Poster #121:

A non-animal toolbox informed by pulmonary toxicity adverse outcome pathways (AOPs): a next-generation risk assessment (NGRA) approach for human inhalation safety

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Early Investigators Flash Presentations
(Theme: Human Health - Replacement)

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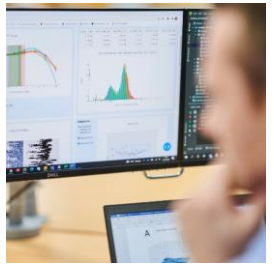

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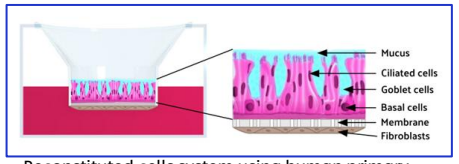
Human-relevant strategy for selecting NAMs for lung toxicity NGRA

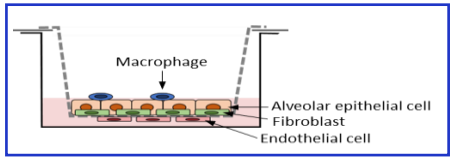
Broad coverage of bioactivity readouts relevant to inhalation hazards which can provide *in vitro* PoDs

Upper respiratory tract: MucilAir™-HF



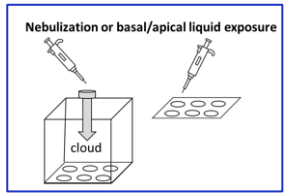
Reconstituted cells system using human primary bronchial cell cocultured with human airway fibroblast.

Lower respiratory tract: EpiAlveolar™



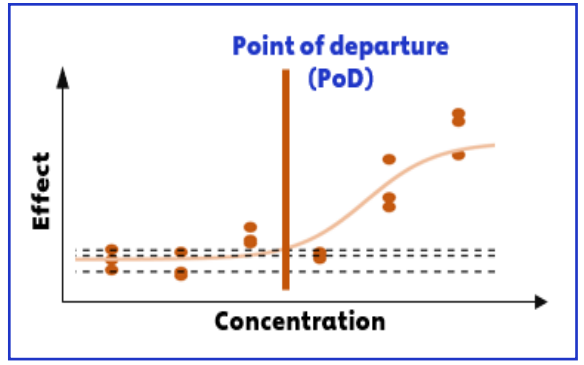
Primary human alveolar epithelial cells, pulmonary endothelial cells and monocyte-derived macrophages

12-day exposure scheme for both tissue models:

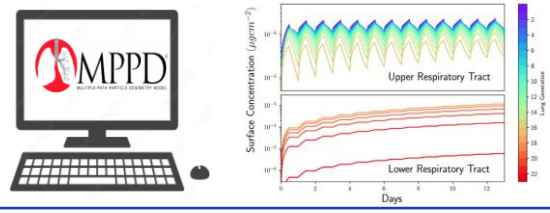


Bioactivity readouts investigated:

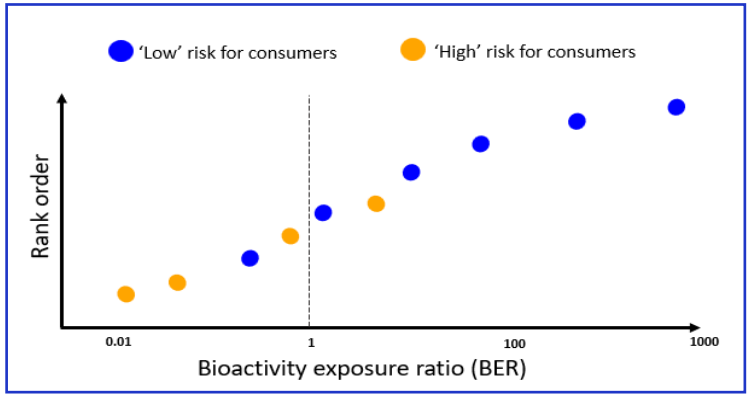
- Histology and immunohistochemistry analyses
- Tissue integrity loss (TEER)
- Tissue functionality: mucociliary clearance (MCC), cilia beating frequency (CBF) and mucin secretion
- Cytokine/chemokine secretion:
  - Inflammation response: CCL2, CCL7, CCL26, CXCL10, CXCL11, ICAM-1, IL-1α, osteopontin, IFN-γ, TNF-α, IL-6, and IL-8
  - degradation of extracellular matrix/fibrosis: MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1, uPAR, uPA, serpin E1, and TGF-β1
  - anti-inflammatory response: IL-1ra
- High-throughput transcriptomics analysis



Exposure data: inhalation particle deposition or literature plasma C_{max}



Bioactivity exposure ratio (BER):
the ratio between the *in vitro* PoD and predicted human exposure



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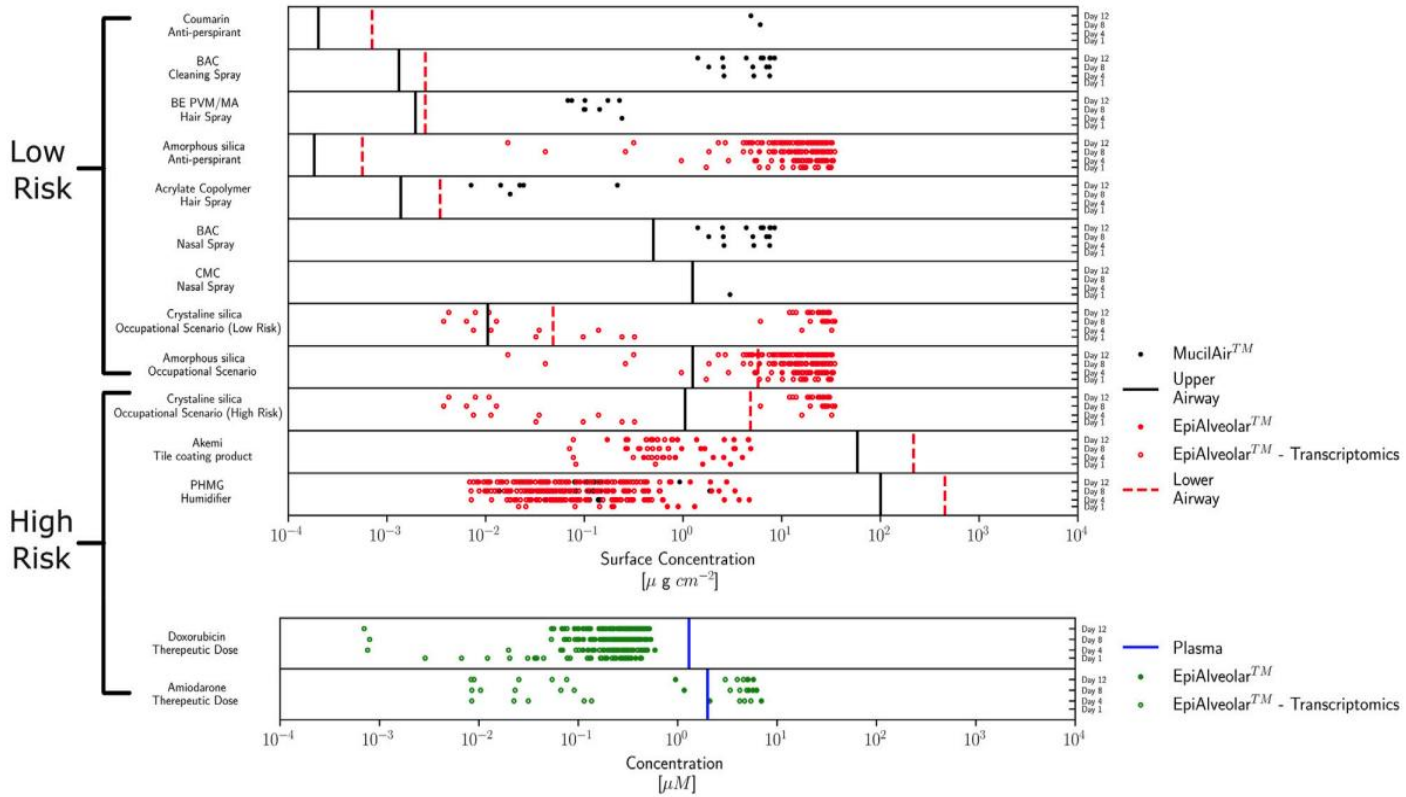
In general, for high-risk exposure-chemical scenarios *in vitro* PoDs were lower than the predicted exposure

11 benchmark chemicals investigated in
14 human low- or high risk exposure scenarios

Some examples	Risk classification	Risk classification reasoning	Scenario
Coumarin	Low	Safe use in cosmetic products	Anti-perspirant
Crystalline silica	Low	Safe under permissible exposure limit	Occupational scenario
	High	Silicosis after cumulative exposure	Occupational scenario
Doxorubicin	High	Interstitial lung disease in cancer patients	Therapeutic dose

- The obtained PoDs were combined with exposure estimates to calculate BER values
- BER is able to separate the low- and high-risk benchmark exposure scenarios for 12 out of the 14 scenarios
  - ✓ **Low-risk:** PoDs occurred at higher concentrations than the corresponding human exposure values. **Except:** crystalline and amorphous silica occupational scenarios
  - ✓ **High-risk:** clear overlap between the PoDs and human exposure (lung deposited mass or Cmax)



Defining a safe threshold: animal testing *versus* non-animal NAMs

- Traditional Margin of Safety ($MoS_{animal\ data}$ for local lung effects) > 25* → **low risk**

  *Uncertainty safety factor of 25 to account for uncertainties related to interspecies (animal-to human: 2.5-safety factor) and inter-individual (human-to human: 10-safety factor) variabilities[1]

- *In vitro* Bioactivity Exposure Ratio ($BER_{NAM\ data}$) > 3 → **low risk (?)**

  *Uncertainty safety factor of 3 applied in the chlorothalonil acute inhalation risk assessment to cover potential variation in sensitivity among human population (intraspecies)[2]

- **$BER_{NAM\ data}$ > 3 would be protective for all benchmark chemicals**, particularly driven by the transcriptomics PoDs for the high-risk exposure scenarios, e.g., Amiodarone and Crystalline silica

Amiodarone - high risk therapeutic dose

Day	Min PoD	Biomarker	BER	Risk
4	6.95	Cytokine: MMP-1 (Lab 2)	3.47	Low
	0.0084	Transcriptomics: LV30	0.0042	High
8	1.31	Cytokine: ICAM-1 (Lab 1)	0.65	High
	5.20	Cytokine: ICAM-1 (Lab 2)	2.60	High
	0.0084	Transcriptomics: LV30	0.0042	Low
12	0.97	Cytokine: ICAM-1 (Lab 1)	0.48	High
	5.03	Cytokine: ICAM-1 (Lab 2)	2.51	High
	0.0083	Transcriptomics: LV30	0.0041	High

Crystalline silica – high risk occupational scenario

Day	Min PoD	Biomarker	BER	Risk
1	0.032	Transcriptomics: LV131	0.071	High
4	0.0075	Transcriptomics: LV110	0.0041	High
8	34.53	Cytokine: MMP-7 (Lab 2)	11.14	Low
	0.0037	Cytokine: LV110 (Lab 2)	0.0012	High
12	30.51	Cytokine: MMP-7 (Lab 2)	6.32	Low
	0.0042	Transcriptomics: 110	0.00087	High

- Note some differences in EpiAveolar PoDs among Laboratories 1 and 2

[1]ECHA (2012). Guidance on information requirements and chemical safety assessment: chapter R.8: characterisation of dose [Concentration]-Response for human health.
[2]EPA (2021). Document ID: EPA-HQ-OPP-2011-0840-0080. Available at https://www.regulations.gov/document/EPA-HQ-OPP-2011-0840-0080

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Concluding remarks

- Strategy of selecting **non-animal NAMs informed by AOPs** associated with pulmonary toxicity can provide relevant biological coverage
- **Benchmarking decision outcomes provides an alternative to the traditional validation of NAMs:**
  apical effects in rodent studies *versus* NAMs in the context of making protective safety decisions

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Poster #121

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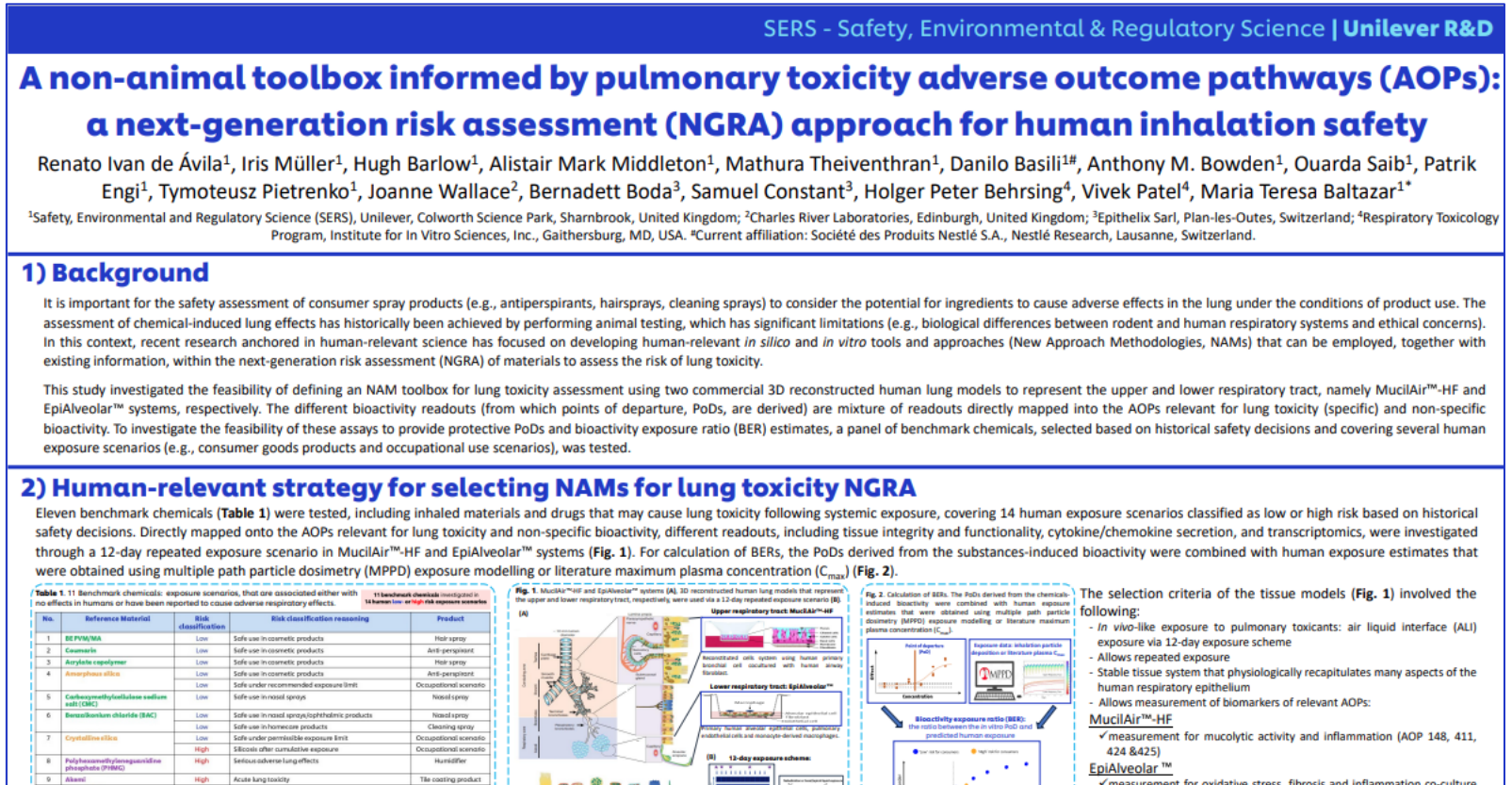

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A non-animal toolbox informed by pulmonary toxicity adverse outcome pathways (AOPs): a next-generation risk assessment (NGRA) approach for human inhalation safety

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1) Background

It is important for the safety assessment of consumer spray products (e.g., antiperspirants, hairsprays, cleaning sprays) to consider the potential for ingredients to cause adverse effects in the lung under the conditions of product use. The assessment of chemical-induced lung effects has historically been achieved by performing animal testing, which has significant limitations (e.g., biological differences between rodent and human respiratory systems and ethical concerns). In this context, recent research anchored in human-relevant science has focused on developing human-relevant *in silico* and *in vitro* tools and approaches (New Approach Methodologies, NAMs) that can be employed, together with existing information, within the next-generation risk assessment (NGRA) of materials to assess the risk of lung toxicity.

This study investigated the feasibility of defining an NAM toolbox for lung toxicity assessment using two commercial 3D reconstructed human lung models to represent the upper and lower respiratory tract, namely MucilAir™-HF and EpiAlveolar™ systems, respectively. The different bioactivity readouts (from which points of departure, PoDs, are derived) are mixture of readouts directly mapped into the AOPs relevant for lung toxicity (specific) and non-specific bioactivity. To investigate the feasibility of these assays to provide protective PoDs and bioactivity exposure ratio (BER) estimates, a panel of benchmark chemicals, selected based on historical safety decisions and covering several human exposure scenarios (e.g., consumer goods products and occupational use scenarios), was tested.

2) Human-relevant strategy for selecting NAMs for lung toxicity NGRA

Eleven benchmark chemicals (**Table 1**) were tested, including inhaled materials and drugs that may cause lung toxicity following systemic exposure, covering 14 human exposure scenarios classified as low or high risk based on historical safety decisions. Directly mapped onto the AOPs relevant for lung toxicity and non-specific bioactivity, different readouts, including tissue integrity and functionality, cytokine/chemokine secretion, and transcriptomics, were investigated through a 12-day repeated exposure scenario in MucilAir™-HF and EpiAlveolar™ systems (**Fig. 1**). For calculation of BERs, the PoDs derived from the substances-induced bioactivity were combined with human exposure estimates that were obtained using multiple path particle dosimetry (MPPD) exposure modelling or literature maximum plasma concentration (C_{max}) (**Fig. 2**).

Table 1. 11 Benchmark chemicals: exposure scenarios, that are associated either with no effects in humans or have been reported to cause adverse respiratory effects. 11 benchmark chemicals investigated in 14 human low- or high risk exposure scenarios

No.	Reference Material	Risk classification	Risk classification reasoning	Product
1	BEPVM/MA	Low	Safe use in cosmetic products	Hair spray
2	Coumarin	Low	Safe use in cosmetic products	Anti-perspirant
3	Acrylate copolymer	Low	Safe use in cosmetic products	Hair spray
4	Amorphous silica	Low	Safe use in cosmetic products	Anti-perspirant
		Low	Safe under recommended exposure limit	Occupational scenario
5	Carboxymethylcellulose sodium salt (CMC)	Low	Safe use in nasal sprays	Nasal spray
6	Benzalkonium chloride (BAC)	Low	Safe use in nasal spray/ophthalmic products	Nasal spray
		Low	Safe use in homecare products	Cleaning spray
7	Crystalline silica	Low	Safe under permissible exposure limit	Occupational scenario
		High	Silicosis after cumulative exposure	Occupational scenario
8	Polyhexamethyleneguanidine phosphate (PHMG)	High	Serious adverse lung effects	Humidifier
9	Akemi	High	Acute lung toxicity	Tile coating product

The selection criteria of the tissue models (**Fig. 1**) involved the following:

- *In vivo*-like exposure to pulmonary toxicants: air liquid interface (ALI) exposure via 12-day exposure scheme
- Allows repeated exposure
- Stable tissue system that physiologically recapitulates many aspects of the human respiratory epithelium
- Allows measurement of biomarkers of relevant AOPs:

MucilAir™-HF
✓measurement for mucolytic activity and inflammation (AOP 148, 411, 424 &425)

EpiAlveolar ™
✓measurement for oxidative stress, fibrosis and inflammation co-culture