sondern auch die zeitsparende und kostengünstige Bestimmung von toxikologischen Effekten ermöglichen. Da die Exposition gegenüber inhalierbaren Stoffen wie Fasern oder Feinstäuben mit der Ausbildung von pulmonaler Fibrose oder kardiovaskulären Erkrankungen assoziiert wird, wurden im Rahmen dieser Arbeit verschiedene *in vitro*-Zellmodelle entwickelt, welche in der Lage sind mechanistische Veränderungen, die im Zusammenhang mit der Ausbildung derartiger Gesundheitseinschränkungen stehen, zu ermitteln. Insbesondere Epithelzellen, Immunzellen und Fibroblasten sind stark involviert in die Reaktion auf fremdartige Stoffe, welche bis in die Lungenbläschen, und von dort sogar in das Bindegewebe und den Blutkreislauf, vordringen können. Die zuvor genannten Zelltypen, aber auch Endothelzellen, welche die Blutgefäße bilden, weisen eine ständige Kommunikation auf, welche durch physikalische oder chemische Einflüsse substantiell gestört werden und somit zu genetischen oder zellulären Defekten führen kann. Letztere können für schwerwiegenden Folgen wie Lungenkrebs oder -fibrose verantwortlich sein.

Verschiedene Zellmodelle wurden durch die Exposition gegenüber verschiedener Luftschadstoffe hingehend ihrer Fähigkeit untersucht, Fibrose-relevante Veränderung zu detektieren. Dazu wurde die Freisetzung von Partikeln beim Trockenschneiden von (thermisch behandeltem) Carbonbeton physikochemisch und toxikologisch in kokultivierten Lungenepithelzellen und Fibroblasten analysiert. Insbesondere das thermisch behandelte Material, hat Fasern freigesetzt, welche durch die WHO als gefährlich eingeschätzte Dimensionen aufwiesen. In dem angewandten Zellmodell rief dieses Material eine akute Entzündungsreaktion und (sekundäre) DNA-Schäden hervor. Des Weiteren zeigten Transkriptomanalysen, dass diese Partikel pro-fibrotische Signalwege beeinflussen und somit möglicherweise zur Entstehung von Lungenfibrose beitragen. Eine weitere Studie konnte unter Verwendung von Epithelzellen, Makrophagen und Fibroblasten zeigen, dass die inflammatorische Signalübertragung eine wichtige Rolle in der Ausbildung eines pro-fibrotischen Phänotyps einschließlich des epithelial-mesenchymalen Übergangs sowie der sekundären Genotoxizität spielt und deshalb in toxikologischen Untersuchungen nicht vernachlässigt werden sollte. Des Weiteren sind Makrophagen in der Eliminierung von Schadstoffen involviert, was zu einer geringeren Zytotoxizität führen kann. Somit reflektiert solch ein Zellmodell die Vorgänge in der Lunge präziser und kann das Gesundheitsrisiko genauer einschätzen. Auch ein Zellmodell bestehend aus Epithel- und Endothel-zellen hat erste Anzeichen für pro-fibrotische Veränderungen nach Exposition gegenüber sekundärer organischer Aerosol aufgezeigt.

Alle Zellmodelle, welche für diese Arbeit entwickelt und angewandt wurden, sind einfach und kostengünstig in ihrer Herstellung und könnten zur Reduktion von Tierversuchen beitragen. Die Untersuchungen haben gezeigt, dass die Modelle dafür geeignet sind mechanistische Vorgänge induziert durch Luftschadstoffe in Bezug auf die Entwicklung von Lungenfibrose zu erfassen und somit die Toxizitätseinschätzung zu erleichtern.

Abstract

Due to growing industrialization and an increased production of nanomaterials, humans are increasingly exposed to the health hazards of respirable contaminants. In order to better assess the resulting risk and to meet the growing ethical requirements for testing methods, there is a need to develop alternative testing strategies to animal testing. With regard to inhalation toxicology, these should not only reflect the anatomical and physiological conditions in the human lung, but also allow the time-saving and cost-effective determination of toxicological effects. Since exposure to inhalable agents such as fibers or fine dusts is associated with the development of pulmonary fibrosis or cardiovascular disease, this work developed several *in vitro* cell models capable of detecting mechanistic changes associated with the development of such health impairments. In particular, epithelial cells, immune cells and fibroblasts are strongly involved in the response to foreign substances, which can penetrate into the alveoli, and from there further into the connective tissue and circulation. The aforementioned cell types, as well as endothelial cells that form blood vessels, exhibit constant communication, which can be substantially disrupted by physical or chemical influences, leading to genetic or cellular defects. The latter can lead to serious consequences such as lung cancer or fibrosis.

In this work, various cell models were investigated by exposure to different air pollutants with respect to their ability to detect fibrosis-relevant changes. For this purpose, the release of particles during dry cutting of (thermally treated) carbon concrete was physicochemically and toxicologically analyzed in co-cultured lung epithelial cells and fibroblasts. Especially the thermally treated material, released fibers possessing dimensions considered hazardous by the WHO. In the applied cell model, this material evoked an acute inflammatory response and (secondary) DNA damage. Furthermore, transcriptome analyses showed that these particles affect pro-fibrotic signaling pathways and thus possibly contribute to the development of pulmonary fibrosis. Another study, using epithelial cells, macrophages, and fibroblasts, demonstrated that inflammatory signaling plays an important role in the formation of a pro-fibrotic phenotype, including epithelial-mesenchymal transition, as well as secondary genotoxicity, and therefore should not be neglected in toxicological studies. Moreover, macrophages are involved in the elimination of contaminants, which may result in lower cytotoxicity. Thus, such cell model more accurately reflects processes in the lung and can more accurately assess the health risk. Also a cell model consisting of epithelial and endothelial cells has shown first signs of pro-fibrotic changes after exposure to secondary organic aerosol. All cell models developed and applied for this work are simple and low-cost to produce and could contribute to the reduction of animal testing. The studies have shown that the models are suitable to capture mechanistic processes induced by air pollutants in relation to the development of pulmonary fibrosis and thus to facilitate comprehensive toxicity assessment.

Table of contents

Abbreviations

α-SMA	alpha smooth muscle actin
βPIN	beta pinene
ALI	air-liquid interface
AHR	aryl hydrocarbon receptor
AO	adverse outcome
AOP	adverse outcome pathway
ARDS	acute respiratory distress syndrome
BaP	benzo[*a*]pyrene
BER	base excision repair
BMP4	bone morphogenetic protein-4
BPA	bisphenol A
BSA	bovine serum albumin
C^3	carbon concrete
CC	co-culture
CDH1/2	cadherin-1/2
cDNA	complementary DNA
CF	carbon fiber
CFE	colony forming efficiency
CLDN2	claudine-2
CNT	carbon nanotube
COPD	chronic obstructive pulmonary disease
CR	carbon rod
CTGF	connective tissue growth factor
CYP450	cytochrome P450
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDX	energy dispersive X-ray
EH	epoxide hydrolase
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EndMT	endothelial-mesenchymal transition
EPCAM	epithelial cell adhesion molecule
FADH	flavin adenine dinucleotide
FN1	fibronectin 1
HARM	high aspect ratio materials
GCxGC-MS	comprehensive two-dimensional gas chromatography mass spectrometry
GC-MS	gas chromatography-mass spectrometry

GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
IARC	International Agency for Research on Cancer
IATA	Integrated Approaches for Testing and Assessment
IL	interleukin
IPF	idiopathic pulmonary fibrosis
ISTD	internal standard
KE	key event
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
MC	monoculture
MDA	malondialdehyde
MeOH	methanol
MIE	molecular initiating event
miniCAST	miniature combustion aerosol standard soot generator
MMMF	man-made mineral fiber
MWCNT	multi-walled carbon nanotube
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NAM	new approach methodologies
NAP	naphthalene
NEM	*N*-ethylmaleimide
NER	nucleotide excision repair
NM	nanomaterial
Nrf2	nuclear factor erythroid 2-related factor 2
PAH	polycyclic aromatic hydrocarbon
PCLS	precision cut lung slice
PGE_2	prostaglandin E_2
PM	particulate matter
PMA	phorbol-12-myristate-13-acetate
POA	primary organic aerosol
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RNA	ribonucleic acid
RNAseq	RNA sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
RS	reactive species
SNAI1	zinc finger protein SNAI1
SOA	secondary organic aerosol
SP	soot particle

SVOC	semi-volatile organic compounds
TC	triple culture
TMB	3,3′,5,5′-Tetramethylbenzidine
ttC³	thermally treated C³
TGFβ	transforming growth factor beta
TWIST2	twist-related protein 2
UFP	ultrafine particle
UV	ultraviolet
VOC	volatile organic compound
WHO	world health organization

List of figures

1. Motivation & Scope

There are concerning and undeniable evidences that ambient (outdoor) and indoor air pollution is severely impacting human health by causing acute and chronic diseases like COPD (chronic obstructive pulmonary disease), heart diseases and strokes, lower respiratory tract infections, lung fibrosis and cancer [1-3]. The World Health Organization (WHO) claims that approximately 7 billion premature deaths per year occur due to air pollutants and states that, in 2019, 99 % of the global population lived in areas not fulfilling WHO air quality guidelines levels [4], of which an enormous proportion appears to live in low- and middle-income countries (WHO, Air pollution data portal, 2022). Driving sources of air pollution are of anthropogenic as well as natural origins (**Figure 1**). Whereas windblown dusts, wildfires, volcanic activities and plants release particulate matter (PM) of varying physical and chemical characteristics, as well as compounds such as trace gases and volatile organic compounds (VOCs) naturally [5], mankind contributes tremendously to the emission of such materials through the industrial, agricultural as well as energy and mobility sectors [6-8].
Even though the world's population is continuously exposed to a polluted environment and its association with severe diseases is well known, there are large knowledge gaps regarding specific inhalable pollutants and their interaction with the human body, their metabolism, modes-of-action and influence on the development of deleterious outcomes [9, 10]. Especially for regulatory purposes, the need for new approach methodologies (NAMs) is unavoidable due to the rapidly growing industrial sector, which brings more and more innovative materials of unknown toxicological properties to the market [11]. With the European Union compliance on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), which came into force in 2007, new manufactured or imported products (≥ 1 ton/year) have to be registered and require the communication of information concerning consumer safety [12, 13]. This state of affairs poses great challenges; as regulatory requirements can hardly be met by the sole use of animal testing. To investigate mechanisms of underlying toxicity, estimate health risks and to protect from harmful substances such as airborne compounds, the development of advanced cell models is inevitable. This approach not only allows the avoidance or reduction of animal experiments in consent with the 3R principle to reduce, refine or replace animal experiments [14], but also the provision of an efficient and physiologically relevant high-throughput screening methodology [15-17].

With respect to the urgent need for NAMs, also the evaluation of secondary (genotoxicity) mechanisms-of-action is giving important information on toxicological pathways and should be considered in the development of new *in vitro* test systems. Especially nanomaterials are known to exert secondary organ toxicity, whether due to direct or indirect mechanisms of actions, and are associated with increased health burden such as cardio-vascular or neurological disorders [18, 19]. Advanced cell culture techniques with separate cultivation of representative cell types allow for dedicated investigation of secondary organ effects. Such methods were successfully applied in the publications listed above and which are highlighted in this work, investigating indirect secondary effects occurring due to cell-cell interaction. Moreover, this dissertation presents the relevance and the development of three different

multicellular *in vitro* systems, which are able to highlight the possible involvement of primary and secondary mechanisms of genotoxicity in pulmonary fibrosis.

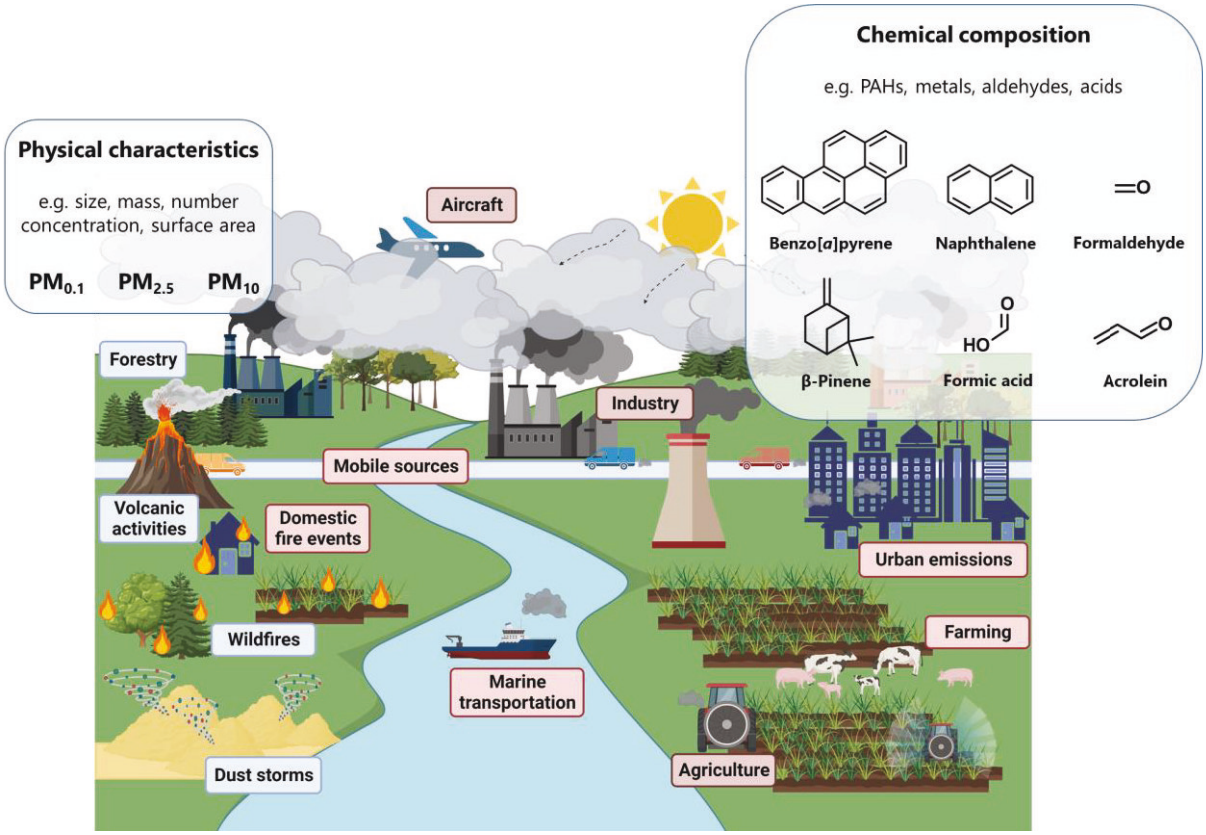


Figure 1: Sources of air pollution and physico-chemical properties influencing related toxicity. Anthropogenic sources (red) like industry, agriculture, farming, housing and the mobility sector as well as biogenic sources (blue) like wildfires, volcanic activities, plant emissions and dust storms are contributing tremendously to outdoor, but also indoor air pollution. The degree and the mechanisms of toxicity greatly depends on the physico-chemical properties of the aerosols such as the size, mass, number concentration or surface area. Moreover, chemical compounds present in environmental aerosols like e.g. PAHs, metals, aldehydes or acids may tremendously impact human health. Created with BioRender.com.

2. Introduction

2.1 Pulmonary fibrosis

2.1.1 Disease phenotype

Lung fibrosis is an irreversible, progressive disorder of the lower respiratory tract, which may follow a tissue damage through a chemical or physical insult, besides a possible hereditary disease formation. It involves a scarring of the alveoli through excessive extracellular matrix (ECM) deposition such as collagen and is caused by a non-physiological wound healing response [20, 21]. The alteration of the tissue results in stiffness and thickening of the lungs and manifests itself in shortness of breath, dry coughing and fatigue syndrome [22, 23]. Until to date, there is no cure for pulmonary fibrosis and the only treatment is to ease the symptoms of the patient and slow down the progression of the disease by antifibrotic drugs [24]. In the majority of the cases, the origin of the disease is barely known and comes with an average life expectancy of 3 to 4 years after diagnosis [25]. In such cases it is referred to idiopathic pulmonary fibrosis (IPF). It is estimated that the latter could be linked to several factors like exposure to dusts or fumes, smoking, viral infections, genetic predispositions or aging, but there are no evidences for a direct relationship between the mentioned sources and IPF formation [23].

2.1.2 Mechanistic background

Even though there are indications for a vast amount of signaling pathways involved in disease pathology, cellular and molecular mechanisms of onset and progression of pulmonary fibrosis need to be further unraveled [26]. In pro-fibrotic and fibrotic conditions several cell types undergo fundamental changes in morphology, location, function and secretion pattern influencing each other's fate [27-29]. The multifaceted cell-to-cell interplay is a challenging factor in defining a precise and comprehensive disease pathway.

Molecular pathways associated with fibrosis development have been widely researched in the past years [21, 26, 30]. It is assumed, that upon physical or chemical insult of environmental stressors on the alveolar epithelial cell layer (**Figure 2**), as first response residential cells like alveolar macrophages are activated and try to clear the pathogen from the injured alveolar tissue [31]. To support clearance and wound repair mechanisms, macrophage activation is followed by a release of pro-inflammatory and pro-fibrotic mediators, which leads to subsequent recruitment of additional pro-inflammatory cells such as neutrophils. The latter participate in the wound healing process by the release of pro-fibrotic cytokines playing a role in ECM deposition and remodeling [26]. In addition to that, epithelial type II cells become activated and undergo epithelial-mesenchymal transition (EMT) by losing their cell polarity and cell-cell adhesion in response to the inflammatory cytokine burst [32]. Epithelial cells gain a fibroblast-like mesenchymal phenotype achieving invasive and migratory properties to participate at the site of action in wound closure processes [21, 23]. Moreover, upon excessive ECM deposition, fibroblasts of the connective tissue get activated and differentiate into star- or web-shaped myofibroblasts which stimulate further ECM production and are able to contract the edges of the wound due to their contractile power [21, 33, 34].

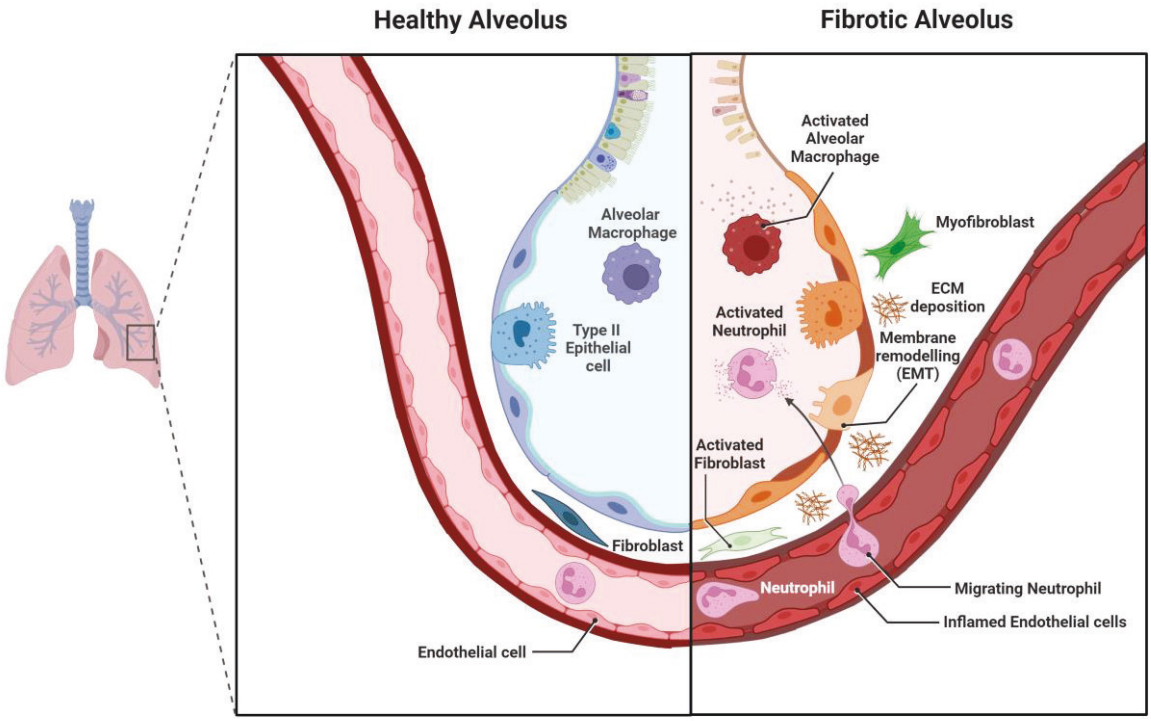


Figure 2: Cellular changes and interactions in pulmonary fibrosis. Schematic depiction of alveolar differences in healthy (left) or diseased (right) conditions. In fibrotic tissue, epithelial cells (orange) and fibroblasts (green) show morphological changes (EMT and fibroblast-myofibroblast transition), accompanied by extracellular matrix deposition (ECM) and an infiltration of inflammatory cells such as neutrophils (pink) into the alveolus. Adapted from "Acute Respiratory Distress Syndrome (ARDS)", by BioRender.com (2022).

All processes mentioned display physiological events of the wound healing response and do not necessarily mean an establishment of fibrotic conditions. Under physiological circumstances, following an acute and transient impact, injured tissue is repaired and myofibroblasts, immune cells as well as excessive ECM are removed from the wound site [35]. Contrariwise, a permanent persistence of myofibroblasts and scarred tissue caused by a repeated or sustained insult favoring chronic inflammation is associated with an aberrant wound healing response and a progression of the disease. In pulmonary fibrosis the complex intercellular communication is imbalanced and subject to the dysfunction of individual cell types, which makes the investigation of the etiology of the disease and the development of proper healing agents enormously challenging [20, 21, 34].

2.2 Primary and secondary genotoxicity and its role in fibrosis

Recent research indicates a strong correlation between fibrosis and chronic inflammation, as well as inflammation and DNA damage [36-39]. Given that we are continuously exposed to genotoxic pollutants via inhalation, it could be assumed that an increased DNA damage or a disturbance of DNA damage response pathways is driving pulmonary fibrosis onset and progression. A great amount of environmental agents is known to follow a genotoxic mode of action (**Figure 3**) via different

mechanisms involving primary genotoxicity in the target cell itself or secondary genotoxicity in cells not directly exposed to the toxicant [40, 41]. Direct primary genotoxicity can be caused by several factors. DNA may be oxidized by elevated levels of reactive oxygen (ROS) or nitrogen (RNS) species initiated by a reactive particle surface or particle-bound metals [38, 42, 43]. Furthermore, genotoxic compounds like polycyclic aromatic hydrocarbons (PAHs) adsorbed to the particle surface can directly bind to the DNA forming stable DNA adducts [44, 45]. Moreover, there is the possibility for small particles such as ultrafine particles (UFP) to translocate to the nucleus and directly interact with the DNA [40, 46-48]. Apart from that, primary genotoxicity can get indirectly induced due to a disturbed mitochondrial metabolism [49-51], a disruption of the cell division and repair machinery [52-54] or an imbalance in the antioxidative defense system [55-57].

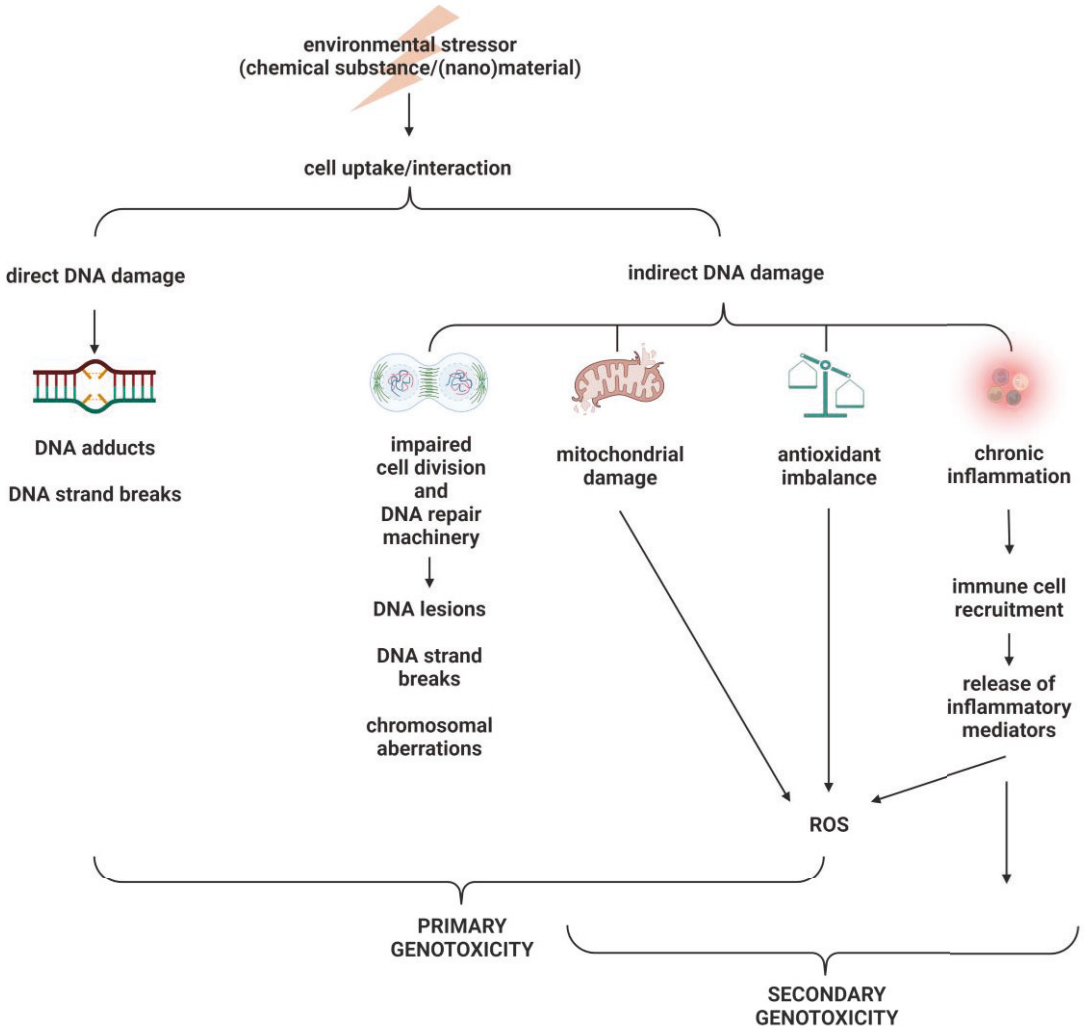


Figure 3: Mechanisms of primary and secondary genotoxicity. Primary genotoxicity occurs through direct interaction of the target cell with the toxicant or indirectly due to disturbed molecular mechanisms. Secondary genotoxicity is the result of elevated levels of reactive oxygen species (ROS), most often following a chronic inflammatory response. Created with BioRender.com.

Secondary genotoxicity mostly occurs in the form of DNA oxidation and is primarily evoked by the formation of ROS induced as result of the clearance-related phagocytic oxidative burst of macrophages resulting in pro-inflammatory signaling [47, 58]. Furthermore, it could derive from the target cell's metabolism or the genotoxicant itself. Generally, the human body is able to cope with endogenous and low levels of exogenous DNA damage through a DNA repair machinery comprising a complex network of DNA damage recognition and repair enzymes to restore genomic stability [59]. In case of excessive levels of DNA damage, impaired defense or repair mechanisms, DNA lesions become persistent and possibly resulting in gene mutations [60, 61]. Unresolved DNA damage is known to be involved in processes like epithelial dysfunction, abnormal fibroblast proliferation, EMT or myofibroblast differentiation [62], all of which display key events in the course of pulmonary fibrosis development.

2.3 Fibrosis-related adverse outcome pathways

As consequence of a constantly growing production and identification of (nano)materials (NM) a testing strategy apart from time- and resource-consuming animal testing is required to assess the hazard of hundreds newly developed or identified materials released to the environment [16, 63]. For this reason, the adverse outcome pathway (AOP) concept was established, reflecting a systematic mechanistic overview of a certain adverse outcome (AO) and its formation due to a molecular initiating event (MIE), which is ideally well-defined. However, in the case of NM it could be a challenge to assign a certain MIE, since not all NM involve specific molecular actions with cellular components as observed for chemical exposures [63, 64]. Nevertheless, the AOP approach can be applied by either describing non-specific interactions, or by defining selected possible MIEs [65]. This primary biological interference is followed by a causative chain of key events (KE) resulting in a certain AO. Such linear approach allows for defined targeting of relevant cellular or molecular events in toxicity assessment and poses a guidance for tiered safety assessment approaches [63, 66, 67]. As a single AOP reflects only a particular MIE associated with the formation of a specific outcome, a connection of several AOPs in the frame of an AOP network mirrors the outcome development more precisely and might enable a more comprehensive and effective testing strategy [68].

Pulmonary fibrosis is known to be induced through several MIEs depending on the physical and chemical properties of the external stressor. Whereas most AOPs describe a more generalized compound or nanomaterial unspecific interaction with residential cell membrane components leading to a series of key events (**Figure 4**), there are AOPs existing which involve compound class specific MIEs and pathways [68]. Especially organic, nonpolar chemical compounds like PAHs dissociated from the particle surface are readily taken up by the cell through diffusion and interact with the cell by *aryl hydrocarbon receptor (AHR)* dependent or independent mechanisms. The mechanism of AHR activation is well understood and is associated with the induction of various signaling pathways such as the *AHR*, *transforming growth factor beta (TGF-β)*, *interleukin (IL) 6* or *p53* pathways (AOP 415) [69, 70].

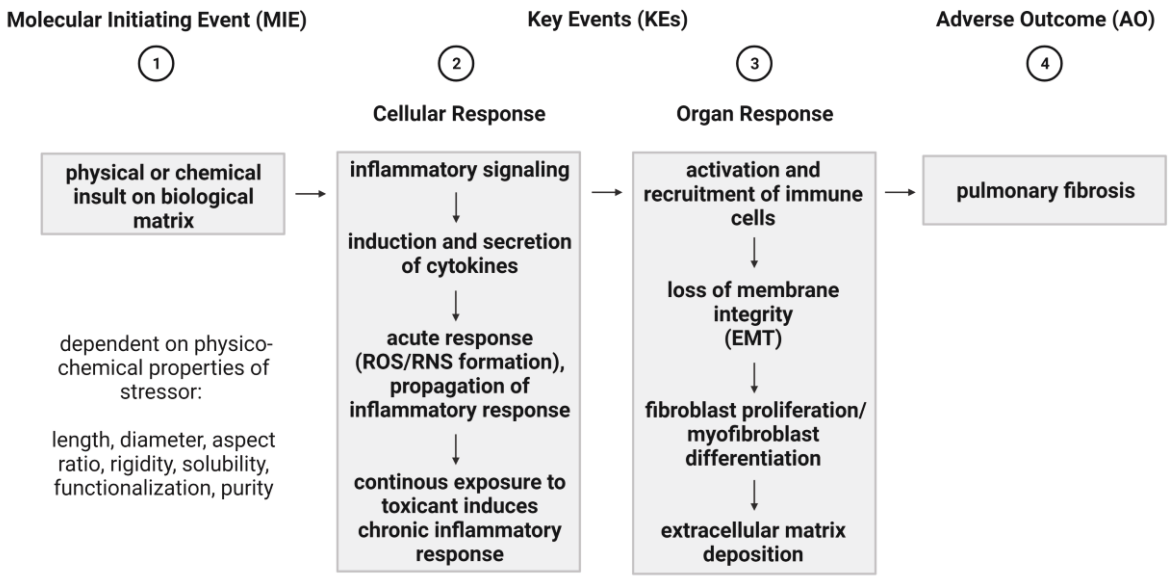


Figure 4: General AOP of pulmonary fibrosis formation. The adverse outcome (AO) pulmonary fibrosis is induced by a molecular initiating event (MIE) followed by a series of key events (KEs) on cellular level involving inflammatory signaling and ROS/RNS release, which induces morphological (epithelial-mesenchymal transition (EMT)) and cellular compositional changes like fibroblast proliferation and differentiation into myofibroblasts followed by extracellular matrix deposition on organ level. Created with BioRender.com.

This activation could, on the one hand, be a potential cause for fibrosis-development itself due to the enhancement of inflammation, proliferation or loss of cell-cell adhesion [71], but, on the other hand, could also lead to the activation of phase I metabolizing enzymes such as *cytochrome P450* (CYP450) family members CYP1A1 or CYP1B1 or epoxide hydroxylase (EH, **Figure 5**). Even though phase I metabolism aims to detoxify xenobiotics by adding functional groups to the rather nonpolar compounds, it might lead to further toxification in the first step [44]. Commonly known pro-carcinogens such as benzo[*a*]pyrene (BaP) are metabolized to reactive electrophilic radical cations, diol epoxides or quinones, which should ideally be conjugated by phase II metabolism enzymes to endogenous, highly hydrophilic compounds facilitating a fast excretion of the toxicant from the body. However, such reactive metabolites are short-lived and particularly prone to react with macromolecules such as DNA, proteins or lipids, leading to an altered gene regulation and molecular function, which could be associated with lung cancer or fibrosis development [44, 72].

Besides chemical interaction, also physical particle characteristics play a pivotal role in the development of fibrosis-associated outcomes. Exposure to high aspect ratio materials (HARMs) like asbestos fibers, carbon fibers (CFs) or multi-walled carbon nanotubes (MWCNTs) is associated with the event of frustrated phagocytosis, which involves the rupture of the engulfing cells membrane due to a particle size exceeding the size of the cell. This is followed by cytokine and reactive species (RS) release, interconnected with lung cancer formation (AOP 303), but could also be associated with fibrosis development [63, 68]. In addition to that, it should be noted, that nanoparticles such as UFPs

are able to translocate to the nucleus and might induce pathways through direct interference with the genetic material or cell division machinery like the mitotic spindle [46, 53].

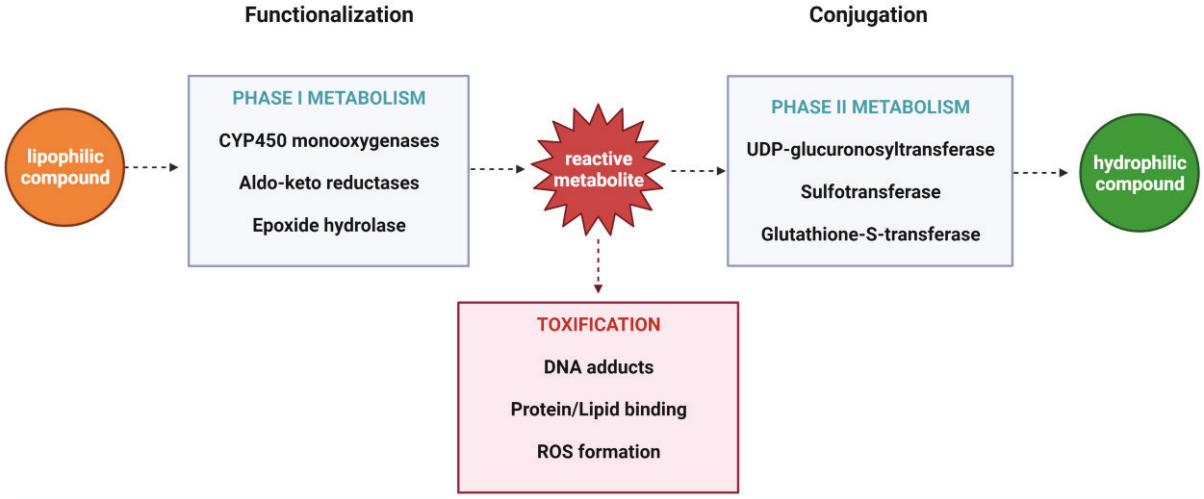


Figure 5: Xenobiotic metabolism. Lipophilic compounds easily enter the cell and are metabolized by phase I and II metabolism enzymes. Oxidoreductases such as cytochrome P450-dependent monooxygenases or aldo-keto reductases as well as hydrolases such as epoxide hydrolase increase the reactivity of nonpolar compounds through the addition of functional groups. This can lead to conjugation reactions with endogenous hydrophilic compounds facilitated by UDP-glucuronosyltransferases, sulfotransferases or glutathione-S-transferases enabling excretion, or result in a toxification of the compound, which can lead to DNA/protein/lipid adduct or ROS formation.

2.4 Model systems to address pulmonary fibrosis

Due to the complex interplay of various cell types like epithelial cells, macrophages and fibroblasts as well as cross-organ signaling, the most common way to assess systemic mechanism of pulmonary fibrosis induced by toxicants usually involved animal organisms [73]. Owing to dissimilar anatomy and physiology of humans and commonly used test animals such as mice and rats (**Figure 6, H**), especially of the inhalation tract [74], but also to fulfill ethical goals of abolishing or reducing animal testing, the development of physiologically relevant *in vitro* models is of great concern. Furthermore, human *ex vivo* precision cut lung slices (PCLS, **I**) may be used to predict respiratory toxicology [75], but require expert knowledge and are not feasible for high-throughput screening. Particularly for the investigation of secondary effects further development of advanced non-animal systems is crucial [76]. Even though test systems comprising a single cell type [77] are able to give a first impression of local toxicity, mutual intercation of different cell types and their intercellular signaling cannot be considered (**A**) compared to direct co-cultures (**B**) culturing multiples cell types in one compartment [78]. Another approach involves the treatment of secondary cells with the conditioned cell culture medium of already exposed cells (**C**) [79]. In such case, however, the secondary cells are solely exposed to the medium of an accumulated and time-delayed snapshot, which does not allow for kinetic studies. Moreover, usually sample media are stored for investigation at a later time point, which can influence sample media composition and the loss of signaling molecules due to usually prolonged freezing storage. Another important factor is that simple test systems are often applied under submerged conditions (**A-E**).

However, since the epithelial layer in the lung is exposed to aerosols containing air, transferable inserts have been developed, which allow the construction of organotypic cultures on a membrane with the apical side facing the air and the basolateral side supplied with nutrients contained in the medium, referred to as air-liquid interface (ALI, **F+G**) [75, 80]. Furthermore, this support enables the cultivation and cell-specific investigation of secondary cells of interest on the downside of the membrane permitting direct cell-cell contact (**F**) [76, 81, 82] and further addition of cells on the bottom of the well (**G**). As complexity of the model systems is increasing, the more time-consuming and cost-intensive the preparation becomes, nevertheless, physiological relevance and the quality of data is rising as well [75].

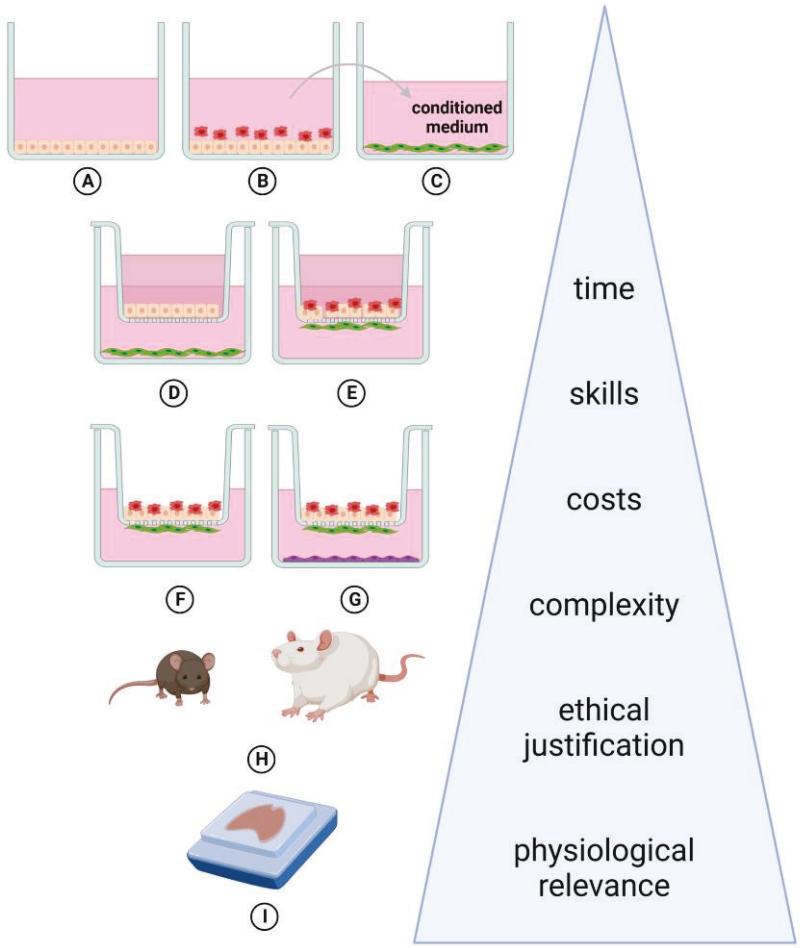


Figure 6: Model systems for toxicity testing. Simple test systems as mono (A) or direct co-cultures (B) pose easy-to-use high-throughput test systems but do not allow for cell-specific secondary effects as conditioned cultures (C) or organotypic insert supported indirect co-cultures (D-G). Cultures gain in physiological relevance when apical cells are cultured at the air-liquid interface (F+G). If in vitro tests are not sufficient, animal testing (H) or ex vivo tissue slices (I) become the tool of choice. Created with BioRender.com.

3. Aims of the study

As we are continuously exposed to airborne particles and particle-bound chemical compounds through outdoor and indoor air pollution, the assessment of associated toxicological effects is of great concern. A vast amount of studies deals with the exposure to anthropogenic aerosol, investigating, amongst others, health-related effects of aerosols deriving from traffic [77, 83], industry [84] or residential heating [39, 85]. Of major concern is the inhalation of fine particulate matter ($PM_{2.5}$, diameter < 2.5 µm) [86, 87] and ultrafine particulate matter (UFP, diameter < 100 nm) [88, 89], the toxicity of which strongly depends on its physico-chemical composition. The latter is influenced by environmental factors such as atmospheric aging, which involves an (photo-)oxidative or photolytic transformation of volatile organic compound containing primary organic aerosols (POA) to secondary organic aerosols (SOA) [82, 83, 90].

Apart from this, indoor air pollution may pose an even greater health risk, as concentrations often exceed those of ambient air [91, 92]. Nevertheless, there is less knowledge on specific mechanisms of toxicity of indoor PM. Most research is done in the area of workplace safety, especially in the field of engineered nanotechnology [37, 93, 94], construction and recycling materials [95-97]. Probably the most studied particle and its health-related effects is asbestos, which has caused a certain sensitization to the toxic potential of fiber-like materials, especially those possessing a length > 5 µm, a diameter < 3 µm and a length-to-diameter ratio > 3 and which are thereby considered as hazardous by the World Health Organization (WHO; IARC Monographs - 100C). Man-made mineral fibers (MMMFs) like carbon nanotubes (CNTs) or carbon fibers are of particular interest, as they pose a high tensile strength and durability, which makes them an attractive polymer composite [98] or composite construction material in the building sector [99, 100] or in wind turbine rotor blades [101]. On the other hand, it is exactly these properties, which increase the durability and biopersistence of the material and make it hazardous when releasing inhalable particles.

This study (**Figure 7**) reports the development of multicellular *in vitro* models, comprising epithelial cells and fibroblasts or epithelial cells, macrophages and fibroblasts, all of which display an important function in the development of pulmonary fibrosis. Furthermore, the thesis aims to unravel the toxicological potential of various air pollutants like fibrous construction materials such as (thermally treated) carbon-reinforced concrete ((tt)C^3) or carbon rods (CR) as well as MWCNTs focusing on secondary genotoxicity and pro-fibrotic events. Additionally, similar research was performed for anthropogenic (naphthalene) and biogenic (β-pinene) SOA, assessing the potential of secondary toxicity on endothelial cells to address cardiovascular impairment in the frame of fibrosis.

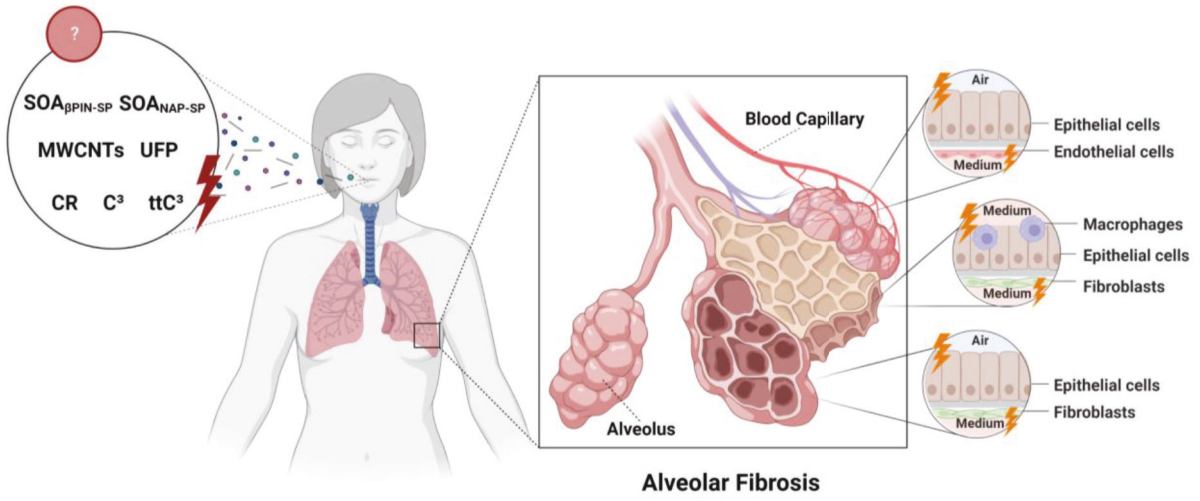


Figure 7: Aims of the thesis. The study aimed to develop fibrosis-specific model systems to assess primary and secondary toxicity of multi-walled carbon nanotubes (MWCNTs), UFP, carbon rods (CR), (thermally treated) carbon fiber-reinforced concrete ((tt)C³) as well as biogenic (SOA$_{\beta PIN-SP}$) and anthropogenic (SOA$_{NAP-SP}$) secondary organic aerosols. The model systems contained fibrosis-associated cell types such as epithelial cells, macrophage and, fibroblasts as well as endothelial cells. Created with BioRender.com.

Specified description of aims:

i. Development of multicellular test systems comprising main cell types known to participate in lung fibrosis

ii. Elaborate chemical, morphological and toxicological differences of particles released from dry-cutting of different carbon-based construction materials as risk estimation in domestic fire events and the assessment of primary and secondary toxicity of the materials regarding fibrosis formation

iii. Validation of the developed triple culture (TC) system with MWCNTs as benchmark particle and the comparison of the TC with less complex cell systems to analyze the influence of macrophages on cytotoxicity, (secondary) genotoxicity as well as pro-fibrotic outcomes

iv. Development of a sensitive detection method of glutathione and its disulfide by liquid chromatography coupled with tandem mass spectrometry for the use in multicellular test systems

v. Investigation of UFP containing aerosols with low and high organic content regarding their acute (geno-)toxic potential

vi. Assessment of primary and secondary toxicological potential of biogenic (β-pinene) and anthropogenic (naphthalene) SOA (SOA$_{NAP-SP}$ and SOA$_{\beta PIN-SP}$) and the possible involvement in pro-fibrotic conditions

4. Fundamentals of applied methods

4.1 Model development

To address the toxicity induced by airborne pollutants, several *in vitro* test systems of varying complexity were established. The studies were conducted in co-cultures of epithelial cells (A549) as well as fibroblasts (MRC-5) or endothelial cells (EA.hy926) at the ALI to investigate the effects of exposure to the aforementioned construction materials or SOA, respectively. In addition to that, a physiologically more appropriate triple culture model of epithelial cells, macrophages (dTHP-1) and fibroblasts was established and compared to a co-culture of epithelial cells and fibroblasts and a monoculture of epithelial cells exposed to MWCNTs under submerged conditions.

In order to create the best test conditions, some pretesting had to be performed. For all multicellular culture systems, different culture media were tested for the different cell lines to assure a suitable medium for all cell lines, allowing both, rapid growth and proper proliferation of all cell types. Furthermore, the seeding order of cells was investigated, as well as suitable cell densities and ratios in order to gain viable and physiologically relevant cell models. The triple culture deserved further testing to achieve proper fibroblast growth of cells in a uniform and not confluent single cell layer, which required an adequate coating of the transwell insert membrane. Furthermore, in this model, THP-1 cells are differentiated from monocytes to macrophages using phorbol-12-myristate-13-acetate (PMA), which needed the examination of different PMA concentrations as well as different differentiation durations to receive the best differentiation and adhesion on the epithelial layer of macrophages. Once all conditions were set to optimum, the assembly of the cultures on the transwell inserts could be accomplished.

As depicted in **Figure 8**, for the combination of the cells, first, epithelial cells are seeded on the upside (apical) of the membrane and maintained in cell culture medium. The next day, the insert is inverted and either MRC-5 or EA.hy926 cells are seeded in a bubble of cell culture medium on the downside (basolateral) of the (pre-coated) membrane for one hour. After turning back the inserts, the triple culture requires further seeding of differentiated THP-1 cells on top of epithelial cells before being exposed to MWCNTs under submerged conditions two days after. The co-culture and monocultures systems involved in this study, were built in the same way as the triple culture, even if no cells were added, to achieve the highest comparability. The co-culture models used in the studies of construction materials or SOA, are lifted to ALI the day after inverted seeding, followed by exposure to $PM_{2.5}$ of CR, C^3 and ttC^3 or $SOA_{NAP\text{-}SP}$ and $SOA_{\beta PIN\text{-}SP}$ the next day.

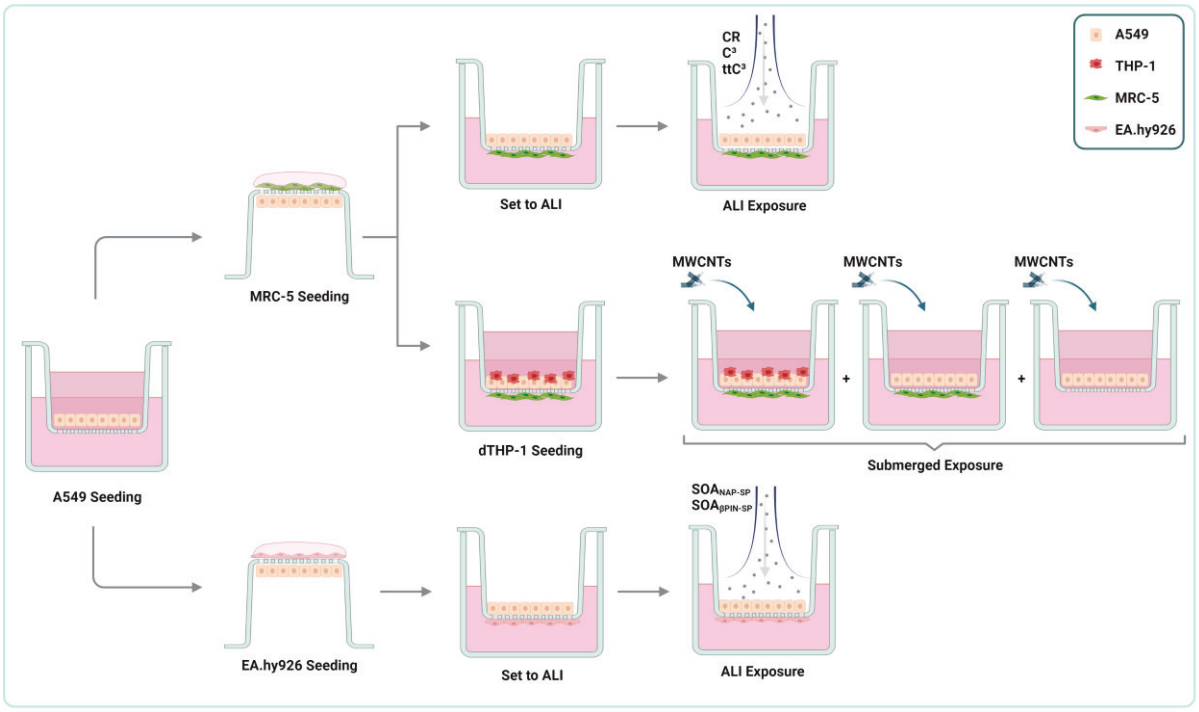


Figure 8: Seeding schemes of the used cell model systems. All cell models used required an inversion of the insert membrane containing epithelial cells (A549) with subsequent seeding of fibroblasts (MRC-5) or endothelial cells (EA.hy926). The addition of macrophages (dTHP-1) resulted in a fibrosis-relevant cell model, which was exposed under submerged conditions to MWCNTs and compared to less complex systems. Co-cultures containing either MRC-5 or EA.hy926 were exposed at air-liquid interface (ALI) to carbon rods (CR) and (thermally treated) carbon concrete ((tt)C³) or naphthalene and β-pinene derived secondary organic aerosols ($SOA_{NAP\text{-}SP}$/$SOA_{\beta PIN\text{-}SP}$).

4.2 Cytotoxicity assessment

Before performing in-depth toxicological characterization of potential genotoxicity, inflammation or gene expression changes, a comprehensive cytotoxicity assessment is required in order to avoid misinterpretation of data, evoked due to cell death-related signaling or disintegration of cellular components such as DNA. The release of lactate dehydrogenase (LDH) into the cell culture medium or the metabolic activity are generally reliable measures of cell viability. However, both assays can be negatively influenced by physical interaction of particles with LDH or an interference of particles with the absorbance or fluorescence of the detected dyes, which might result in false or inconsistent data [102, 103]. Therefore, especially for nanomaterial testing, the use of at least two cell viability tests, proper interference controls or the use of non-colorimetric or non-fluorescent endpoints such as cell count or colony forming efficiency (CFE) are recommended [104, 105].

4.2.1 Lactate dehydrogenase release

The fairly stable enzyme LDH is increasingly released into the cell culture medium when cells undergo cell death, which is accompanied by a rupture of their cell membrane releasing cytosolic constituents into the surroundings. The assay (**Figure 9**) uses the reduction activity of LDH converting lactate to

pyruvate by producing the reducing agent nicotinamide adenine dinucleotide (phosphate) NAD(P)H. The latter is consumed by a reaction of yellow tetrazolium salt to the red formazan dye displaying an absorbance at λ=490 nm, which is quantifiable and proportional to the released LDH and thereby the amount of damaged cells, reflecting cytotoxicity [106].

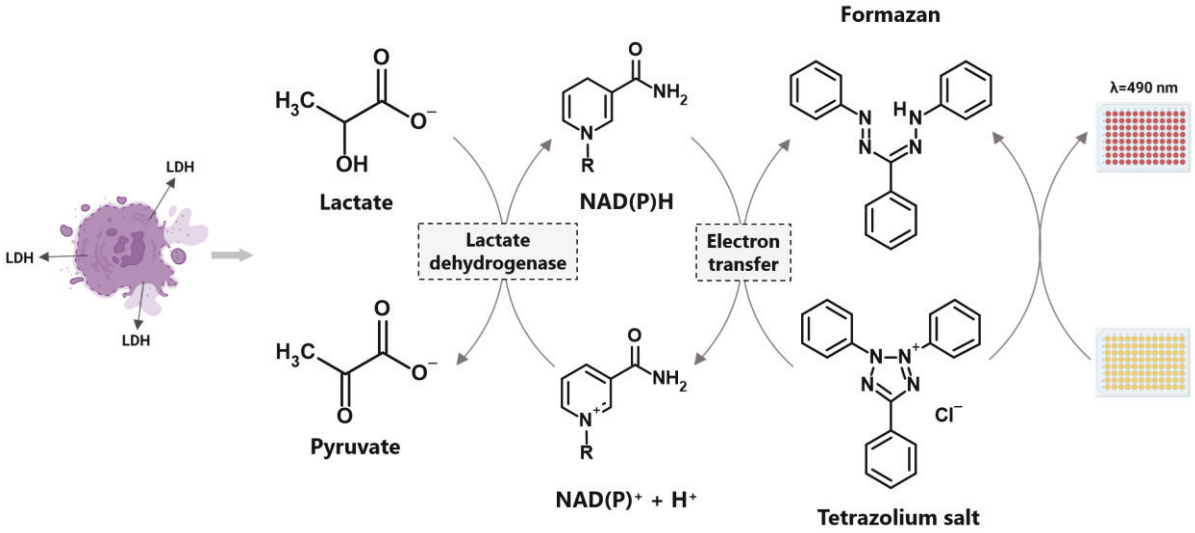


Figure 9: Principle of the LDH assay. Lactate dehydrogenase (LDH) is released into the sample medium when the cell membrane is damaged. LDH oxidizes lactate to pyruvate, which favors the color reaction of a tetrazolium salt to formazan through the production of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H). The absorbance can be quantified and is proportional to cytotoxicity at λ=490 nm. Created with BioRender.com.

4.2.2 Metabolic activity

The resazurin reduction assay (**Figure 10**) was used to assess cell viability by means of metabolic activity and is based on the fluorometric redox-reaction of the blue non-fluorescent dye resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) to its highly fluorescent reduced counterpart, the pink resorufin (7-hydroxy-3H-phenoxazin-3-one) dye. Both indicators are cell membrane permeable, which enables the intracellular reduction of resazurin to resorufin by NAD(P)H or flavin adenine dinucleotide (FADH) deriving from aerobic respiration processes, and the subsequent release of the reduced resorufin into the reagent solution. Resazurin may be reduced by mitochondrial or cytosolic reductases such as NADH-ubiquinone reductase 1 or flavin reductase, which makes the redox-pair good metrics for the cellular metabolism status by measuring the fluorescence of resorufin at λ=570 nm [105].

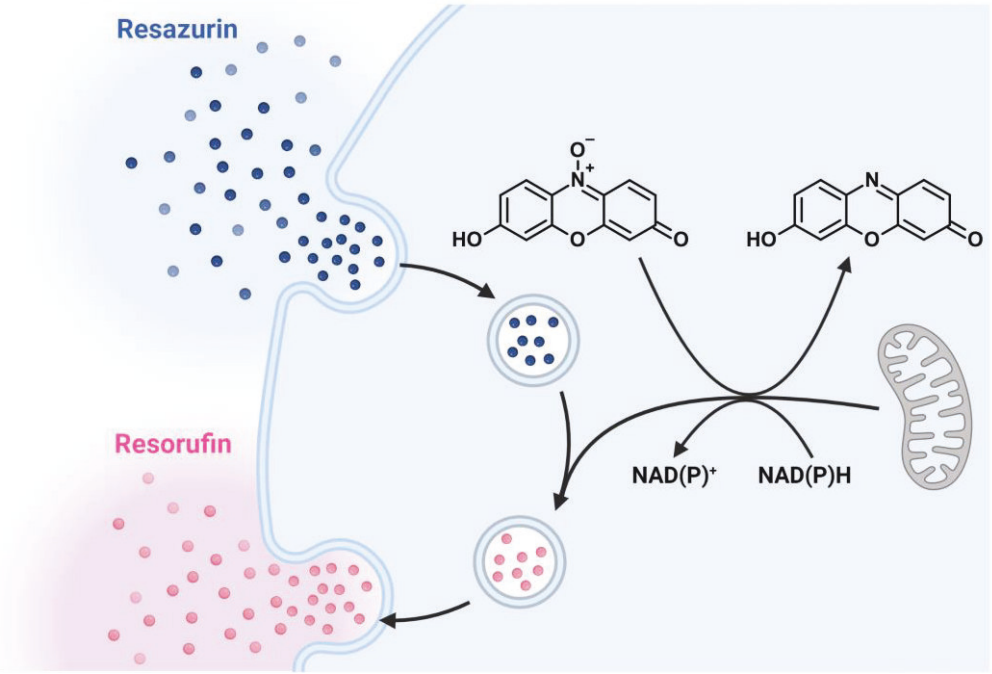


Figure 10: Principle of the resazurin reduction assay. Resazurin is taken up by the cell and metabolized by reducing agents such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) produced by cytosolic or mitochondrial metabolism. Resorufin is released by the cell and its fluorescence can be detected and is proportional to the metabolic activity indicating cell viability. Created with BioRender.com.

4.2.3 Cell count and colony forming efficiency

Fluorescence- and absorbance-independent cytotoxicity assays are particularly important when testing (MW)CNTs, since they have frequently shown in previous studies to interfere with the applied detection agents [102, 107]. For this reason, a combination of cell count and CFE was added to the spectrum of cytotoxicity tests used in the present study. After treatment, cells are harvested and counted by suspending them with trypan blue dye, which allows to discriminate between alive and dead cells. Since the azo dye is not cell membrane permeable, only dead cells with disrupted membranes are internalizing the dye [108]. A decreased microscopic live cell count or increasing amount of stained cells therefore indicates cell death or cytotoxicity, respectively.

A subsequent examination of CFE is conducted by re-plating the harvested cells into culture dishes at a single-cell density and following-up their ability to proliferate and to form colonies. After approximately one week the colonies are fixed, visualized with Giemsa stain and counted manually. The colony forming efficiency is than calculated according to the following equation [109]:

$$\text{CFE}\ [\%] = \left(\frac{\text{Average number of colonies of exposed cells}}{\text{Average number of colonies of control cells}}\right) \times\ 100$$

Advantageously, the percentage of CFE is not only describing cytotoxic effects, but shows also an impact on proliferation, why it is often referred to as clonogenicity, which describes the potential of

cells to divide. Not necessarily a decreased CFE represents cytotoxic events, but could also display a deceleration of the cell cycle as response to increased DNA damage or due to alteration of cell cycle controlling enzymes. In pro-fibrotic conditions an increased CFE could be observed due to enhanced fibroblast proliferation [34]. Moreover, often it is observed that nanomaterials induce proliferation at low particle concentrations, which might be related to the induction of repair or clearance mechanisms [110, 111].

4.3 Inflammatory potential - interleukin 8 release

The presence of inflammatory mediators such as interleukin 8 (IL-8) in the exposure medium is commonly assessed by an enzyme-linked immunosorbent assay (ELISA) [112]. In this study we used the principle of a sandwich ELISA, in which the wells of a 96 well plate are coated with an antigen-specific capture antibody (**Figure 11, 1**), which strongly binds the antigen of interest (**2**, e.g. IL-8). In the next step, a biotinylated detection antibody recognizes the bound analyte (**3**), which is followed by binding of a streptavidin-horseradish peroxidase conjugate, which catalyzes an enzymatic color reaction of a chromogenic substrate such 3,3′,5,5′-Tetramethylbenzidine (TMB, **4**). Subsequently, the absorbance can be measured and the exact concentration of the analyte is calculated by using a standard curve.

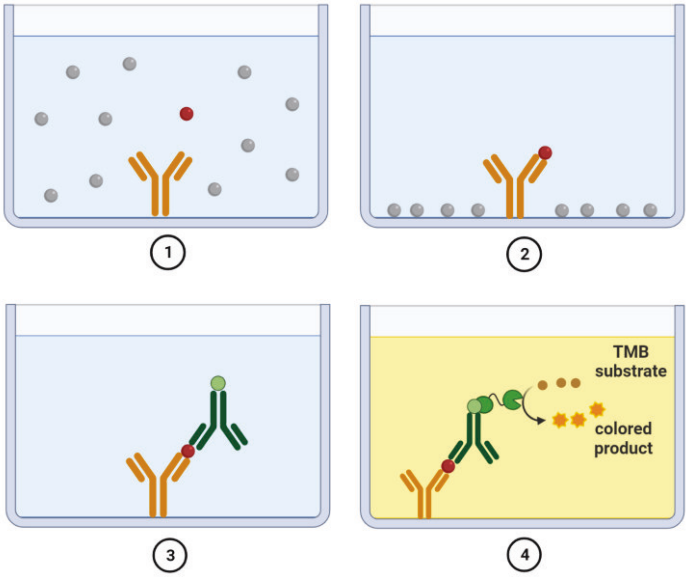


Figure 11: Principle of the sandwich ELISA. A 96 well plate is coated with a capture antibody (1) binding the agent of interest (2), which binds a biotinylated detection antibody (3). The latter is bound itself by a streptavidin-horseradish peroxidase conjugate, which promotes the color reaction of a chromogenic substrate (TMB, 4), the absorbance of which can be detected and quantified. Adapted from “Sandwich ELISA”, by BioRender.com (2023).

4.4 Indirect ROS measurement by malondialdehyde

Malondialdehyde (MDA), one of the end-products of lipid peroxidation, is commonly used as a biomarker for oxidative stress since it can be the end product of free radical toxicity [113]. Free radicals can either covalently bind to macromolecules such as DNA, proteins and lipids or initiate a chain reaction of lipid peroxidation of unsaturated fatty acids, as found in the lipid bilayer of the cell membrane. The degradation of fatty acids is propagated as radicals rapidly react with non-radicals, again forming highly reactive radicals. However, this chain reaction is terminated when two radicals react with each other to form a non-radical product. To finalize the chain reaction, the probability that two radicals react with each other must be very high, which implies that also concentrations of free radicals must be high. Considering that the cell is scavenging a certain amount of reactive species through antioxidative detoxifying systems such as superoxide dismutase or glutathione, radical concentrations inside the cell must be already remarkably high when MDA is formed [114, 115].
In this study, MDA released into the exposure medium is analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [116]. Since MDA itself is a highly reactive electrophilic compound it requires derivatization to detect and measure it. For this purpose, 2,4-Dinitrophenylhydrazine is used, which is a nucleophilic compound specifically reacting with ketones and aldehydes such as MDA. Owing to the increased reactivity, MDA concentrations quantified might underestimate the level of ROS, since the measured fraction only represents free MDA and not the fraction that might formed covalently bound adducts with proteins or DNA, which also makes MDA a possible genotoxicant [113].

4.5 Genotoxicity assessment

To assess possible direct or indirect DNA damage caused by the physical characteristics of particles or their chemical constituents, the alkaline mini-gel version of the comet assay (**Figure 12**) was performed [93]. This method detects single and double strand breaks as well as alkali labile sites such as apurinic or apyrimidinic sites.
For the assessment, cells are detached from both sides of the membrane by trypsin and single cells are embedded in agarose mini-gels on a microscope slide. While the cells are lysed, the nascent DNA is set free and gets unwind through alkaline treatment. In the next step, the nucleoids are exposed to an electric field, which makes pieces of DNA strands, occurring due to strand breaks, move towards the anode, since the DNA is negatively charged. DNA without strand breaks does not move during electrophoresis, which results in the formation of comet-shaped nucleoids, bearing intact parts of DNA in the head and broken parts in the tail in function of their size. Electrophoresis is followed by a neutralization of the slides with subsequent drying overnight. During the whole process it is of great importance that the handling is performed under cold (4°C) conditions and that the DNA is not exposed towards light. Handling at room temperature does not inhibit DNA repair enzymes and could lead to an underestimation of DNA damage. Exposure to ultraviolet (UV) light-emitting sources, on the other hand, might increases DNA strand breaks [117]. For quantification, comets are visualized by a fluorescent nucleic acid straining dye and are microscopically evaluated. The percentage of DNA in the tail is proportional to the degree of DNA damage.

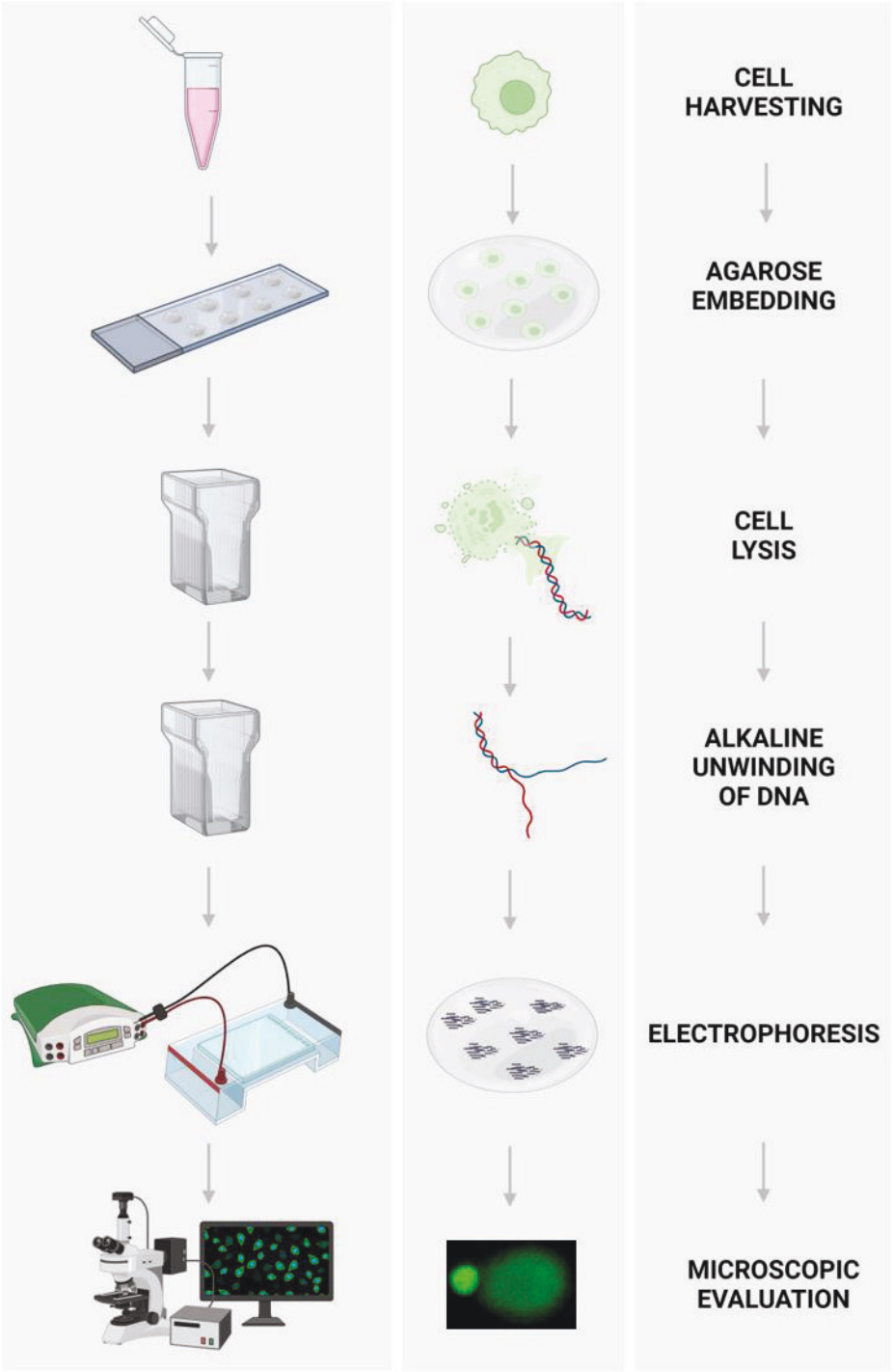


Figure 12: Principle of the alkaline version of the comet assay. To quantify DNA damage, cells are harvested from both sides of the insert membrane, and single cell suspensions are embedded in agarose mini-gels. After breaking down the cell membrane in a jar containing lysis solution, an alkaline treatment helps to unwind the nascent DNA, which is afterwards exposed to an electric field in an electrophoresis chamber. During this process, damaged DNA migrates towards the anode, whereas intact DNA remains in the head of the nucleoid. This formation of a comet is visualized by a fluorescent nucleic acid staining and manually evaluated trough fluorescence microscopy. Created with BioRender.com

4.6 Immunostaining

The immunohistochemical staining follows a similar principle as described for the ELISA. The goal is to target specific antigens such as vimentin or α-smooth muscle actin and to optically analyze their changes in protein expression pattern after treatment compared to control cells [118].
For this purpose, the insert membranes are fixed with paraformaldehyde, and the cell membrane is permeabilized by a detergent such as Triton-X to enable the antibodies to enter the cell. Subsequently, reactive sites are blocked by agents such as serum or bovine serum albumin (BSA) to avoid unspecific binding of the antibodies, which would increase the background signal while imaging. In the next step, the primary unlabeled antibodies bind to the targeted antigens, followed by incubation with the secondary antibody, which is labeled with a fluorophore and binds itself to the primary antibody. During this step, counterstaining with the DNA intercalating dye 4′,6-diamidino-2-phenylindole (DAPI) or phalloidin, which binds to actin filaments, is performed. This additional staining makes the cell nuclei and the cytoskeleton visible, which gives important information on cell morphology or cell division processes. As a last step, the membrane is transferred to a microscope slide and covered with a cover glass to examine the stainings at their specific wavelengths with a fluorescence microscope. To enable staining of both sides of the insert membrane, half of the membrane can be scraped removing cells from one side, followed by cutting the membrane and inverting one half for staining of the downside.

4.7 Gene expression analysis by the use of microarrays

The use of microarrays for transcriptome analysis allows the assessment of the expression of thousands of genes in parallel by requiring low amounts of RNA. Therefore, it is an ideal tool to get an overview about regulatory changes induced on gene level following external stimuli such as particle exposure [119]. In principle (**Figure 13**), microarrays are most often glass slides, usually called “chips”, which are spiked with specific nucleic acid fragments on certain locations, which can be assigned later-on. The assay uses the hybridization of complementary nucleic acid strands, for which cells are harvested and the RNA isolated. The latter is then purified, reversely transcribed to cDNA, cut into fragments and fluorescently labeled. In the next step, the microarray is incubated with the sample allowing the isolated cDNA to react with their complementary target strand on the chip by forming hydrogen bonds between their nucleotide base pairs. Subsequently, remaining unbound strands are removed by washing, which leaves behind only strongly bound strands possessing a high amount of hydrogen bonds. Finally, the fluorescence of the target DNA along with its complementary labeled strand can be detected and quantified [119, 120].
In contrast to a method called RNA sequencing, microarrays are cost-effective and time-efficient tools to analyze gene expression patterns. However, this technique often displays an unspecific cross-hybridization background signal and lower resolution compared to RNAseq. The latter gene expression method uses next-generation sequencing, which determines the nucleotide order of sample nucleic acids strands, which are then compared to a reference transcript. Advantageously, this method allows for high-throughput screening by performing transcriptome-wide analysis of high specificity at even lower amounts of sample RNA compared to microarray analysis [121].

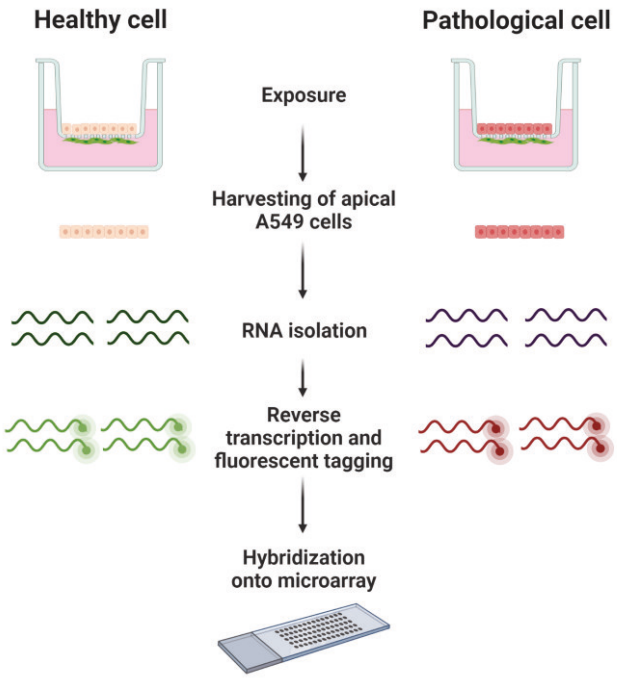


Figure 13: Principle of the gene expression analysis by microarray. Cells are harvested and the RNA is isolated, purified and reversely transcribed. Furthermore, it is cut into fragments and fluorescently labeled, before hybridizing the sample cDNA strands to the complementary target strands on the microarray chip. Afterwards, the fluorescence of bound DNA is detected and quantified. Adapted from "DNA Microarray", by BioRender.com (2022).

Both, for microarray and RNAseq analysis, gene expression results may be statistically evaluated and investigated by assigning the regulated genes to biological processes by data-based gene-ontology analysis [122] complemented by an ingenuity pathway analysis (IPA). The latter sets the observed expression profiles into a biological context by unraveling pathways, which seem to be associated with certain gene regulation patterns [123].

4.8 Glutathione analysis by LC-MS/MS

Glutathione (GSH) is a nucleophilic constituent of the antioxidative defense systems, which either scavenges RS directly, conjugates with electrophilic xenobiotics or participates in the antioxidant capacity of glutathione peroxidase (GPx, **A**). The latter can be found in the mitochondria as well as the cytosol, making it an important regulator of the antioxidative response by catalyzing the reduction of hydro peroxides such as lipid or hydrogen peroxides, which are commonly formed following exposure to PM or chemical compounds like PAHs. During this reaction, reduced GSH is oxidized to its disulfide form (GSSG), which might be recycled by glutathione reductase (GR) by consuming NADPH [124, 125].

The ratio of intracellular GSH to GSSG can be used to determine levels of oxidative stress and was examined in this study by applying LC-MS/MS (**Figure 14, B+C**). In contrast to colorimetric GSH

assay, this method might be used for high-throughput applications, involving a fast and cost-effective sampling, resulting in higher sensitivity and lower auto-oxidation of GSH compared to color-based kits [126, 127]. Moreover, the developed method enables the investigation of GSH levels in model systems at the air-liquid interface, considering the depletion of the antioxidant marker in primary and secondary cells. In general, exposed cells are immediately incubated with a cold solution of the alkylating agent *N*-ethylmaleimide (NEM) in methanol (MeOH), spiked with deuterated internal standards (ISTD) of GSSG and GS-NEM. NEM derivatizes free GSH by binding to the thiol groups and prevents further oxidation during the sampling procedure (GS-NEM, **B**). Moreover, the cold milieu blocks the enzymatic activity of GR, thereby inhibiting the recycling of GSSG formed during the exposure. In the next step, cells are scraped from both sides of the membrane, collectively harvested in a tube, vortexed and centrifuged at high speed (10,000 x g, 7 min) to ensure a complete extraction of GS-NEM and GSSG markers. After evaporation of the precipitation and extraction solution (supernatant) under a nitrogen stream, the analytes are dissolved in the mobile phase A (0.1% formic acid in H_2O/MeOH), applied for separation on a C18-column (**C**). Subsequently, GSSG (m/z 613) and GS-NEM (m/z 433), representative for GSH, are detected by targeted mass spectrometry using multiple reaction monitoring in positive ionization mode and quantified according to internal calibration applying ISTD and GS-NEM/GSSG standard compounds.

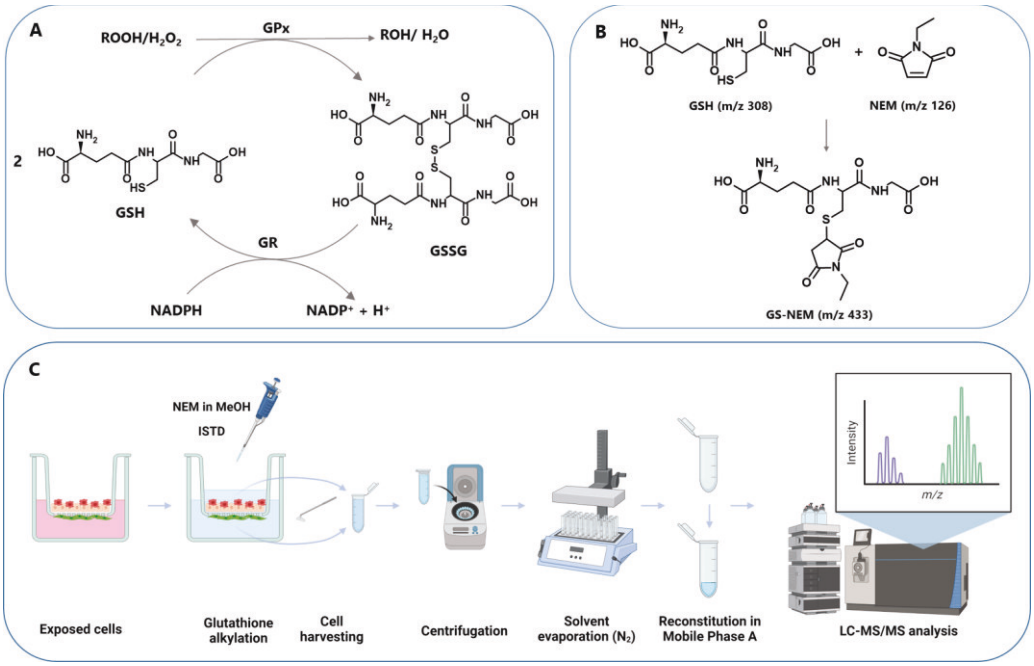
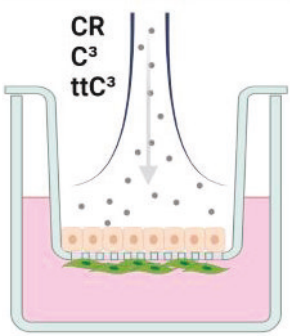


Figure 14: Glutathione analysis by LC-MS/MS. Glutathione levels are used as indicator for oxidative stress. (A) The reduced form of GSH is oxidized to the disulfide GSSG through glutathione peroxidase (GPx), enabling the removal of reactive species such as hydro peroxides, e.g. lipid peroxides and hydrogen peroxides. GSSG can be recycled by glutathione reductase (GR) consuming NADPH. By derivatization of GSH with *N*-ethylmaleimide (NEM, B), GSH levels present after exposure can be quantified by LC-MS/MS (C). For this purpose, GSH is alkylated with NEM and the analytes are extracted from apical and basolateral cells. The extraction solvent is evaporated under a nitrogen stream and the analytes are reconstituted in the mobile phase A, followed by separation and detection via LS-MS/MS. Created with BioRender.com.

5. Results & Discussion

5.1 Co-culture of epithelial cells and fibroblasts



A co-culture system of alveolar epithelial cells (A549) and fibroblasts (MRC-5) was developed involving two main cell types in close proximity, which are known to be especially important in wound healing processes and to influence each other substantially driving the progression of pulmonary fibrosis. An enhanced accumulation of fibroblasts as well as ECM is driven by a recurrent or persistent damage and therefore subsequent signaling of epithelial cells [27]. Harmed alveolar epithelial cells release numerous mediators such as transforming growth factor beta (TGF-β) [128-130] or connective tissue growth factor (CTGF) [27, 131], which initiate the activation and proliferation of fibroblasts and the differentiation into myofibroblasts. On the other hand, under physiological conditions, epithelial cells synthesize prostaglandin E_2 (PGE_2) [132, 133] or bone morphogenetic protein-4 (BMP4) [134], which suppresses fibroblast proliferation and was shown to be reduced in IPF. Furthermore, in IPF, an increased amount of epithelial cells seems to follow an apoptotic cell death pathway, which is amplified by activated fibroblast signaling releasing pro-apoptotic mediators such as angiotensin II and ROS and resulting in a negative feedback loop. As there is a strong bilateral interconnection between the two cell types [27, 135], deviant events such as genotoxicity or mutations, may lead to a changed gene expression pattern and an imbalance in the complex signaling with subsequent initiation of aberrant wound healing and fibrosis progression. For this reason, it is extremely important to perform toxicity tests of inhalable materials with models that consider outcome-relevant cell types and their direct communication. The technique to co-culture different cell types on separate sides of a membrane, enables the easy investigation of distinct cell types without losing direct cell-cell communication.

5.1.1 Application (1)

This co-culture model was applied at the ALI to investigate how different exposure scenarios to fiber-containing construction materials could induce genotoxicity as well as pro-fibrotic pathways after 2 h exposure and after 2 h exposure plus 22 h post-incubation time, referred to as 24 h exposure. Not only workers who come into contact with both the raw materials and the composite materials during building or dismantling at construction sites can be exposed to hazardous fiber dusts, but also the civil population is subjected to the latter when it comes to the demolition of houses or domestic fire events. Moreover, the latter could pose a great health risk for fire fighters, who might be directly exposed to dusts, even though not in a continuous manner. The mentioned scenarios were mimicked by dry-cutting of the reinforcement material CR, the reinforced carbon concrete C^3 as well as the thermally treated C^3 (700 °C), which resembles the released fibers occurring in house fires.

5.1.2 Physico-chemical aerosol characterization

SEM imaging of the particulate matter (PM_{10}; PM ≤ 10 µm) fractions (**Figure 15, A**) revealed, that dry-cutting of CR as well as C³ released fibers with median diameters of 6.9 and 6.6 µm (**Figure 15, B**), respectively and are therefore not respirable according to WHO. The dry-cutting of ttC³ on the other hand, released fibers with a median diameter of 2.7 µm meeting WHO criteria of respirable fibers and for this reason, should be treated with particular caution. For chemical and toxicological analysis, the $PM_{2.5}$ fraction was investigated. The overall number concentration (**Figure 15, C**) was the lowest for particles released by cutting of CR, but derived mainly from those below a particle diameter of 0.5 µm with the highest concentration at~0.2 µm. C³ and ttC³ showed a comparable particle distribution pattern with the highest number concentration of~ 0.4 µm, nonetheless ttC³ generally showed a lower concentration than C³. From the results obtained, it can be concluded that only for ttC³ WHO fibers are contained in the $PM_{2.5}$ fraction guided to the cells, whereas all materials release non-fibrous particles, released from the reinforcement material, the epoxy resin or the concrete material. Particles deposited on the epithelial layer are in the low microgram range ranging from 3.5 µg/cm² for CR and 45 µg/cm² for ttC³ up to 75 µg/cm² for C³, displaying a realistic abrasion scenario but not an equal deposition.

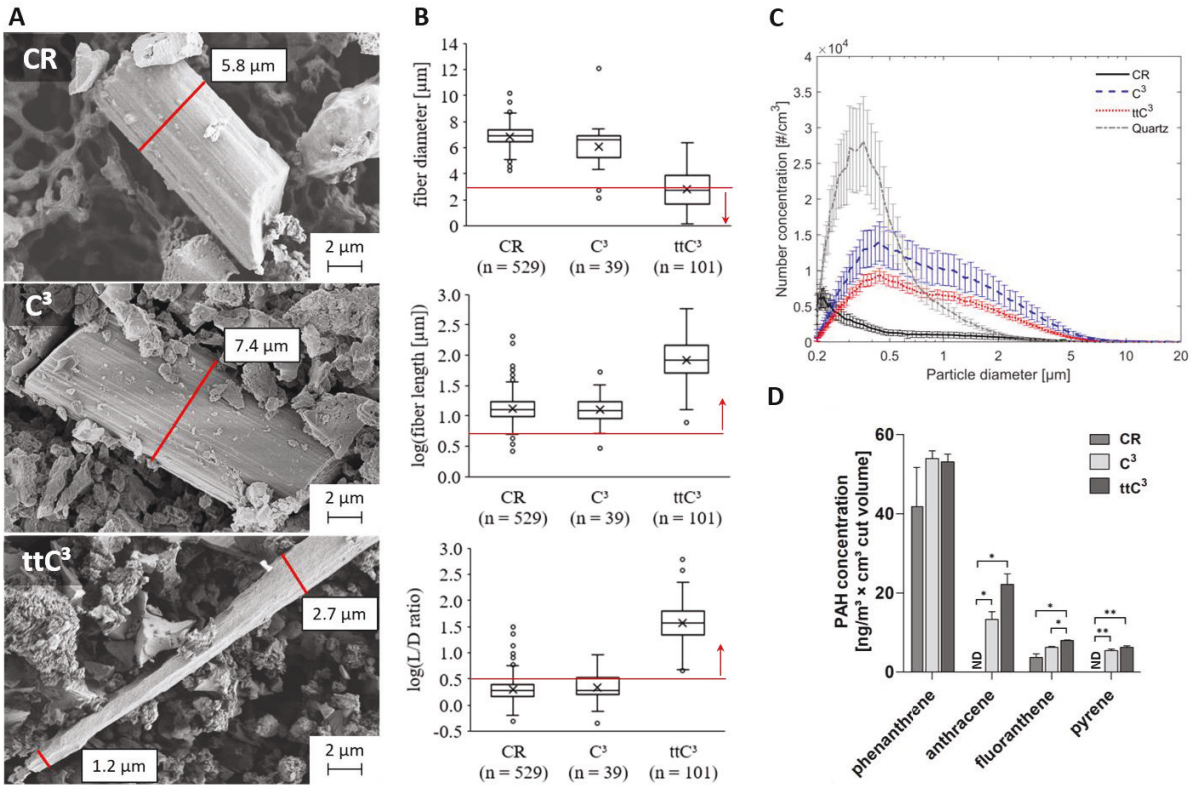


Figure 15: Physico-chemical characterization of the dry-cut construction materials. A) SEM micrographs of fibers released by dry-cutting of CR, C³, and ttC³ shows that ttC³ released fibers of lower diameter meeting WHO specifications. B) The fiber diameter is decreasing, whereas fiber length and length-to-diameter (L/D) ratio of ttC³ is increasing. C) The particle distribution is comparable for C³ and ttC³, nevertheless CR is shifted to smaller size and shows a lower number concentration. D) PAHs can be found in all materials, even though to a decreased extent in CR particles.

A chemical analysis of the fine particulate matter ($PM_{2.5}$; PM ≤ 2.5 µm) fraction of the dry-cutting products revealed pronounced concentrations of bisphenol A (BPA; ≤ 383 µg/mg PM) and its derivatives in CR [100], most likely deriving from the pyrolytic degradation of the epoxy resin coating at the saw blade edge. Furthermore, the PAHs phenanthrene and fluoranthene could be found in the released particles of all materials, whereas anthracene and pyrene were only present in the concrete materials, with anthracene being more concentrated in ttC^3 particles (**Figure 15, D**).

5.1.3 Toxicological assessment

The analysis of the $PM_{2.5}$ fraction showed significant differences between the materials tested with ttC^3 and CR showing the strongest effects on cell viability, inflammation and primary as well as secondary genotoxicity. An increase in cytotoxicity (**Figure 16, a+b**) could be observed for all materials after 2 h (a), but especially after 24 h (b) exposure. The higher effects of CR and ttC^3 after 24 h exposure compared to C^3 could be attributed to the presence of BPA [136, 137] and PAHs [138, 139] known to be cytotoxic on A549 as well as MRC-5 cells. In addition, ttC^3 might induce phagosome rupture due to the presence of fibers with a needle-like shape, which leads to an acidification of the cytosol initiating necrosis and an increased release of LDH into the medium. Another marker indicating viability of the cells is the metabolic activity (**Figure 16, c+d**), which revealed slight reductions after 2 h exposure (c). Those effects seem to be restored after 24 h exposure (d) for all materials with ttC^3 even increasing the cells metabolism. The observed metabolism pattern points towards an early oxidative stress response of the tissue, which depletes reducing agents important for the reduction of the resazurin dye. A central co-factor, which is consumed in such condition is NAD(P)H + H^+, which recycles antioxidative enzymes like glutathione reductase, but could also be diminished due to respiratory burst to produce RONS as immunological defense or signaling molecules. A delayed increased synthesis of mediators could restore the antioxidant imbalance and increase metabolic activity after 24 h. Moreover, the enhanced activity following prolonged ttC^3 exposure, might be associated with an increased proliferation of fibroblasts or an acceleration of metabolic processes to meet the energy demand needed for fibroblast activation, proliferation and differentiation in pro-fibrotic conditions [140].

Since $PM_{2.5}$ is known to initiate a strong inflammatory response, the release of the inflammatory marker IL-8 into the cell culture medium was analyzed. A pronounced release could be detected for all materials after 2 h exposure (**Figure 16, e**), which could be restored to untreated control levels after 24 h exposure (**Figure 16, e**) in case of the concrete materials. The early inflammatory response could be caused by the particle-bound chemicals BPA [141, 142] and its derivatives as well as the detected PAHs [139, 143-145], all of which have shown in previous studies to induce an inflammatory cytokine release including IL-8, interleukin-6, cyclooxygenase-2 or tumor necrosis factor-α. Moreover, the physical structure of the particle itself and its interaction with cell constituents may induce a transient inflammation, which could become persistent when particles get smaller in size as it could be the case for CR. The exposure to UFP might even lead to the formation of fibroblastic foci [146, 147] or cancer formation [148].

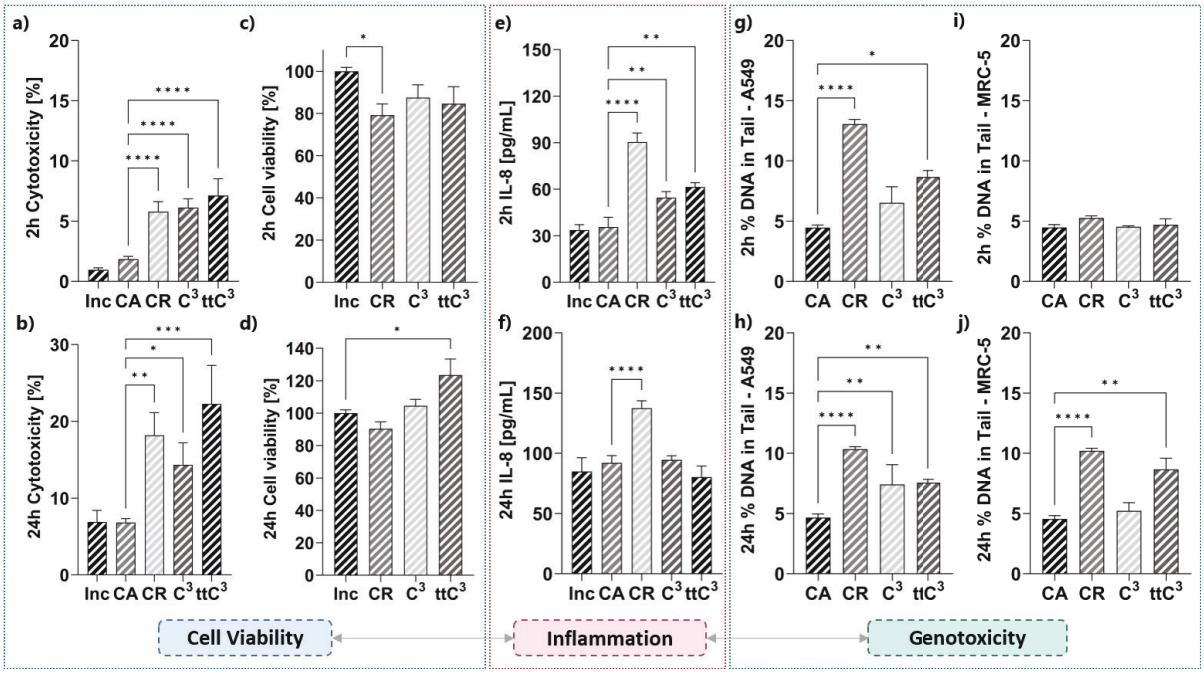
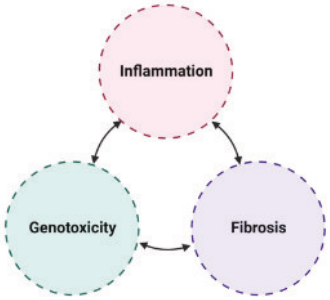


Figure 16: Toxicological analysis. LDH release revealed increased cytotoxicity after 2 h (a) and 24 h (b) exposure of co-cultured A549 and MRC-5 cells for all materials. The metabolic activity slightly decreased after CR treatment. Moreover, the inflammatory potential was assessed via the release of interleukin 8 (IL-8) and showed a strong induction after CR exposure and slightly after C³ and ttC³ treatment. Primary genotoxicity (g+h) was observed for all materials after 24 h exposure, while secondary genotoxicity (i+j) was only induced by CR and ttC³, comparable to the DNA damage occurring after 2 h in epithelial cells.



Inflammation is a process that is receiving more and more attention as a driving force in the development of serious diseases caused by particle exposure [36, 148]. It seems to be strongly involved in lung fibrosis as well as cancer development, all linked by genotoxicity.

Primary genotoxicity on epithelial A549 cells was detected for CR and ttC³ after 2 h exposure (**Figure 16, g**), and for all materials after 24 h exposure (**Figure 16, h**). Since % DNA in tail is slightly lowered again for CR and ttC³ after prolonged exposure, it seems like the damaged DNA could be partially repaired. Secondary genotoxicity on MRC-5 fibroblasts (**Figure 16, i+j**) was only observed after 24 h exposure (j) to CR and ttC³. There are various explanations where DNA damage might originate from. Primary genotoxicity could be caused by enhanced oxidative stress due to the formation of ROS [38, 42, 149], which could subsequently lead to an increased inflammatory response as observed for C3 and ttC³ after 2 h exposure and strongly for CR after 24 h exposure, which is in accordance to previous *in vivo* as well as *in vitro* studies implicating a pronounced association of inflammation and genotoxicity [36]. Moreover, fibrous materials were proven to interact directly with the DNA, inducing stand breaks [53, 150]. Also, the chemical composition of the particles may play a crucial role as BPA [137, 151] as well as PAHs [44, 152] have been shown to possess the potential to cause genotoxic lesions. The latter do

not seem to be the cause for the observed secondary genotoxicity, since C^3 is not showing any induction in DNA damage. ttC^3 but especially CR possibly exert indirect genotoxicity via inflammatory pathways [36, 67, 153], which are induced as an early response to the materials and seems to become persistent for CR. For ttC^3 on the other hand, it might be more realistic that fibroblasts are affected by an increased formation of ROS [47, 153], causing oxidative DNA damage and an antioxidant depletion, which correlates with an increased mitochondrial metabolic activity to counteract this imbalance [154].

To shed light on a possible interconnectivity of inflammation, genotoxicity and the development of fibrosis, a microarray analysis was performed and revealed significantly regulated pathways on gene expression level. Whereas for C^3 almost no distinct regulation was observed (not further discussed), ttC^3 (**Figure 17, left**) and CR (**Figure 17, right**) showed a distinctively regulated profile, particularly after 2 h (**Figure 17, top**) and 24 h (**Figure 17, bottom**) exposure, respectively. Especially the pathways intensely upregulated after ttC^3 treatment are strongly related to pro-fibrotic conditions. Following 2 h exposure, the fibrosis-associated pathways 'Pulmonary Fibrosis Idiopathic Signaling', 'Wound Healing Signaling Pathway' and 'ID1 Signaling Pathway' [155, 156] are induced, whereas after 24 h, the 'Sirtuin Signaling Pathway' [62] is attenuated and the 'Phagosome Formation' [157] increased, as frequently observed in fibrotic diseases. The conspicuous relationship between ttC^3 exposure and the induction of pro-fibrotic pathways might be caused by the size and shape of the fibers fulfilling the hazardous characteristics of WHO fibers. In addition to that, also the cell cycle and division machinery is affected, shown by a decrease of the 'Cell Cycle: G2/M DNA Damage Checkpoint Regulation' pathway and an induction of the 'Kinetochore Metaphase Signaling Pathway', possibly responsible for the increased genotoxicity and enhanced metabolism following ttC^3 treatment [158, 159].
While fibrosis-related pathways seem to be downregulated after 2 h exposure of epithelial cells to CR, especially after 24 h exposure pathways regulating DNA repair ('Cell Cycle Control of Chromosomal Replication', 'NER (Nucleotide Excision Repair, Enhanced Pathway)' and 'BER (Base Excision Repair) Pathway') and favoring tumor formation ('RHOGDI Signaling', 'HOTAIR Regulatory Pathway', 'Autophagy', 'IL-17 Signaling') are predominantly regulated. The up- and downregulation of pathways associated with distinctively expressed genes following ttC^3 and CR exposure, implicates different mechanisms-of-action for the materials.

This study proved that the developed cell model is able to elaborate complex mechanisms of different airborne materials and show differences in terms of adverse outcome. Not only primary effects on epithelial cells could be assessed with the model, but also secondary effects on fibroblasts, which increases the comprehensiveness and significance of the study. It could be shown that thermal treatment of carbon concrete results in the release of hazardous fibers, which are able to initiate pro-fibrotic signaling pathways and induce secondary genotoxicity. Moreover, the study revealed that also the exposure to the reinforcement material itself could lead to a severe burden of the lung tissue.

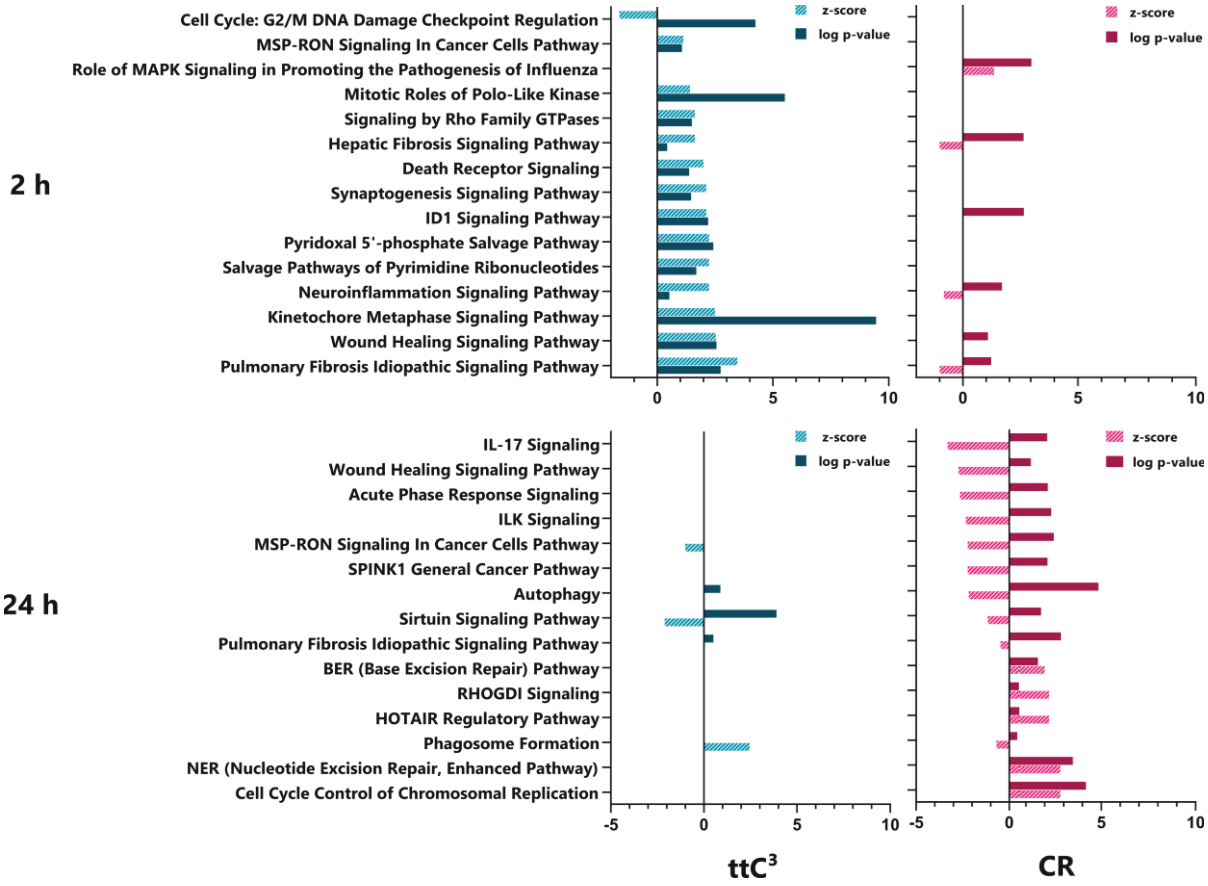
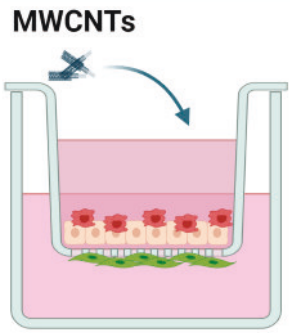


Figure 17: Gene expression analysis. Microarray analysis showed a pronounced regulation of fibrosis-associated pathways by ttC³ treatment, especially after 2 h exposure, whereas CR treatment showed a strong effect on DNA repair and tumor-related pathways following the prolonged 24 h exposure, indicating different modes-of-action of the materials.

5.2 Triple culture of epithelial cells, macrophages and fibroblasts



As already highlighted above, inflammation and the activation of immune cells such as macrophages are crucial events in the defense of the respiratory system against pathogens and toxicants. For potentially pro-fibrotic particles, it is critical for the outcome whether those cells are able to internalize the stressor for tissue clearance and to which extent an inflammatory response is induced, leading to a cascade of mediator release and recruitment of other cell types supporting for example to keep antioxidant balance and to participate in wound healing at the site of action. For toxicity testing, therefore, it is recommended to consider the influence of immune cells on the toxicological outcome. Especially fibrotic tissue in diseases like IPF is associated with elevated levels of inflammatory markers such as IL-8 and the infiltration of neutrophils into the affected areas [160, 161]. Furthermore, there seems to be a strong correlation of inflammatory signaling and increased levels of DNA damage [36], which could additionally contribute to the

progression of fibrosis. To evaluate the interplay of the main cell types known to be involved in pro-fibrotic conditions following particle exposure, an easy-to-use cell model comprising epithelial cells and macrophages on the top of the insert membrane as well as fibroblasts cultured on the downside of the membrane was developed. Advantageously, in contrast to the co-culture system introduced before, the contribution of immune cells phagocytic processes and inflammatory signaling is not missed and can influence the response to the exposure of the other cell types. Importantly, the separate cultivation of cells enables an individual investigation of fibroblasts including secondary genotoxicity, which might be substantially influenced by the presence of macrophages and could contribute to the manifestation of fibrosis.

5.2.1 Application (2)

The functionality of the established cell model was comprehensively toxicologically assessed by submerged exposure to different concentrations of commercial MWCNTs for 24 h and 48 h. The latter are known to possess a fiber-like structure and are estimated to exert similar effects like MMMFs such as asbestos due to their high biopersistence as well as their small size enabling them to penetrate deeply into the alveolar region. Not only their impact on cell viability, inflammation, genotoxicity and fibrosis development was investigated in the triple culture system (TC), but also differences in response compared to less advanced systems such as a monoculture (MC) of epithelial cells and the aforementioned co-culture (CC) of epithelial cells and fibroblasts. For the sake of comparability all cell models were treated in the same way at any time independent of their complexity.

5.2.2 Physico-chemical characterization of MWCNTs

The high-purity MWCNTs possessed the size specifications of WHO fibers with an average length of 10-30 µm, a diameter of 25 nm and an aspect ratio > 3, which made them particularly suitable for the model validation. According to the manufacturer's information, the dry powder was of > 99.9 % purity. It was assumed that all effects observed derived from the physical properties of the particles only, as a contamination of metal catalysts and endotoxins could also be excluded by energy dispersive X-ray (EDX) spectroscopy and endotoxin analysis. Suspended particles in cell culture medium revealed an average diameter of 267.7 nm at the highest concentration tested as determined by dynamic light scattering. The MWCNTs were applied in concentrations of 0, 2.2, 4.5, 11.2 and 22.3 µg/cm² corresponding to 0, 5, 10, 25 and 50 µg/mL.

5.2.3 Toxicological assessment

The toxicological investigation revealed more pronounced and differential effects between the systems following prolonged exposure, which is why the following description is focused on the effects observed after 48 h only.

Cytotoxicity assessment, including the cell count (**Figure 18, a+b**) as well as the release of LDH into the cell culture medium (**Figure 18, c)**, showed, that the simpler cell models are more prone to MWCNTs-induced necrotic cell death of epithelial cells than the TC, which becomes evident by a

substantially decreased cell number and increased levels of LDH in the MC and CC compared to their respective untreated control cells. However, in the CC fibroblasts seem to attenuate the membrane damage slightly, even though not as much as the TC. The reduced necrotic cell death in the CC could be attributed to enhanced fibroblast signaling, as the latter have shown to promote a pro-apoptotic milieu and signaling in IPF [162, 163]. Contrastingly, protective effects could be observed by immune cells already before [164], and might be associated with the engulfment of the material by macrophages, thereby avoiding direct contact of the tubes with the epithelial layer, explaining lower toxicity in the TC [165, 166]. Since fibroblasts do not show a direct reduction in cell count, neither in the CC, nor in the TC, it can be assumed that they are not directly contributing to the LDH release.
In contrast to the aforementioned findings, the clonogenicity is the most affected in the co-culture system and displays also a substantial reduction in the triple culture (**Figure 18, d**). CC-effects might occur due to pro-apoptotic signaling of affected fibroblasts as mentioned before, whereas the lowered clonogenicity of epithelial cells in the TC might only partly be explained by apoptosis, rather than by a possible arrest of the cell cycle caused by immune cell signaling [167].

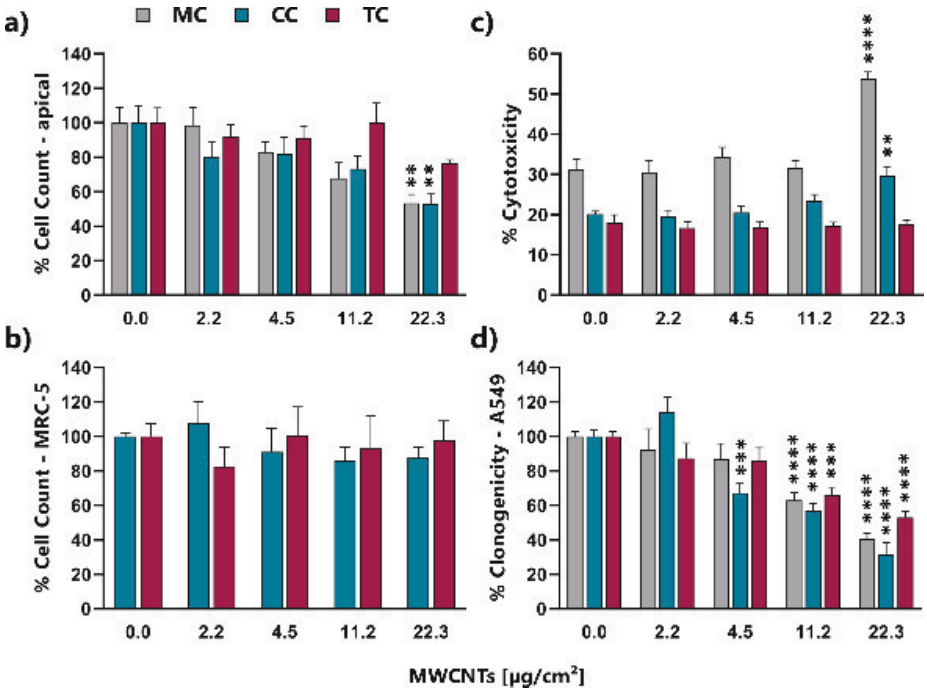


Figure 18: Cytotoxicity assessment. Investigation of the cell count of the apical cells (a) revealed a reduction in the monoculture (MC) and co-culture (CC), but not in the triple culture (TC), whereas the fibroblast cell count (b) was not affected. A significantly increased LDH release (c), implying necrotic cell death, was detected in the less complex systems, but not in the TC, indicating protective effects of macrophages. However, the clonogenic potential of epithelial cells (d) was substantially reduced in all model systems, indicating apoptotic cell death or a decreased cell cycle progression, which can be influenced by the presence of macrophages.

A central objective of this study was to unravel primary, but especially secondary mechanisms of genotoxicity occurring in the multicellular test systems and their potential involvement in the

development of pro-fibrotic conditions following MWCNT exposure. Genotoxicity was mainly observed in the TC, with a slight increase in epithelial cells (**Figure 19, a**), but especially in fibroblasts (**Figure 19, b**), showing for the first time secondary genotoxicity in fibroblasts caused by MWCNTs. Considering aberrant fibroblast signaling in fibrotic conditions, a manifestation of genetic mutations occurring due to oxidative stress, altered DNA repair mechanisms or paracrine signaling, might explain an unresolved wound healing response, accumulating ECM deposition and dysfunction of the affected tissue. An increase of unrepaired DNA damage might induce programmed apoptosis or senescence to avoid proliferation of such persistently altered cells [168]. Remarkably, senescence of fibroblasts as well as myofibroblasts is frequently observed in IPF-derived fibroblasts [169, 170] and linked to a disrupted wound repair, chronic inflammation as well as a decreased regeneration of the tissue [171, 172]. Another interesting observation made during genotoxicity assessment, are the generally lower levels of spontaneous DNA damage in the triple culture compared to the other models. This finding could be explained by a deceleration of the cell cycle allowing for enhanced DNA repair and is in accordance with the suppressed clonogenicity discussed above. In accordance with the known interconnection of inflammation, oxidative stress and genotoxicity [36], the observed DNA damage is accompanied by elevated levels of ROS indicated by increased MDA concentrations (**Figure 19, d**) found in the exposure medium, but especially by enormously increased levels of the marker IL-8 (**Figure 19, c**), detected in the medium of the TC. It can be estimated that the high levels of this inflammatory mediator are caused by macrophages present in the triple culture, since the other models uncover comparably low amounts of it. Furthermore, it was previously shown that co-cultures including macrophages react more sensitively regarding their inflammatory response compared to cultures without [173, 174], which might be attributed to the fact that macrophages are the first cells in line to defend against the pollutant and are able to generate high amounts of cytokines in a short time to initiate defense mechanisms [175, 176]. This process might also involve the production of ROS [177, 178], as slightly detected for the TC and could lead to oxidative DNA lesions in primary and secondary tissue [38, 47]. The increased MDA levels detected in the MC might occur due to direct interaction of MWCNTs with cellular components of the epithelial cells.

The inflammatory mediator IL-8 is used as a biomarker in IPF diagnosis and is negatively linked to lung function [160]. Moreover, abnormal fibroblast signaling, possibly linked to genetic lesions, is commonly found in diseased lung tissue of IPF patients, which made the examination of potential pro-fibrotic lesions inevitable.

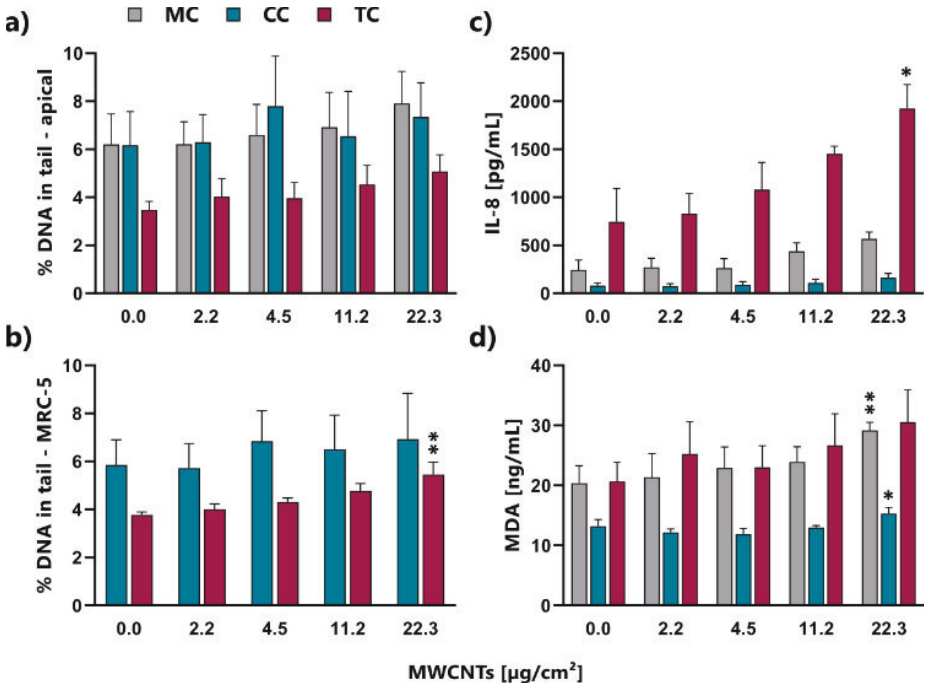


Figure 19: Analysis of the genotoxic, inflammatory and oxidative stress-associated potential. DNA damage induced in epithelial cells (a) and fibroblasts (b), was mainly prevalent in the TC. Genotoxicity was accompanied by high levels of the inflammatory marker interleukin 8 (IL-8, c), possibly released by macrophages, and increased ROS formation indicated by rising MDA concentrations (d). The latter was especially pronounced in the MC, most likely due to direct contact of the epithelial layer with MWCNTs.

In a morphological assessment of cells treated with the highest concentration used (**Figure 20, A**), significant differences between the epithelial layers of the different models became evident. While, the MC showed a substantial loss of membrane integrity combined with dead cells, CC and TC showed an intact layer, but with enlarged and dilated cell bodies. Interestingly, in the TC, cells lost their cuboidal shape to a spindle-like morphology containing stress fibers. These features are typical for EMT and are highly associated with fibrosis-related outcomes [179, 180]. Furthermore, EMT was also reflected by the expression of the marker E- and N-cadherin in all models (not shown, found in [17]). Another fibrosis-associated hallmark, possibly induced by paracrine EMT signaling [180] and activated macrophages [181], is the differentiation of fibroblasts into myofibroblasts, which could be detected by an upregulation of vimentin and α- smooth muscle actin (α-SMA) in both, the CC as well as the TC, revealing slight changes already in control cells of the co-culture, indicating altered behavior already in untreated conditions (**Figure 20, B**).

Taken together, this study describes the successful development of a multicellular model system, which could represent a useful test system reflecting simple lung physiology and avoiding animal testing in early screening procedures following the 3R principle of refinement, reduction and replacement of animal experiments. The established model is a reproducible, cost- and time-effective tool by overcoming ethical justification required for animal testing, which moreover, is often not properly suitable due to different respiratory tract anatomy, breathing patterns as well as clearance mechanisms

of humans and animals [74]. The model is able to indicate cytotoxic, inflammatory, genotoxic and pro-fibrotic conditions in epithelial cells as well as fibroblasts under consideration of intercellular cross-talk. Our investigations have shown, that both, the presence of macrophages as well as fibroblasts, is significantly influencing the outcome of the treated model and should not be excluded from toxicity testing, especially when fibrotic outcomes are of particular interest. Even though, the TC has shown a stronger protective response regarding cytotoxic mechanisms, the inflammation and genotoxicity assessment displayed a greater sensitivity of the advanced cell model over the simpler models. The strong interconnection of inflammation and genotoxicity, became evident in this study and could be linked to pro-fibrotic conditions. Progressive EMT as well as an aberrant fibroblast function could be induced by genetic alterations due to primary genotoxicity in epithelial cells as well as secondary genotoxicity in fibroblasts.
Future studies using such model, also applying it at the ALI under more realistic and toxicologically relevant conditions, could help to further unravel mechanistic fundamentals of fibrosis development and the role of genotoxicity in it. Furthermore, the model is suited to perform early-stage toxicity testing, which becomes increasingly relevant due to the permanently ongoing development of HARMs and recycling processes of materials containing potentially inhalable and hazardous particles and fibers, especially in the construction and wind energy sector, which are faced with a tremendous amount of materials that are approaching the end of their service lifes.

5.2.4 Application (3)

A common result of combustion processes or industrial emissions is the release of UFP. Due to their high surface area, which increases the concentrations of potentially adsorbed compounds, their high reactivity and their low aerodynamic diameter, allowing the particles to penetrate deeply into the small airways and adjacent tissues, the exposure to UFP is of great concern [182, 183]. Nevertheless, whether the physical characteristics of UFP are the main drivers of toxicity or whether the particles primarily act as carriers of toxic chemicals requires further investigation. To examine the impact of the latter, two chemically distinct UFP aerosols with low or high content of semi-volatile organic compounds (SVOC) but comparable sizes were produced by a miniature combustion aerosol standard soot generator (miniCAST) and investigated regarding their acute (geno)-toxic potential in the developed triple culture system at the ALI.

Since exposure to air pollutants such as UFP is often associated with the production of RS, a direct measure of the antioxidant capacity gives a more detailed mechanistic background in comparison to the analysis of lipid peroxidation markers only. Glutathione is an important factor of the defense machinery [184, 185]. Decreased levels of intracellular GSH are not only indicating an enhanced redox cycling due to RS scavenging that is coming along with increased GSSG concentrations, but might also be attributed to an enhanced conjugation of xenobiotic compounds like PAHs, such as BaP [186, 187] or naphthalene [188], or bisphenol A [189](**Figure 22, A**).

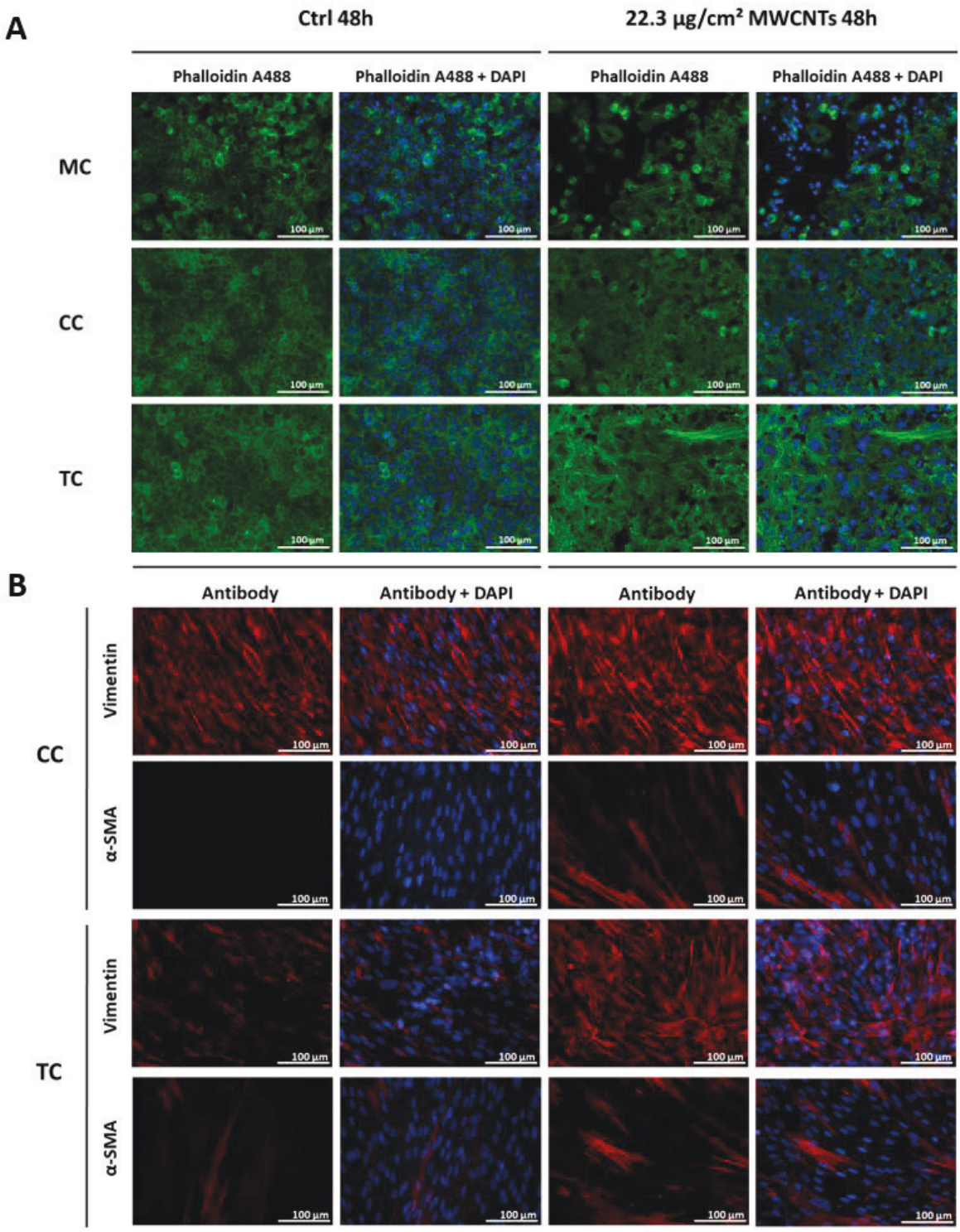


Figure 20: Analysis of epithelial morphology and expression of fibrosis-related markers. A staining of the cytoskeleton (A, green) showed a disruption of the MC cell layer and increased cell death indicated by round, bright blue nuclei after treatment with MWCNTs, whereas the CC and TC revealed dilated cell bodies with stress-fiber formation in the TC, acquiring a mesenchymal spindle-like morphology. Expression analysis of fibrosis-related markers vimentin and α- smooth muscle actin (α-SMA) showed an upregulation of markers in the TC and CC.

5.2.5 Physico-chemical aerosol characterization

Ultrafine soot particles were generated with a miniCAST. Organic compounds were removed by a catalytic stripper (CS) along with carbon denuders to produce an UFP aerosol of low SVOC content. The applied approach allowed for the generation of two different UFP aerosols, which showed similar physical characteristics but different chemical compositions. In-depth chemical and physical investigations indicated significant differences in organic content while displaying no significant differences in particle mass (90-110 µg/m³), particle number concentration (4.9 -5.3e10^5 #/cm³) and particle size (35-45 nm). The high SVOC aerosol showed pronounced levels of PAHs analyzed by gas chromatography - mass spectrometry (GC-MS), such as pyrene, benzo[*a*]pyrene, benzo[*ghi*]perylene, or benzofluoranthenes (**Figure 21**) [personal communication by Anusmita Das], which could contribute to respiratory toxicity through activation of those compounds by xenobiotic CYP450 metabolism.

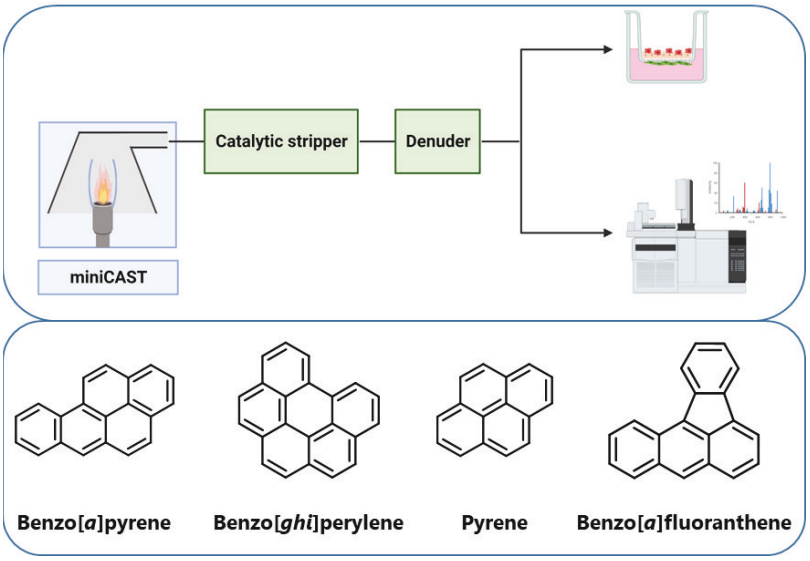


Figure 21: Generation of chemically distinctive UFP aerosols potentially forming PAHs. Ultrafine soot particles were generated with a miniCAST and for the low SVOC the organic fraction was removed by a catalytic stripper and a denuder. The high SVOC aerosol contained sustained amounts of PAHs such as benzo[a]pyrene, benzo[ghi]-perylene, pyrene, or benzo-fluoranthens.

5.2.6. GSH method validation and toxicological assessment

For this study, a LC-MS/MS method was established, which enables the precise detection of intracellular GSH and GSSG levels. It can be applied to aerosol-exposed multicellular systems and was used for the investigation of the toxicity induced by low and high SVOC UFP aerosols. Advantageously, this technique requires only low amounts of sample material, enables the investigation of unlimited sample sizes with a high sensitivity (GS-NEM: LOQ = 0.3 ng/mL; GSSG: LOQ = 7 ng/mL) and possesses the possibility to store the samples for later analysis. Moreover, the usage of NEM minimizes sampling artifacts, since it results in a low auto-oxidation of GSH. Furthermore, NEM is a highly cell membrane permeable compound and able to inhibit the enzymatic activity of GR, which achieves a more accurate estimation of GSH and GSSG levels [126, 127].

To validate the functionality of the method, the aforementioned triple culture was incubated with 100 µM solution of menadione (**Figure 22, B**), which has shown before to induce ROS in lung epithelial cells [190]. For the treated cells, elevated levels of the glutathione disulfide with almost 30 % GSSG

of the total intracellular glutathione concentration could be detected. The untreated incubator controls on the other hand was displaying GSSG levels below 0.1 %, which can be attributed to the fact, that the cell is trying to keep GSH levels as high as possible for antioxidative defense and therefore recycles generated GSSG relatively fast [124, 184].

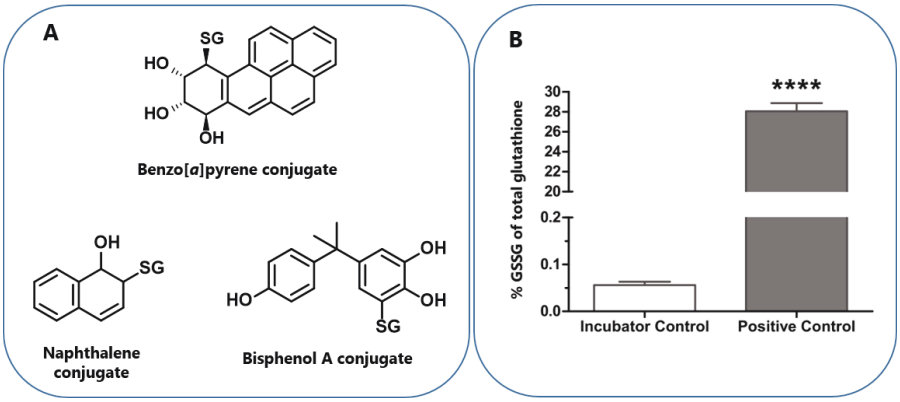


Figure 22: Glutathione (GSH) as defense marker in air pollutant toxicity. GSH possess a reactive thiol group, which makes it a strong nucleophile, able to react with possibly toxic electrophilic metabolites of air pollutants such as benzo[a]pyrene, naphthalene or bisphenol A, resulting in less toxic glutathione conjugates (A), which are more readily excreted. The developed LC-MS/MS method enables the detection of increased GSSG levels (% GSSG of total glutathione) following exposure to ROS-inducing compounds like menadione (positive control), while displaying low amounts of GSSG in control cells such as incubator control cells (B).

In the future, this method will contribute to the investigation of mechanisms-of-action no matter which kind of compound might be tested. It takes into account the depletion of GSH in primary and secondary cells. Moreover, the method could be adopted for combined investigation of glutathione levels as well as cell uptake studies of organic compounds entering the cell. Due to its cost- and time-efficient applicability, it might be considered as lower tier test in compound screening, giving first insight in possible mechanisms of toxicity. Additionally, it is a sensitive method, which is able to show minor changes in GSH balance and is therefore a suitable technique for aerosol exposure at the ALI, since exposure relevant concentrations of outdoor and indoor air pollution are most often rather low.
For those reasons, the method was applied to investigate the two chemically different UFP aerosols, besides a cell viability and genotoxicity assessment (**Figure 23,** unpublished data). Briefly, low SVOC UFP induced an increased cytotoxicity accompanied by a decreased metabolic activity of fibroblasts. Furthermore, a depletion of intracellular glutathione could be observed, which might be attributed to an increased ROS release. The latter might be causative for the observed secondary genotoxicity in fibroblasts, exclusively detected after exposure to low organic content UFP. High SVOC UFP did not impact cell viability, but an increased DNA damage was observed in epithelial cells, which could be caused by DNA-adduct formation of PAHs. All in all, compared to the high SVOC aerosol, UFP with low organic content seem to induce a stronger acute cytotoxic and oxidative stress response in epithelial cells, which might cause secondary effects in fibroblasts. This observation could be related to different cell uptake mechanisms by macrophages and epithelial cells. A previous study by Liu *et al*. 2021

showed, that fine particles coated with BaP are rather taken up by macrophages than by epithelial cells and particles with no or lower BaP coating showed a faster cell uptake by epithelial cells [191]. Therefore, low SVOC UFP might directly interact with epithelial cells, exerting immediate toxicity, while high SVOC could be initially internalized by macrophages, possibly resulting in a protective response or delayed toxicity.

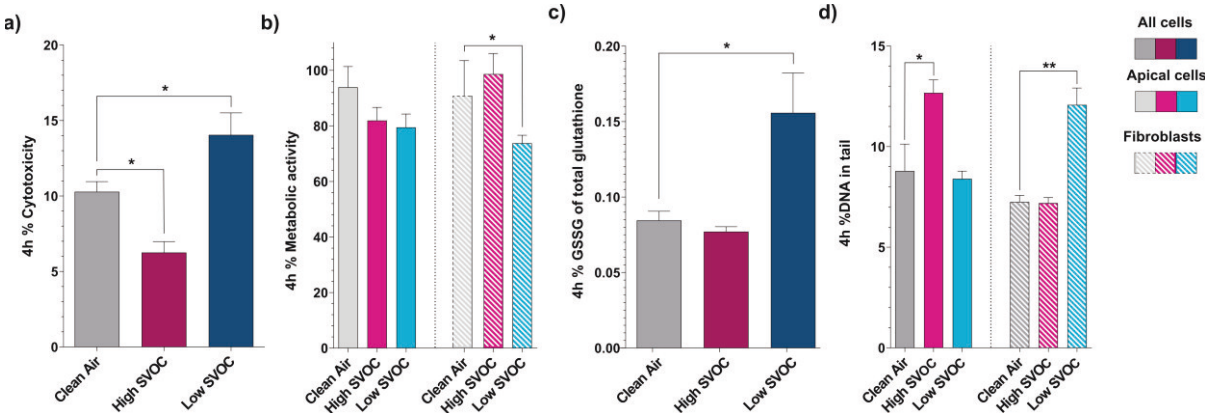
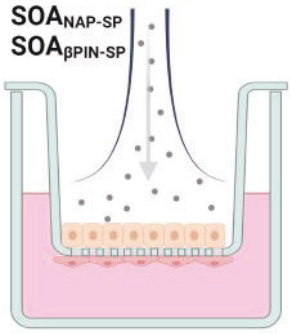


Figure 23: Toxicological assessment of UFP aerosols. The developed triple culture system showed increased cytotoxicity after exposure to low SVOC UFP (a) accompanied by a decreased metabolic activity of fibroblasts (b). The observed toxicity might be attributed to ROS formation leading to a depletion of GSH and an increase in GSSG (d), which might be causative for secondary genotoxicity in fibroblasts after low SVOC UFP exposure. High SVOC UFP aerosol did not cause acute cytotoxicity after 4 h exposure, but showed an increased DNA damage in epithelial cells. Unpublished data.

5.3 Co-culture of epithelial cells and endothelial cells



Not only the connective tissue is a secondary target of airborne particulate matter but also the cardiovascular system is of great vulnerability, especially when exposed to particles that are able to penetrate deeply into the alveolar system and translocate into blood capillaries. The endothelial lining of the latter can be affected through direct contact with the material but also via secondary effects, such as oxidative stress and inflammatory or pro-fibrotic signaling leading to vascular remodeling and injury [192, 193]. Furthermore, endothelial cells might contribute themselves to the progression of fibrosis via endothelial-mesenchymal transition (EndMT) to myofibroblasts [192, 194], inflammatory mediator secretion [28] or maintenance of enhanced ECM deposition [28, 192]. To more comprehensively understand the interplay of epithelial and endothelial cells in diseases associated with air pollution such as cardiovascular diseases [195, 196] or pulmonary fibrosis, a cell model comprising both cell types was established, including epithelial cells on the apical side of the membrane and endothelial cells on the basolateral side. Again, the latter allows for separate investigation of genotoxicity and gene expression levels of both cell types.

5.3.1 Application (4)

In this cell model, two model aerosols were investigated mimicking exposure to different dilutions (1:30, 1:10, 1:3, undiluted) of anthropogenic (naphthalene, NAP) and biogenic (β-pinene, βPIN) emissions altered in the atmosphere through aging processes. For this purpose, freshly generated soot particles were coated with naphthalene or β-pinene VOC precursors ($SOA_{NAP\text{-}SP}$ and $SOA_{\beta PIN\text{-}SP}$) in an oxidation flow reactor, leading to distinct condensed secondary organic aerosols containing ultrafine particles with a diameter of approx. 115 nm [82]. Even though comprehensive data is lacking to clearly unravel mechanisms-of-toxicity, SOA are estimated to exert a stronger toxicity compared to their precursors including tissue injury and inflammation, which might lead to a decrease in lung capacity and fibrosis development [197, 198]. Accordingly, a more in-depth toxicological analysis of such aerosols in relevant model systems is essential.

5.3.2 Physico-chemical aerosol characterization

The generated model aerosols possessed comparable physical properties such as mass, size distribution or particle number, but showed a distinctive chemical pattern, which allowed for determination of toxicological effects induced by the chemical characteristics only. Whereas in $SOA_{NAP\text{-}SP}$ especially aromatic compounds were detected, comprehensive two-dimensional gas chromatography mass spectrometry (GCxGC-MS) analysis revealed a high amount of predominantly cyclic and acyclic volatile compounds for $SOA_{\beta PIN\text{-}SP}$ [82]. Aromatic structures, as prevalent in $SOA_{NAP\text{-}SP}$, are well-known products of photochemical aging, and are estimated to exert toxic effects, especially with increased oxidation status (**Figure 24)**, on the respiratory system, involving genotoxicity and mutagenicity, but could also be causative for the induction of fibrosis.Further analysis showed, that $SOA_{NAP\text{-}SP}$ contained higher levels of hydrogen peroxides, implying an increased prevalence of ROS, which might occur due to the higher photochemical oxidation of $SOA_{NAP\text{-}SP}$ compared to $SOA_{\beta PIN\text{-}SP}$. Since ROS are a crucial stressor for lung residential cells, this observation might contribute substantially to the formation of the adverse outcomes mentioned before.

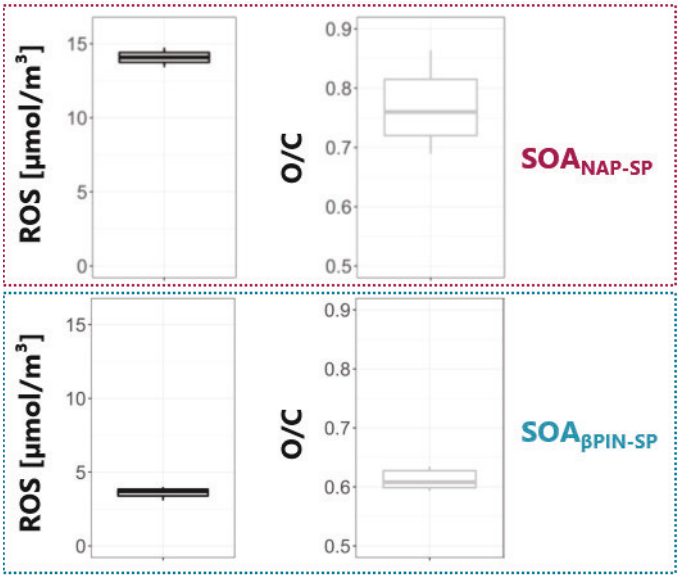


Figure 24: Oxidation state of $SOA_{NAP\text{-}SP}$ and $SOA_{\beta PIN\text{-}SP}$. (Left) Measurements of H_2O_2 show a higher prevalence of reactive oxygen species (ROS) in $SOA_{NAP\text{-}SP}$ than in $SOA_{\beta PIN\text{-}SP}$. Moreover, the determined O/C ratios (right) revealed an increased oxidation state of $SOA_{NAP\text{-}SP}$ compared to $SOA_{\beta PIN\text{-}SP}$, which could be causative for stronger toxicological effects.

5.3.3 Toxicological assessment

Even though the two aerosols displayed very different chemical characteristics, the cytotoxicity assessment (**Figure 25, a**) showed similar effects for $SOA_{NAP\text{-}SP}$ and $SOA_{\beta PIN\text{-}SP}$. Interestingly, both aerosols seemed to induce comparable cytotoxic effects following exposure to the undiluted aerosol, which is unexpected considering that aromatic compounds and ROS, both known prerequisites for cytotoxicity, are more pronounced in $SOA_{NAP\text{-}SP}$. This assumption becomes evident in the further investigation of inflammation, oxidative stress as well as primary and secondary genotoxicity. A substantial release of the inflammatory marker IL-8 could be detected for both aerosols (**Figure 25, b**), nevertheless in higher concentrations after exposure to $SOA_{NAP\text{-}SP}$. The results are in accordance to previous studies, especially for exposure to anthropogenic SOA, which has shown to induce inflammation in epithelial cells and to activate nuclear factor erythroid 2-related factor 2 (Nrf2) signaling [199, 200], which regulates the antioxidative response. The latter is also activated through the release of ROS by the aerosol or as cell response to injury helping to maintain MDA, the final product of free radical-induced lipid peroxidation, could be observed for both aerosol (**Figure 25, c**), yet again showing a stronger oxidative stress response due to the anthropogenic SOA. It can be assumed, that the strong genotoxic potential derives from DNA adduct formation of naphthalene and its metabolites such as naphthoquinone as previously shown [201, 202], as well as ROS contained in the aerosol or generated during inflammatory and oxidative stress response, leading to DNA oxidation [38]. Noticing that secondary genotoxicity is exclusively occurring after $SOA_{NAP\text{-}SP}$ exposure, and assuming that no particles translocate to the downside of the membrane due to its low pore size of 0.4 µm [203], oxidative stress signaling might be as well causative for indirect DNA damage detected in EA.hy926 cells (**Figure 25, d**).

Given that inflammation and oxidative stress represent key events in the formation of fibrosis, an induction of fibrosis-relevant genes for both aerosols is suggestive. Gene expression analysis [personal communication by Svenja Offer) reveals, that early mediators of EMT, but also EndMT, such as the *zinc finger protein SNAI1 (SNAI1)* [204, 205] and *twist-related protein 2 (TWIST2)* [206, 207], as well as IPF associated markers like *epithelial cell adhesion molecule (EPCAM)* [208], *claudin-2 (CLDN2)* [209] and *fibronectin 1 (FN1)* [210] are upregulated, especially after $SOA_{NAP\text{-}SP}$ exposure. On the other hand, it should be noted that important late-stage E(nd)MT related markers like *cadherin-1 (CDH1)* and *cadherin-2 (CDH2)* are inversely regulated of what is known from E(nd)MT [211, 212], implicating that pro-fibrotic conditions are not manifested yet and might be resolved. Nevertheless, the initiation of the cadherin switch is strongly time-dependent [213], and requires a preceding activation of several signaling pathways leading to an increased transcription and expression of e.g. *SNAI1* [214] or *TWIST* [215, 216], which are significantly involved in the switch, and might need to be expressed above a certain threshold to induce the latter. This study successfully investigated the different toxicological effects exerted by an anthropogenic SOA deriving from condensed soot particle-bound naphthalene as well as a biogenic SOA generated from condensed soot particle-bound β-pinene. Cytotoxicity, inflammation, oxidative stress as well as primary and secondary genotoxicity were examined in a co-culture comprising alveolar epithelial and endothelial cells, enabling cross-talk between the distinct cell types. In general, $SOA_{NAP\text{-}SP}$ induced stronger effects compared to $SOA_{\beta PIN\text{-}SP}$, which might be related to the pronounced levels of aromatic

compounds, higher oxidation status and higher prevalence of ROS. As in the studies before, also in this study, secondary DNA damage was detected and there are indications for the induction of pro-fibrotic pathways. Nevertheless, longer or repeated exposures are required to, firstly, determine whether the altered inflammatory and oxidative stress signaling as well as genotoxicity become persistent and result in mutagenic or fibrotic conditions and to, secondly, understand how endothelial cells are affected and contribute to the formation of such adverse outcomes.

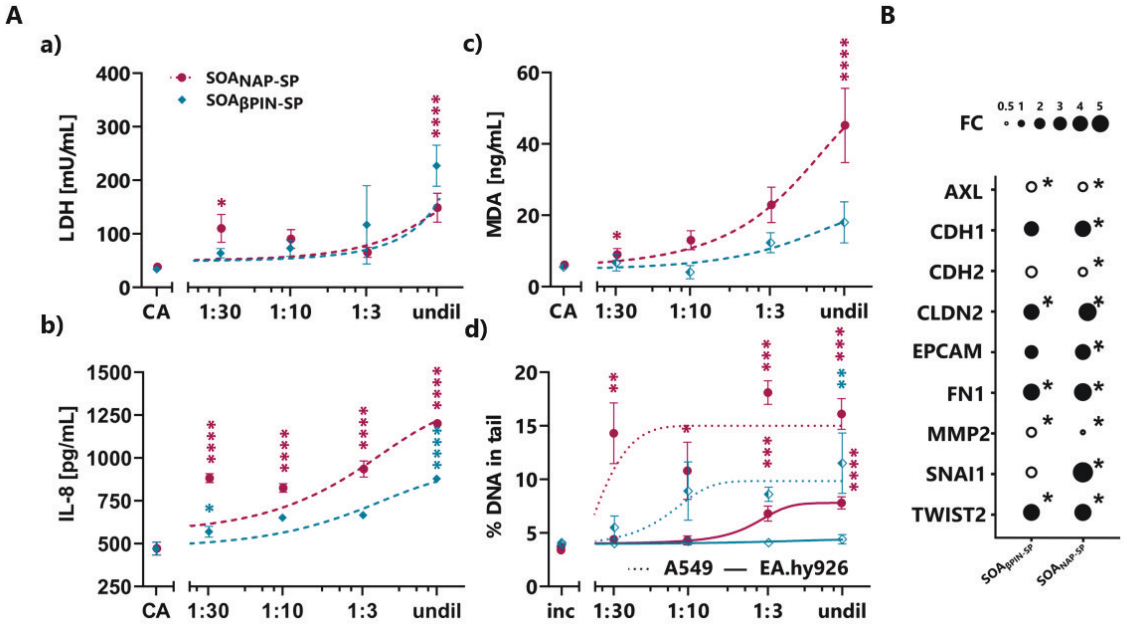


Figure 25: Toxicological assessment. A) The LDH release was similarly increased after 4 h exposure of co-cultured epithelial (A549) and endothelial (EA.hy926) cells exposed to $SOA_{\beta PIN\text{-}SP}$ and $SOA_{NAP\text{-}SP}$ (a). The inflammatory marker IL-8 (b) and the oxidative marker MDA (c) on the other hand, were more strongly induced by exposure to the "anthropogenic" $SOA_{NAP\text{-}SP}$. Primary genotoxicity was increased through both aerosols, nevertheless to a greater extent by $SOA_{NAP\text{-}SP}$, which additionally lead to increased secondary genotoxicity in endothelial cells. B) Gene expression analysis indicates a regulation of pro-fibrotic markers, even though, once more, to a higher degree by $SOA_{NAP\text{-}SP}$.

6. Conclusion & Outlook

This work presents the successful development of different multicellular *in vitro* test systems, all constituted of competent cell types, which are commonly known to be involved in pulmonary diseases such as fibrosis. The applied co-culturing technique allowed the investigation of multiple cell types, in compliance with the 3Rs, exposed to various air pollutants like (thermally treated) fiber-containing construction materials, MWCNTs as well as secondary organic aerosols derived from soot particles coated by naphthalene or β-pinene. Nevertheless, cell-cell communication was constantly maintained due to the spatial proximity of epithelial cells, macrophages, fibroblasts or endothelial cells. It was comprehensively demonstrated that the latter were strongly affected by the different aerosol exposures and exhibited adverse conditions, which are associated with the formation of fibrosis. The findings highlight a strong interconnection of fibrosis, inflammation and genotoxicity, especially in the cell model containing immunocompetent cells, which are of great importance for inflammatory signaling. Interestingly, our investigations revealed at all times the involvement of indirect secondary DNA damage, which is usually not considered as a key event in fibrosis-specific AOP design as presented in **Figure 4**. Nevertheless, this genetic alteration could represent, according to our studies, a common denominator of several key events such as loss of membrane integrity, EMT and EndMT, or fibroblast function. The implementation of secondary genotoxicity could help to derive AOPs of increased accuracy or AOP networks as presented in **Figure 26**, allowing for more precise and effective tiered toxicity testing. In addition to that, an advanced and highly sensitive method for the assessment of intracellular glutathione levels of multicellular test systems was established. This LC-MS/MS technique enables a more detailed understanding of mechanisms of toxicity induced by environmental pollutants It involves not only the detection of redox cycling compounds participating in the antioxidative defense of the cell, which could be altered by RS signaling and is known to be strongly involved in secondary modes-of-action. Furthermore, the method could also unravel xenobiotic conjugation mechanisms.

Future studies could increase their relevance, quality and operating range by using multicellular systems at the ALI for repeated or long-term exposures with physiologically relevant cell lines, treated at exposure-relevant concentrations, depending on the hypothesis or aim. A harmonization of cell culture protocols between different laboratories could lead to a higher reproducibility and acceptance of *in vitro* models as replacement for animal experimentation. Moreover, such methods may find application in the development of integrated approaches for testing and assessment (IATAs), which consider information on chemical properties, exposure scenarios, and the relationships of the latter with the formation of adverse outcomes, by compiling data gained from *in vivo*, *in silico*, *in vitro* as well as *in chemico* studies. This procedure is more time- and cost-effective compared to traditional toxicity testing and could lead to the further reduction of animal testing [217, 218].

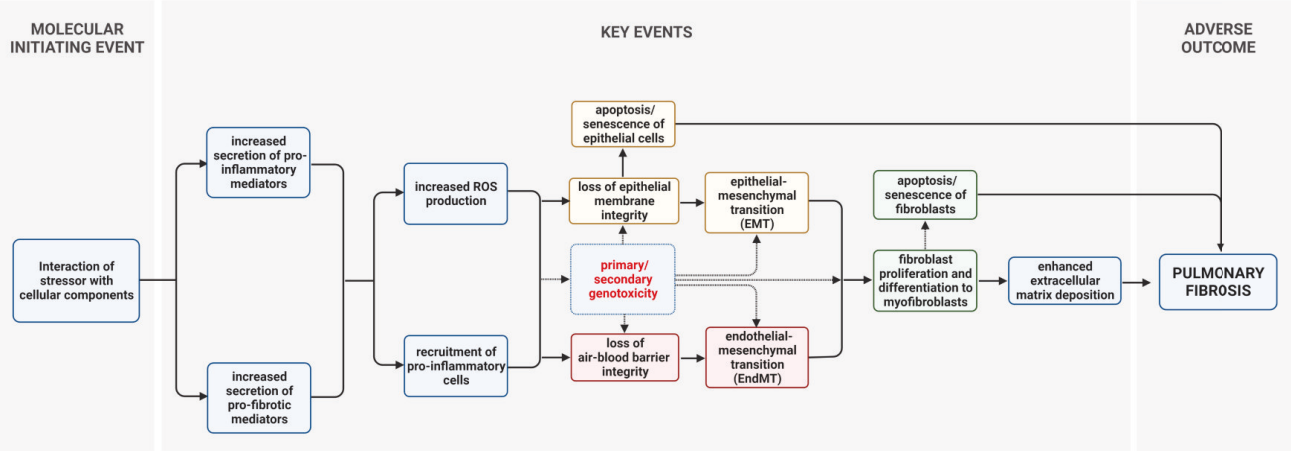


Figure 26: Putative adverse outcome pathway (AOP) network of pulmonary fibrosis development. Following chemical or particle exposure, the stressor interacts with cellular components as initiating event, which activates the secretion of pro-inflammatory and/or pro-fibrotic mediators, leading to the recruitment of inflammatory cells and a potential increase in ROS production, which could also be induced by the stressors characteristics itself. Those key events can lead to primary and/or secondary genotoxicity, which can be, but not necessarily alone, responsible for the loss of epithelial membrane and air-blood barrier integrity, possibly leading to epithelial (EMT) or endothelial (EndMT) mesenchymal transition. Fibroblast function and differentiation into myofibroblasts might be altered directly by genotoxicity or through E(nd)MT, which results in increased, unresolved extracellular matrix deposition and the formation of pulmonary fibrosis. The adverse outcome could also be induced by apoptosis or senescence of involved cell types and their insufficient clearance. Blue: events influencing all cell types; red: events affecting the vascular system; green: events influencing fibroblast homeostasis.

References

1. Almetwally, A.A., M. Bin-Jumah, and A.A. Allam, *Ambient air pollution and its influence on human health and welfare: an overview.* Environ Sci Pollut Res Int, 2020. **27**(20): p. 24815-24830.
2. Manisalidis, I., et al., *Environmental and Health Impacts of Air Pollution: A Review.* Front Public Health, 2020. **8**: p. 14.
3. Turner, M.C., et al., *Outdoor air pollution and cancer: An overview of the current evidence and public health recommendations.* CA Cancer J Clin, 2020.
4. World Health Organization, *WHO global air quality guidelines. Particulate matter ($PM_{2.5}$ and PM_{10}), ozone, nitrogen dioxide, sulfur dioxide and carbon monoxide.* 2021.
5. Pai, S.J., et al., *Updated World Health Organization Air Quality Guidelines Highlight the Importance of Non-anthropogenic PM2.5.* Environmental Science & Technology Letters, 2022. **9**(6): p. 501-506.
6. Osipov, S., et al., *Severe atmospheric pollution in the Middle East is attributable to anthropogenic sources.* Communications Earth & Environment, 2022. **3**(1): p. 203.
7. Daellenbach, K.R., et al., *Sources of particulate-matter air pollution and its oxidative potential in Europe.* Nature, 2020. **587**(7834): p. 414-419.
8. Liu, X., et al., *Levels and drivers of urban black carbon and health risk assessment during pre- and COVID19 lockdown in Augsburg, Germany.* Environmental Pollution, 2023. **316**: p. 120529.
9. Kelly, F.J. and J.C. Fussell, *Toxicity of airborne particles-established evidence, knowledge gaps and emerging areas of importance.* Philos Trans A Math Phys Eng Sci, 2020. **378**(2183): p. 20190322.
10. Longhin, E.M., P. Mantecca, and M. Gualtieri, *Fifteen Years of Airborne Particulates in Vitro Toxicology in Milano: Lessons and Perspectives Learned.* Int J Mol Sci, 2020. **21**(7).
11. Nymark, P., et al., *Toward Rigorous Materials Production: New Approach Methodologies Have Extensive Potential to Improve Current Safety Assessment Practices.* Small, 2020. **16**(6): p. e1904749.
12. Isigonis, P., et al., *Risk Governance of Emerging Technologies Demonstrated in Terms of its Applicability to Nanomaterials.* Small, 2020. **16**(36): p. e2003303.
13. Williams, E.S., J. Panko, and D.J. Paustenbach, *The European Union's REACH regulation: a review of its history and requirements.* Crit Rev Toxicol, 2009. **39**(7): p. 553-75.
14. Olsson, A., et al., *The 3Rs Principle–Mind the Ethical Gap!* 2012.
15. Zavala, J., et al., *New Approach Methods to Evaluate Health Risks of Air Pollutants: Critical Design Considerations for In Vitro Exposure Testing.* Int J Environ Res Public Health, 2020. **17**(6).
16. Clippinger, A.J., et al., *Expert consensus on an in vitro approach to assess pulmonary fibrogenic potential of aerosolized nanomaterials.* Arch Toxicol, 2016. **90**(7): p. 1769-83.
17. Pantzke, J., et al., *An alternative in vitro model considering cell-cell interactions in fiber-induced pulmonary fibrosis.* Toxicology Mechanisms and Methods, 2022: p. 1-16.
18. Schraufnagel, D.E., et al., *Air Pollution and Noncommunicable Diseases: A Review by the Forum of International Respiratory Societies' Environmental Committee, Part 2: Air Pollution and Organ Systems.* Chest, 2019. **155**(2): p. 417-426.
19. Oberdorster, G., et al., *Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy.* Part Fibre Toxicol, 2005. **2**: p. 8.
20. Diegelmann, R.F. and M.C. Evans, *Wound healing: an overview of acute, fibrotic and delayed healing.* Front Biosci, 2004. **9**: p. 283-9.
21. Talbott, H.E., et al., *Wound healing, fibroblast heterogeneity, and fibrosis.* Cell Stem Cell, 2022. **29**(8): p. 1161-1180.

22. Lederer, D.J. and F.J. Martinez, *Idiopathic Pulmonary Fibrosis.* N Engl J Med, 2018. **378**(19): p. 1811-1823.
23. Phan, T.H.G., et al., *Emerging cellular and molecular determinants of idiopathic pulmonary fibrosis.* Cell Mol Life Sci, 2021. **78**(5): p. 2031-2057.
24. Maher, T.M. and M.E. Strek, *Antifibrotic therapy for idiopathic pulmonary fibrosis: time to treat.* Respir Res, 2019. **20**(1): p. 205.
25. Raghu, G., et al., *Idiopathic pulmonary fibrosis in US Medicare beneficiaries aged 65 years and older: incidence, prevalence, and survival, 2001–11.* The Lancet Respiratory Medicine, 2014. **2**(7): p. 566-572.
26. Moss, B.J., S.W. Ryter, and I.O. Rosas, *Pathogenic Mechanisms Underlying Idiopathic Pulmonary Fibrosis.* Annu Rev Pathol, 2022. **17**: p. 515-546.
27. Sakai, N. and A.M. Tager, *Fibrosis of two: Epithelial cell-fibroblast interactions in pulmonary fibrosis.* Biochim Biophys Acta, 2013. **1832**(7): p. 911-21.
28. Sun, X., et al., *Endothelium-mediated contributions to fibrosis.* Semin Cell Dev Biol, 2020. **101**: p. 78-86.
29. Plante-Bordeneuve, T., C. Pilette, and A. Froidure, *The Epithelial-Immune Crosstalk in Pulmonary Fibrosis.* Front Immunol, 2021. **12**: p. 631235.
30. King, T.E., Jr., A. Pardo, and M. Selman, *Idiopathic pulmonary fibrosis.* Lancet, 2011. **378**(9807): p. 1949-61.
31. Cheng, P., S. Li, and H. Chen, *Macrophages in Lung Injury, Repair, and Fibrosis.* Cells, 2021. **10**(2).
32. Liu, L., et al., *Epithelial-mesenchymal transition in organ fibrosis development: current understanding and treatment strategies.* Burns Trauma, 2022. **10**: p. tkac011.
33. Tai, Y., et al., *Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis.* Biomolecules, 2021. **11**(8).
34. Kendall, R. and C. Feghali-Bostwick, *Fibroblasts in fibrosis: Novel roles and mediators.* Frontiers in pharmacology, 2014. **5**: p. 123.
35. El Ayadi, A., J.W. Jay, and A. Prasai, *Current Approaches Targeting the Wound Healing Phases to Attenuate Fibrosis and Scarring.* Int J Mol Sci, 2020. **21**(3).
36. Di Ianni, E., et al., *In vitro-in vivo correlations of pulmonary inflammogenicity and genotoxicity of MWCNT.* Part Fibre Toxicol, 2021. **18**(1): p. 25.
37. Akerlund, E., et al., *Inflammation and (secondary) genotoxicity of Ni and NiO nanoparticles.* Nanotoxicology, 2019. **13**(8): p. 1060-1072.
38. Moller, P., et al., *Oxidative stress and inflammation generated DNA damage by exposure to air pollution particles.* Mutat Res Rev Mutat Res, 2014. **762**: p. 133-66.
39. Ihantola, T., et al., *Genotoxic and inflammatory effects of spruce and brown coal briquettes combustion aerosols on lung cells at the air-liquid interface.* Sci Total Environ, 2022. **806**(Pt 1): p. 150489.
40. Knaapen, A.M., et al., *Inhaled particles and lung cancer. Part A: Mechanisms.* Int J Cancer, 2004. **109**(6): p. 799-809.
41. Donaldson, K., C.A. Poland, and R.P. Schins, *Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies.* Nanotoxicology, 2010. **4**: p. 414-20.
42. Risom, L., P. Moller, and S. Loft, *Oxidative stress-induced DNA damage by particulate air pollution.* Mutat Res, 2005. **592**(1-2): p. 119-37.
43. Leni, Z., L. Künzi, and M. Geiser, *Air pollution causing oxidative stress.* Current Opinion in Toxicology, 2020. **20-21**: p. 1-8.
44. Moorthy, B., C. Chu, and D.J. Carlin, *Polycyclic aromatic hydrocarbons: from metabolism to lung cancer.* Toxicol Sci, 2015. **145**(1): p. 5-15.
45. Peluso, M., et al., *Analysis of 13 (32)P-DNA postlabeling studies on occupational cohorts exposed to air pollution.* Am J Epidemiol, 2001. **153**(6): p. 546-58.

46. Stone, V., et al., *Nanomaterials Versus Ambient Ultrafine Particles: An Opportunity to Exchange Toxicology Knowledge.* Environ Health Perspect, 2017. **125**(10): p. 106002.
47. Schins, R.P. and A.M. Knaapen, *Genotoxicity of poorly soluble particles.* Inhal Toxicol, 2007. **19 Suppl 1**: p. 189-98.
48. Geiser, M., et al., *Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells.* Environ Health Perspect, 2005. **113**(11): p. 1555-60.
49. Pardo, M., et al., *Particulate Matter Toxicity Is Nrf2 and Mitochondria Dependent: The Roles of Metals and Polycyclic Aromatic Hydrocarbons.* Chem Res Toxicol, 2020. **33**(5): p. 1110-1120.
50. Jin, X., et al., *Mitochondrial damage mediated by ROS incurs bronchial epithelial cell apoptosis upon ambient PM(2.5) exposure.* J Toxicol Sci, 2018. **43**(2): p. 101-111.
51. Panieri, E. and M.M. Santoro, *ROS homeostasis and metabolism: a dangerous liason in cancer cells.* Cell Death Dis, 2016. **7**(6): p. e2253.
52. Santibanez-Andrade, M., et al., *Particulate matter (PM(10)) destabilizes mitotic spindle through downregulation of SETD2 in A549 lung cancer cells.* Chemosphere, 2022. **295**: p. 133900.
53. Siegrist, K.J., et al., *Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses.* Part Fibre Toxicol, 2014. **11**: p. 6.
54. Gualtieri, M., et al., *Airborne urban particles (Milan winter-PM2.5) cause mitotic arrest and cell death: Effects on DNA, mitochondria, AhR binding and spindle organization.* Mutat Res, 2011. **713**(1-2): p. 18-31.
55. Rao, X., et al., *Effect of Particulate Matter Air Pollution on Cardiovascular Oxidative Stress Pathways.* Antioxid Redox Signal, 2018. **28**(9): p. 797-818.
56. Breton, C.V., et al., *Genetic variation in the glutathione synthesis pathway, air pollution, and children's lung function growth.* Am J Respir Crit Care Med, 2011. **183**(2): p. 243-8.
57. Hatzis, C., et al., *Ambient particulate matter exhibits direct inhibitory effects on oxidative stress enzymes.* Environ Sci Technol, 2006. **40**(8): p. 2805-11.
58. Borm, P.J., L. Tran, and K. Donaldson, *The carcinogenic action of crystalline silica: a review of the evidence supporting secondary inflammation-driven genotoxicity as a principal mechanism.* Crit Rev Toxicol, 2011. **41**(9): p. 756-70.
59. Chatterjee, N. and G.C. Walker, *Mechanisms of DNA damage, repair, and mutagenesis.* Environ Mol Mutagen, 2017. **58**(5): p. 235-263.
60. Shukla, R.K., et al., *Genotoxic Potential of Nanoparticles: Structural and Functional Modifications in DNA.* Front Genet, 2021. **12**: p. 728250.
61. Carriere, M., et al., *Impact of nanoparticles on DNA repair processes: current knowledge and working hypotheses.* Mutagenesis, 2017. **32**(1): p. 203-213.
62. Bindu, S., et al., *SIRT3 blocks myofibroblast differentiation and pulmonary fibrosis by preventing mitochondrial DNA damage.* Am J Physiol Lung Cell Mol Physiol, 2017. **312**(1): p. L68-L78.
63. Halappanavar, S., et al., *Adverse outcome pathways as a tool for the design of testing strategies to support the safety assessment of emerging advanced materials at the nanoscale.* Part Fibre Toxicol, 2020. **17**(1): p. 16.
64. Gerloff, K., et al., *The Adverse Outcome Pathway approach in nanotoxicology.* Computational Toxicology, 2017. **1**: p. 3-11.
65. OECD, *Guidance Document on Developing and Assessing Adverse Outcome Pathways*, in *ENV/JM/MONO(2013)6*, O.f.E.C.-o.a. Development, Editor. 2013.
66. Allen, T.E., et al., *Defining molecular initiating events in the adverse outcome pathway framework for risk assessment.* Chem Res Toxicol, 2014. **27**(12): p. 2100-12.
67. Nymark, P., et al., *Adverse Outcome Pathway Development for Assessment of Lung Carcinogenicity by Nanoparticles.* Front Toxicol, 2021. **3**: p. 653386.

68. Halappanavar, S., et al., *Non-Animal Strategies for Toxicity Assessment of Nanoscale Materials: Role of Adverse Outcome Pathways in the Selection of Endpoints.* Small, 2021. **17**(15): p. e2007628.
69. Jin, Y., et al., *High throughput data-based, toxicity pathway-oriented development of a quantitative adverse outcome pathway network linking AHR activation to lung damages.* J Hazard Mater, 2022. **425**: p. 128041.
70. Wohak, L.E., et al., *Carcinogenic polycyclic aromatic hydrocarbons induce CYP1A1 in human cells via a p53-dependent mechanism.* Arch Toxicol, 2016. **90**(2): p. 291-304.
71. Shi, Y., et al., *The aryl hydrocarbon receptor: An environmental effector in the pathogenesis of fibrosis.* Pharmacol Res, 2020. **160**: p. 105180.
72. Zhang, L., et al., *The Role of Human Aldo-Keto Reductases in the Metabolic Activation and Detoxication of Polycyclic Aromatic Hydrocarbons: Interconversion of PAH Catechols and PAH o-Quinones.* Front Pharmacol, 2012. **3**: p. 193.
73. Clippinger, A.J., et al., *Expert consensus on an in vitro approach to assess pulmonary fibrogenic potential of aerosolized nanomaterials.* Archives of Toxicology, 2016. **90**(7): p. 1769-1783.
74. Mowat, V., D.J. Alexander, and A.M. Pilling, *A Comparison of Rodent and Nonrodent Laryngeal and Tracheal Bifurcation Sensitivities in Inhalation Toxicity Studies and Their Relevance for Human Exposure.* Toxicol Pathol, 2017. **45**(1): p. 216-222.
75. Lacroix, G., et al., *Air-Liquid Interface In Vitro Models for Respiratory Toxicology Research: Consensus Workshop and Recommendations.* Appl In Vitro Toxicol, 2018. **4**(2): p. 91-106.
76. Pantzke, J., et al., *An alternative in vitro model considering cell-cell interactions in fiber-induced pulmonary fibrosis.* Toxicology Mechanisms and Methods, 2022(1537-6524 (Electronic)).
77. Oeder, S., et al., *Particulate Matter from Both Heavy Fuel Oil and Diesel Fuel Shipping Emissions Show Strong Biological Effects on Human Lung Cells at Realistic and Comparable In Vitro Exposure Conditions.* PLOS ONE, 2015. **10**(6): p. e0126536.
78. Kasurinen, S., et al., *Differences between co-cultures and monocultures in testing the toxicity of particulate matter derived from log wood and pellet combustion.* PLOS ONE, 2018. **13**(2): p. e0192453.
79. Hirota, J.A., et al., *PM10-stimulated airway epithelial cells activate primary human dendritic cells independent of uric acid: application of an in vitro model system exposing dendritic cells to airway epithelial cell-conditioned media.* Respirology, 2014. **19**(6): p. 881-90.
80. Bessa, M.J., et al., *In Vitro Toxicity of Industrially Relevant Engineered Nanoparticles in Human Alveolar Epithelial Cells: Air-Liquid Interface versus Submerged Cultures.* Nanomaterials (Basel), 2021. **11**(12).
81. Klein, S.G., et al., *An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung.* Part Fibre Toxicol, 2013. **10**: p. 31.
82. Offer, S., et al., *Effect of Atmospheric Aging on Soot Particle Toxicity in Lung Cell Models at the Air-Liquid Interface: Differential Toxicological Impacts of Biogenic and Anthropogenic Secondary Organic Aerosols (SOAs).* Environ Health Perspect, 2022. **130**(2): p. 27003.
83. Kunzi, L., et al., *Toxicity of aged gasoline exhaust particles to normal and diseased airway epithelia.* Sci Rep, 2015. **5**: p. 11801.
84. Pasanen, K., et al., *Mortality among population with exposure to industrial air pollution containing nickel and other toxic metals.* J Occup Environ Med, 2012. **54**(5): p. 583-91.
85. Ihantola, T., et al., *Influence of wood species on toxicity of log-wood stove combustion aerosols: a parallel animal and air-liquid interface cell exposure study on spruce and pine smoke.* Part Fibre Toxicol, 2020. **17**(1): p. 27.
86. Thangavel, P., D. Park, and Y.C. Lee, *Recent Insights into Particulate Matter (PM(2.5))-Mediated Toxicity in Humans: An Overview.* Int J Environ Res Public Health, 2022. **19**(12).

87. Park, M., et al., *Differential toxicities of fine particulate matters from various sources.* Sci Rep, 2018. **8**(1): p. 17007.
88. Viana, M., et al., *Characterizing the Chemical Profile of Incidental Ultrafine Particles for Toxicity Assessment Using an Aerosol Concentrator.* Ann Work Expo Health, 2021. **65**(8): p. 966-978.
89. Moreno-Ríos, A.L., L.P. Tejeda-Benítez, and C.F. Bustillo-Lecompte, *Sources, characteristics, toxicity, and control of ultrafine particles: An overview.* Geoscience Frontiers, 2022. **13**(1): p. 101147.
90. Pardo, M., et al., *Exposure to naphthalene and beta-pinene-derived secondary organic aerosol induced divergent changes in transcript levels of BEAS-2B cells.* Environ Int, 2022. **166**: p. 107366.
91. Chen, C. and B. Zhao, *Review of relationship between indoor and outdoor particles: I/O ratio, infiltration factor and penetration factor.* Atmospheric Environment, 2011. **45**(2): p. 275-288.
92. Leung, D.Y.C., *Outdoor-indoor air pollution in urban environment: challenges and opportunity.* Frontiers in Environmental Science, 2015. **2**.
93. Di Bucchianico, S., et al., *Genotoxicity of TiO2 nanoparticles assessed by mini-gel comet assay and micronucleus scoring with flow cytometry.* Mutagenesis, 2017. **32**(1): p. 127-137.
94. Jeevanandam, J., et al., *Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations.* Beilstein J Nanotechnol, 2018. **9**: p. 1050-1074.
95. Donaldson, K., et al., *Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences.* Adv Drug Deliv Rev, 2013. **65**(15): p. 2078-86.
96. Felley-Bosco, E. and M. MacFarlane, *Asbestos: Modern Insights for Toxicology in the Era of Engineered Nanomaterials.* Chemical Research in Toxicology, 2018. **31**(10): p. 994-1008.
97. EPA, U.S., *Risk Evaluation for Asbestos, Part I: Chrysotile Asbestos*, O.o.C.S.a.P. Prevention, Editor. 2020, U.S. Environmental Protection Agency: Washington, DC.
98. Bergamaschi, E., et al. *Occupational Exposure to Carbon Nanotubes and Carbon Nanofibres: More Than a Cobweb*. Nanomaterials, 2021. **11**, DOI: 10.3390/nano11030745.
99. Bienkowski, N., et al., *Bearbeitung von Carbonbeton - eine bauverfahrenstechnische und medizinische Betrachtung.* Bauingeneur, 2017. **2017/2018**(10): p. 110-117.
100. Koch, A., et al., *Investigation of Chemical Composition and Fiber-Occurrence in Inhalable Particulate Matter Obtained from Dry Cutting Processes of Carbon Fiber Reinforced Concrete Composite, Concrete and the Carbon Fiber Reinforcement Materials.* Aerosol Science and Engineering, 2021. **5**(3): p. 292-306.
101. Brøndsted, P., H. Lilholt, and A. Lystrup, *COMPOSITE MATERIALS FOR WIND POWER TURBINE BLADES.* Annual Review of Materials Research, 2005. **35**(1): p. 505-538.
102. Worle-Knirsch, J.M., K. Pulskamp, and H.F. Krug, *Oops they did it again! Carbon nanotubes hoax scientists in viability assays.* Nano Lett, 2006. **6**(6): p. 1261-8.
103. Guadagnini, R., et al., *Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests.* Nanotoxicology, 2015. **9 Suppl 1**: p. 13-24.
104. Dusinska, M., et al., *Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST.* Nanotoxicology, 2015. **9 Suppl 1**: p. 118-32.
105. Longhin, E.M., et al., *The alamar blue assay in the context of safety testing of nanomaterials.* Front Toxicol, 2022. **4**: p. 981701.
106. Kumar, P., A. Nagarajan, and P.D. Uchil, *Analysis of Cell Viability by the Lactate Dehydrogenase Assay.* Cold Spring Harbor Protocols, 2018. **2018**(6): p. pdb.prot095497.
107. Rotoli, B.M., et al., *Non-functionalized multi-walled carbon nanotubes alter the paracellular permeability of human airway epithelial cells.* Toxicol Lett, 2008. **178**(2): p. 95-102.
108. Strober, W., *Trypan blue exclusion test of cell viability.* Curr Protoc Immunol, 2001. **Appendix 3**: p. Appendix 3B.

109. Stoccoro, A., et al., *A panel of in vitro tests to evaluate genotoxic and morphological neoplastic transformation potential on Balb/3T3 cells by pristine and remediated titania and zirconia nanoparticles.* Mutagenesis, 2016. **31**(5): p. 511-29.
110. Parak, W.J., et al., *Cell Motility and Metastatic Potential Studies Based on Quantum Dot Imaging of Phagokinetic Tracks.* Advanced Materials, 2002. **14**(12): p. 882-885.
111. Kang, Y., et al., *Quantitative considerations about the size dependence of cellular entry and excretion of colloidal nanoparticles for different cell types.* ChemTexts, 2022. **8**(1): p. 9.
112. DeForge, L.E. and D.G. Remick, *Sandwich ELISA for detection of picogram quantities of interleukin-8.* Immunol Invest, 1991. **20**(1): p. 89-97.
113. Tsikas, D., *Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges.* Anal Biochem, 2017. **524**: p. 13-30.
114. Wood, L.G., P.G. Gibson, and M.L. Garg, *Biomarkers of lipid peroxidation, airway inflammation and asthma.* Eur Respir J, 2003. **21**(1): p. 177-86.
115. El-Beltagi, H.S. and H.I. Mohamed, *Reactive Oxygen Species, Lipid Peroxidation and Antioxidative Defense Mechanism.* Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 2013. **41**(1): p. 44-57.
116. Wu, X., et al., *Determination of air pollution-related biomarkers of exposure in urine of travellers between Germany and China using liquid chromatographic and liquid chromatographic-mass spectrometric methods: a pilot study.* Biomarkers, 2017. **22**(6): p. 525-536.
117. Karlsson, H.L., et al., *Can the comet assay be used reliably to detect nanoparticle-induced genotoxicity?* Environ Mol Mutagen, 2015. **56**(2): p. 82-96.
118. Buchwalow, I.B., E.A. Minin, and W. Boecker, *A multicolor fluorescence immunostaining technique for simultaneous antigen targeting.* Acta Histochem, 2005. **107**(2): p. 143-8.
119. Govindarajan, R., et al., *Microarray and its applications.* J Pharm Bioallied Sci, 2012. **4**(Suppl 2): p. S310-2.
120. Butte, A., *The use and analysis of microarray data.* Nat Rev Drug Discov, 2002. **1**(12): p. 951-60.
121. Mantione, K.J., et al., *Comparing bioinformatic gene expression profiling methods: microarray and RNA-Seq.* Med Sci Monit Basic Res, 2014. **20**: p. 138-42.
122. The Gene Ontology Consortium, *The Gene Ontology resource: enriching a GOld mine.* Nucleic Acids Research, 2021. **49**(D1): p. D325-D334.
123. Werner, T., *Bioinformatics applications for pathway analysis of microarray data.* Curr Opin Biotechnol, 2008. **19**(1): p. 50-4.
124. Gaucher, C., et al., *Glutathione: Antioxidant Properties Dedicated to Nanotechnologies.* Antioxidants (Basel), 2018. **7**(5).
125. Rahman, I. and W. MacNee, *Oxidative stress and regulation of glutathione in lung inflammation.* Eur Respir J, 2000. **16**(3): p. 534-54.
126. Sun, X., et al., *Optimized Protocol for the In Situ Derivatization of Glutathione with N-Ethylmaleimide in Cultured Cells and the Simultaneous Determination of Glutathione/Glutathione Disulfide Ratio by HPLC-UV-QTOF-MS.* Metabolites, 2020. **10**(7).
127. Herzog, K., et al., *An UPLC-MS/MS Assay to Measure Glutathione as Marker for Oxidative Stress in Cultured Cells.* Metabolites, 2019. **9**(3).
128. Sheppard, D., *Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis.* Proc Am Thorac Soc, 2006. **3**(5): p. 413-7.
129. Morishima, Y., et al., *Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding.* Am J Respir Cell Mol Biol, 2001. **24**(1): p. 1-11.
130. Xu, Y.D., et al., *Release of biologically active TGF-beta1 by alveolar epithelial cells results in pulmonary fibrosis.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(3): p. L527-39.

131. Yang, J., et al., *Activated alveolar epithelial cells initiate fibrosis through autocrine and paracrine secretion of connective tissue growth factor.* Am J Physiol Lung Cell Mol Physiol, 2014. **306**(8): p. L786-96.
132. Moore, B.B., et al., *Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE2.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(2): p. L342-9.
133. Elwakeel, E., B. Brune, and A. Weigert, *PGE(2) in fibrosis and cancer: Insights into fibroblast activation.* Prostaglandins Other Lipid Mediat, 2019. **143**: p. 106339.
134. Tan, Q., et al., *Nascent Lung Organoids Reveal Epithelium- and Bone Morphogenetic Protein-mediated Suppression of Fibroblast Activation.* Am J Respir Cell Mol Biol, 2019. **61**(5): p. 607-619.
135. Selman, M. and A. Pardo, *Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder.* Respir Res, 2002. **3**(1): p. 3.
136. Andreescu, S., O.A. Sadik, and D.W. McGee, *Effect of natural and synthetic estrogens on a549 lung cancer cells: correlation of chemical structures with cytotoxic effects.* Chem Res Toxicol, 2005. **18**(3): p. 466-74.
137. Ramos, C., et al., *Cytotoxic and genotoxic effects of environmental relevant concentrations of bisphenol A and interactions with doxorubicin.* Mutat Res Genet Toxicol Environ Mutagen, 2019. **838**: p. 28-36.
138. Rahmatinia, T., et al., *Potential cytotoxicity of PM2.5-bound PAHs and toxic metals collected from areas with different traffic densities on human lung epithelial cells (A549).* J Environ Health Sci Eng, 2021. **19**(2): p. 1701-1712.
139. Zhou, Q., et al., *Toxicity and endocrine-disrupting potential of PM2.5: Association with particulate polycyclic aromatic hydrocarbons, phthalate esters, and heavy metals.* Environ Pollut, 2022. **292**(Pt A): p. 118349.
140. Wang, S., Y. Liang, and C. Dai, *Metabolic Regulation of Fibroblast Activation and Proliferation during Organ Fibrosis.* Kidney Dis (Basel), 2022. **8**(2): p. 115-125.
141. Song, H., et al., *Bisphenol A induces COX-2 through the mitogen-activated protein kinase pathway and is associated with levels of inflammation-related markers in elderly populations.* Environ Res, 2017. **158**: p. 490-498.
142. Song, H., et al., *Korean Red Ginseng suppresses bisphenol A-induced expression of cyclooxygenase-2 and cellular migration of A549 human lung cancer cell through inhibition of reactive oxygen species.* 2021(1226-8453 (Print)).
143. Guo, H., et al., *Low molecular weight-PAHs induced inflammation in A549 cells by activating PI3K/AKT and NF-kappaB signaling pathways.* Toxicol Res (Camb), 2021. **10**(1): p. 150-157.
144. Dilger, M., et al., *Toxicity of wood smoke particles in human A549 lung epithelial cells: the role of PAHs, soot and zinc.* Arch Toxicol, 2016. **90**(12): p. 3029-3044.
145. Rojas, G.A., et al., *Polycyclic Aromatic Hydrocarbons (PAHs) Exposure Triggers Inflammation and Endothelial Dysfunction in BALB/c Mice: A Pilot Study.* Toxics, 2022. **10**(9).
146. Oberdorster, G., J. Ferin, and B.E. Lehnert, *Correlation between particle size, in vivo particle persistence, and lung injury.* Environ Health Perspect, 1994. **102 Suppl 5**(Suppl 5): p. 173-9.
147. Schraufnagel, D.E., *The health effects of ultrafine particles.* Exp Mol Med, 2020. **52**(3): p. 311-317.
148. Borm, P.J.A., et al., *Inflammation as a Key Outcome Pathway in Particle Induced Effects in the Lung.* Front Public Health, 2022. **10**: p. 869041.
149. Quezada-Maldonado, E.M., et al., *Airborne particulate matter induces oxidative damage, DNA adduct formation and alterations in DNA repair pathways.* Environ Pollut, 2021. **287**: p. 117313.
150. Dopp, E. and D. Schiffmann, *Analysis of chromosomal alterations induced by asbestos and ceramic fibers.* Toxicol Lett, 1998. **96,97**: p. 155-162.

151. Khan, N.G., et al., *A comprehensive review on the carcinogenic potential of bisphenol A: clues and evidence.* Environ Sci Pollut Res Int, 2021. **28**(16): p. 19643-19663.
152. Armstrong, B.G. and G. Gibbs, *Exposure–response relationship between lung cancer and polycyclic aromatic hydrocarbons (PAHs).* Occupational and Environmental Medicine, 2009. **66**(11): p. 740.
153. Evans, S.J., et al., *In vitro detection of in vitro secondary mechanisms of genotoxicity induced by engineered nanomaterials.* Part Fibre Toxicol, 2019. **16**(1): p. 8.
154. Mailloux, R.J., *Mitochondrial Antioxidants and the Maintenance of Cellular Hydrogen Peroxide Levels.* Oxid Med Cell Longev, 2018. **2018**: p. 7857251.
155. Wan, H., et al., *Identification of Hub Genes and Pathways Associated With Idiopathic Pulmonary Fibrosis via Bioinformatics Analysis.* Front Mol Biosci, 2021. **8**: p. 711239.
156. Wiercinska, E., et al., *Id1 is a critical mediator in TGF-β–induced transdifferentiation of rat hepatic stellate cells* Hepatology, Liver Biology and Pathobiology, 2006. **43**(5): p. 1032-1041.
157. Suzuki, Y., J. Churg, and T. Ono, *Phagocytic activity of the alveolar epithelial cells in pulmonary asbestosis.* Am J Pathol, 1972. **69**(3): p. 373-88.
158. Jung, Y., et al., *Crosstalk between Plk1, p53, cell cycle, and G2/M DNA damage checkpoint regulation in cancer: computational modeling and analysis.* NPJ Syst Biol Appl, 2021. **7**(1): p. 46.
159. Su, T., et al., *Inhibition of Ganglioside Synthesis Suppressed Liver Cancer Cell Proliferation through Targeting Kinetochore Metaphase Signaling.* Metabolites, 2021. **11**(3).
160. Tsoutsou, P.G., et al., *Cytokine levels in the sera of patients with idiopathic pulmonary fibrosis.* Respir Med, 2006. **100**(5): p. 938-45.
161. Ziegenhagen, M.W., et al., *Serum level of interleukin 8 is elevated in idiopathic pulmonary fibrosis and indicates disease activity.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 762-8.
162. Uhal, B.D., et al., *Fibroblasts isolated after fibrotic lung injury induce apoptosis of alveolar epithelial cells in vitro.* Am J Physiol, 1995. **269**(6 Pt 1): p. L819-28.
163. Barbas-Filho, J.V., et al., *Evidence of type II pneumocyte apoptosis in the pathogenesis of idiopathic pulmonary fibrosis (IFP)/usual interstitial pneumonia (UIP).* J Clin Pathol, 2001. **54**(2): p. 132-8.
164. Barosova, H., et al., *An In Vitro Lung System to Assess the Proinflammatory Hazard of Carbon Nanotube Aerosols.* Int J Mol Sci, 2020. **21**(15).
165. Chortarea, S.Z., F.; Barosova, H.; Septiadi, D. , Clift, M. J.D.; Petri-Fink, A.; Rothen-Rutishauser, B., *Profibrotic Activity of Multiwalled Carbon Nanotubes Upon Prolonged Exposures in Different Human Lung Cell Types.* Applied In Vitro Toxicology, 2019. **5**(1): p. 47-61.
166. Mercer, R.R., et al., *Pulmonary fibrotic response to aspiration of multi-walled carbon nanotubes.* Part Fibre Toxicol, 2011. **8**: p. 21.
167. Mussar, K., et al., *Macrophage/epithelium cross-talk regulates cell cycle progression and migration in pancreatic progenitors.* PLoS One, 2014. **9**(2): p. e89492.
168. Fan, D.N.Y. and C.A. Schmitt, *Genotoxic Stress-Induced Senescence.* Methods Mol Biol, 2019. **1896**: p. 93-105.
169. Yanai, H., et al., *Cellular senescence-like features of lung fibroblasts derived from idiopathic pulmonary fibrosis patients.* Aging (Albany NY), 2015. **7**(9): p. 664-72.
170. Arish, N., D. Petukhov, and S.B. Wallach-Dayan, *The Role of Telomerase and Telomeres in Interstitial Lung Diseases: From Molecules to Clinical Implications.* Int J Mol Sci, 2019. **20**(12).
171. Parimon, T., M.S. Hohmann, and C. Yao, *Cellular Senescence: Pathogenic Mechanisms in Lung Fibrosis.* Int J Mol Sci, 2021. **22**(12).
172. Lin, Y. and Z. Xu, *Fibroblast Senescence in Idiopathic Pulmonary Fibrosis.* Frontiers in Cell and Developmental Biology, 2020. **8**.

173. Meindl, C., et al., *Screening for Effects of Inhaled Nanoparticles in Cell Culture Models for Prolonged Exposure.* Nanomaterials (Basel), 2021. **11**(3).
174. Wottrich, R., S. Diabate, and H.F. Krug, *Biological effects of ultrafine model particles in human macrophages and epithelial cells in mono- and co-culture.* Int J Hyg Environ Health, 2004. **207**(4): p. 353-61.
175. Kunkel, S.L., et al., *Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung.* Exp Lung Res, 1991. **17**(1): p. 17-23.
176. Thorley, A.J., et al., *Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages.* J Immunol, 2007. **178**(1): p. 463-73.
177. Pilette, C., et al., *Oxidative burst in lipopolysaccharide-activated human alveolar macrophages is inhibited by interleukin-9.* Eur Respir J, 2002. **20**(5): p. 1198-205.
178. Brechard, S., J.L. Bueb, and E.J. Tschirhart, *Interleukin-8 primes oxidative burst in neutrophil-like HL-60 through changes in cytosolic calcium.* Cell Calcium, 2005. **37**(6): p. 531-40.
179. Polimeni, M., et al., *Multi-walled carbon nanotubes directly induce epithelial-mesenchymal transition in human bronchial epithelial cells via the TGF-beta-mediated Akt/GSK-3beta/SNAIL-1 signalling pathway.* Part Fibre Toxicol, 2016. **13**(1): p. 27.
180. Yao, L., et al., *Paracrine signalling during ZEB1-mediated epithelial-mesenchymal transition augments local myofibroblast differentiation in lung fibrosis.* Cell Death Differ, 2019. **26**(5): p. 943-957.
181. He, X., et al., *Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF-kappaB signaling, and promoting fibroblast-to-myofibroblast transformation.* Chem Res Toxicol, 2011. **24**(12): p. 2237-48.
182. Saleh, Y., et al., *Exposure to Atmospheric Ultrafine Particles Induces Severe Lung Inflammatory Response and Tissue Remodeling in Mice.* Int J Environ Res Public Health, 2019. **16**(7).
183. Leikauf, G.D., S.H. Kim, and A.S. Jang, *Mechanisms of ultrafine particle-induced respiratory health effects.* Exp Mol Med, 2020. **52**(3): p. 329-337.
184. Kellner, M., et al., *ROS Signaling in the Pathogenesis of Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS).* Adv Exp Med Biol, 2017. **967**: p. 105-137.
185. Ghezzi, P., *Role of glutathione in immunity and inflammation in the lung.* Int J Gen Med, 2011. **4**: p. 105-13.
186. Decker, M., M. Arand, and A. Cronin, *Mammalian epoxide hydrolases in xenobiotic metabolism and signalling.* Arch Toxicol, 2009. **83**(4): p. 297-318.
187. Romero, D.L., et al., *Depletion of glutathione by benzo(a)pyrene metabolites, ionomycin, thapsigargin, and phorbol myristate in human peripheral blood mononuclear cells.* Toxicol Appl Pharmacol, 1997. **144**(1): p. 62-9.
188. Ayala, D.C., D. Morin, and A.R. Buckpitt, *Simultaneous quantification of multiple urinary naphthalene metabolites by liquid chromatography tandem mass spectrometry.* PLoS One, 2015. **10**(4): p. e0121937.
189. Ousji, O. and L. Sleno, *In Vitro Metabolism of Five Bisphenol A Analogs Studied by LC-HRMS/MS.* J Am Soc Mass Spectrom, 2021. **32**(4): p. 847-859.
190. Li, J., et al., *The production of reactive oxygen species enhanced with the reduction of menadione by active thioredoxin reductase.* Metallomics, 2019. **11**(9): p. 1490-1497.
191. Liu, X., et al., *Biochar Fine Particles Enhance Uptake of Benzo(a)pyrene to Macrophages and Epithelial Cells via Different Mechanisms.* Environmental Science & Technology Letters, 2021. **8**(3): p. 218-223.
192. Kato, S., et al., *Changes in pulmonary endothelial cell properties during bleomycin-induced pulmonary fibrosis.* Respir Res, 2018. **19**(1): p. 127.
193. Smadja, D.M., et al., *Imbalance of circulating endothelial cells and progenitors in idiopathic pulmonary fibrosis.* Angiogenesis, 2013. **16**(1): p. 147-57.

194. Martin, M., et al., *Role of endothelial cells in pulmonary fibrosis via SREBP2 activation.* JCI Insight, 2021. **6**(22).
195. Cohen, A.J., et al., *Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015.* Lancet, 2017. **389**(10082): p. 1907-1918.
196. Pope, C.A., 3rd, et al., *Fine particulate air pollution and human mortality: 25+ years of cohort studies.* Environ Res, 2020. **183**: p. 108924.
197. Demeautis, T., et al., *Pathogenic Mechanisms of Secondary Organic Aerosols.* Chem Res Toxicol, 2022. **35**(7): p. 1146-1161.
198. Xu, J., et al., *Photochemical impacts on the toxicity of PM2.5.* Critical Reviews in Environmental Science and Technology, 2022. **52**(1): p. 130-156.
199. Gaschen, A., et al., *Cellular Responses after Exposure of Lung Cell Cultures to Secondary Organic Aerosol Particles.* Environmental Science & Technology, 2010. **44**(4): p. 1424-1430.
200. Chowdhury, P.H., et al., *Exposure of Lung Epithelial Cells to Photochemically Aged Secondary Organic Aerosol Shows Increased Toxic Effects.* Environmental Science & Technology Letters, 2018. **5**(7): p. 424-430.
201. Buchholz, B.A., et al., *Naphthalene DNA Adduct Formation and Tolerance in the Lung.* Nucl Instrum Methods Phys Res B, 2019. **438**: p. 119-123.
202. Carratt, S.A., et al., *Naphthalene genotoxicity: DNA adducts in primate and mouse airway explants.* Toxicol Lett, 2019. **305**: p. 103-109.
203. Geys, J., et al., *In vitro study of the pulmonary translocation of nanoparticles: A preliminary study.* Toxicology Letters, 2006. **160**(3): p. 218-226.
204. Qian, W., et al., *Metastasis-associated protein 1 promotes epithelial-mesenchymal transition in idiopathic pulmonary fibrosis by up-regulating Snail expression.* J Cell Mol Med, 2020. **24**(11): p. 5998-6007.
205. Jayachandran, A., et al., *SNAI transcription factors mediate epithelial-mesenchymal transition in lung fibrosis.* Thorax, 2009. **64**(12): p. 1053-61.
206. Dong, W., et al., *Activation of TWIST Transcription by Chromatin Remodeling Protein BRG1 Contributes to Liver Fibrosis in Mice.* Front Cell Dev Biol, 2020. **8**: p. 340.
207. Chen, Y., et al., *YAP1/Twist promotes fibroblast activation and lung fibrosis that conferred by miR-15a loss in IPF.* Cell Death Differ, 2019. **26**(9): p. 1832-1844.
208. Huang, L., et al., *Functions of EpCAM in physiological processes and diseases (Review).* Int J Mol Med, 2018. **42**(4): p. 1771-1785.
209. Venugopal, S., S. Anwer, and K. Szaszi, *Claudin-2: Roles beyond Permeability Functions.* Int J Mol Sci, 2019. **20**(22).
210. Alsafadi, H.N., et al., *An ex vivo model to induce early fibrosis-like changes in human precision-cut lung slices.* Am J Physiol Lung Cell Mol Physiol, 2017. **312**(6): p. L896-L902.
211. Loh, C.Y., et al., *The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges.* Cells, 2019. **8**(10).
212. Kovacic, J.C., et al., *Endothelial to Mesenchymal Transition in Cardiovascular Disease: JACC State-of-the-Art Review.* J Am Coll Cardiol, 2019. **73**(2): p. 190-209.
213. Bersaas, A., et al., *Epithelial-mesenchymal transition and FOXA genes during tobacco smoke carcinogen induced transformation of human bronchial epithelial cells.* Toxicol In Vitro, 2016. **35**: p. 55-65.
214. Becker, K.F., et al., *Analysis of the E-cadherin repressor Snail in primary human cancers.* Cells Tissues Organs, 2007. **185**(1-3): p. 204-12.
215. Zhuo, X., et al., *Expression of TWIST, an inducer of epithelial-mesenchymal transition, in nasopharyngeal carcinoma and its clinical significance.* Int J Clin Exp Pathol, 2014. **7**(12): p. 8862-8.
216. Alexander, N.R., et al., *N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1.* Cancer Res, 2006. **66**(7): p. 3365-9.

217. Sakuratani, Y., M. Horie, and E. Leinala, *Integrated Approaches to Testing and Assessment: OECD Activities on the Development and Use of Adverse Outcome Pathways and Case Studies.* Basic Clin Pharmacol Toxicol, 2018. **123 Suppl 5**: p. 20-28.
218. Tollefsen, K.E., et al., *Applying Adverse Outcome Pathways (AOPs) to support Integrated Approaches to Testing and Assessment (IATA).* Regul Toxicol Pharmacol, 2014. **70**(3): p. 629-40.

Curriculum Vitae

Jana Pantzke

born February 19th, 1993 in Berlin

Employment history

July 2019 - present

Ph.D. student at Helmholtz Munich and University of Rostock

Education

October 2016 - December 2018

M.Sc. Toxicology, University of Potsdam

October 2012 - March 2016

B.Sc. Chemistry, Free University of Berlin

August 2005 - June 2012

General university entrance certificate (Abitur), Schadow-Gymnasium, Berlin

Conference participation

Oral presentation at the Lung In Vitro event 2022, Nice, France

An alternative *in vitro* model considering cell-cell interactions in fiber-induced pulmonary fibrosis