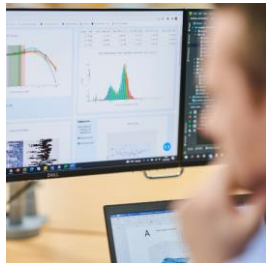
A proof-of-concept to establish a NAM toolbox to be used within a NGRA approach for lung toxicity

Maria Baltazar, PhD

SERS
Safety, Environmental & Regulatory Science

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Assuring inhalation safety:
inhalation exposure depends on product type and habits & practices

Several Unilever products lead to an unintentional inhalation exposure :

Can we safely use x% of ingredient y in product z ?

Household cleaning products

Hairsprays (pump and aerosol)

Shampoos

Anti-perspirant/ deodorant aerosols

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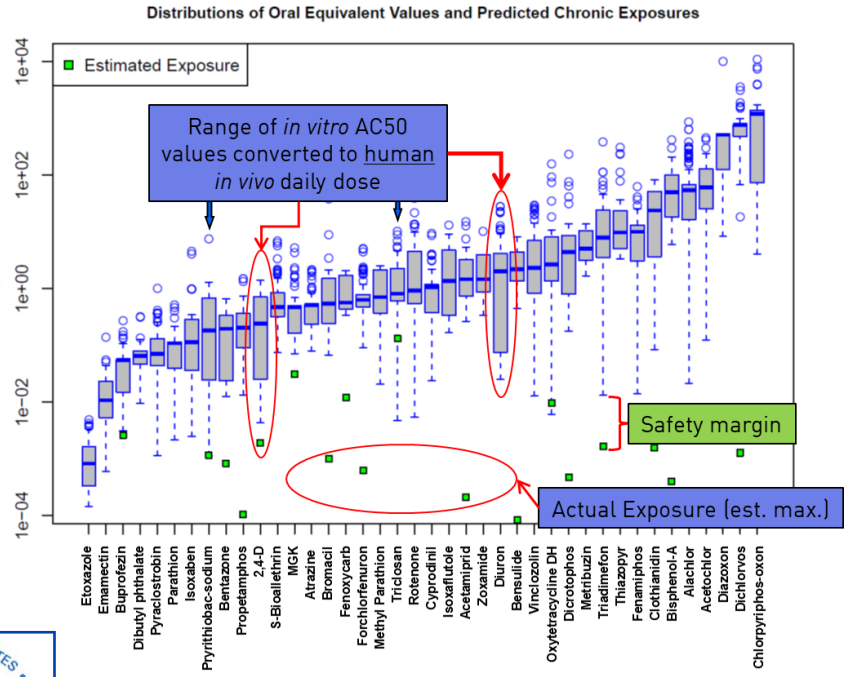
Safety without animal testing - Next Generation Risk Assessment (NGRA)

*NGRA is defined as **an exposure-led, hypothesis-driven** risk assessment approach that **integrates New Approach Methodologies (NAMs)** to assure **safety without the use of animal testing***



Slide from Dr Rusty Thomas, EPA, with thanks

Rotroff, *et al.* Tox.Sci 2010
DOI:10.1093/toxsci/kfq220

The hypothesis underpinning this type of NGRA is that **if there is no bioactivity observed at consumer-relevant concentrations, there can be no adverse health effects.**

Human-relevant strategy for selecting NAMs for lung toxicity NGRA

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Evaluation of a non-animal toolbox informed by adverse outcome pathways for human inhalation safety

Renato Ivan de Ávila[1], Iris Müller[1], Hugh Barlow[1], Alistair Mark Middleton[1], Mathura Theiventhran[1], Danilo Basili[1†], Anthony M. Bowden[1], Ouarda Saib[1], Patrik Engi[1], Tymoteusz Pietrenko[1], Joanne Wallace[2], Bernadett Boda[3], Samuel Constant[3], Holger Peter Behrsing[4], Vivek Patel[4] and Maria Teresa Baltazar[1*]

Hypothetical case study based approach

New ingredients for use in consumer products

Problem formulation: chemistry, physico-chemical properties, potential hazards, existing information

Exposure-led

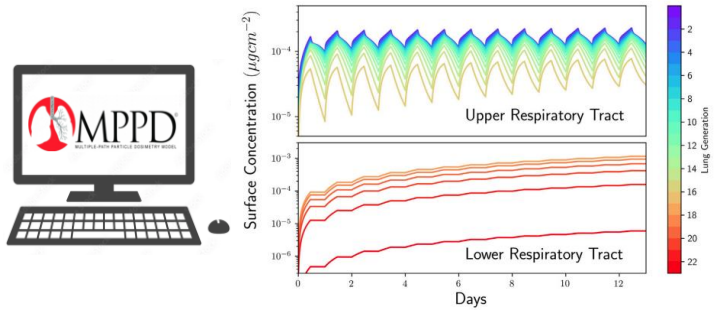
Exposure is calculated using consumer habits and practices. A tiered modelling approach is applied to simulate realistic consumer exposure. Multiple path particle dosimetry (MPPD) model predicts local lung exposure

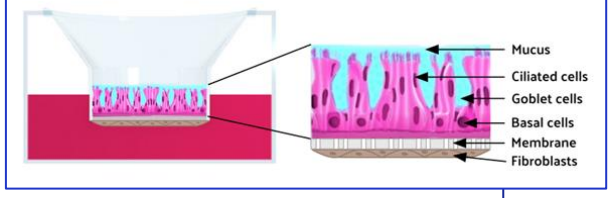
Hypothesis-driven

Proposed NAM toolbox with sufficient biological coverage for the assessment of local lung toxicity for inhaled chemicals

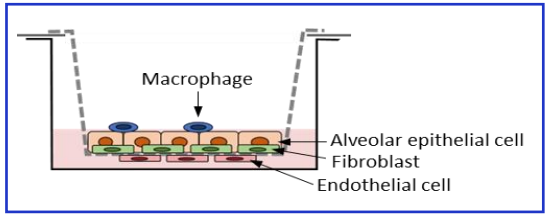
Impairment of MCC Upper airway MucilAir™-HF model (AOP 148)	Lung inflammation and fibrosis Lower airway EpiAlveolar™ model (AOP 173)
Lung surfactant inhibition (AOP 302)	Alveolar clearance (AOPs 173 & 303)

In silico lung dosimetry modelling to estimate inhalation particle deposition in the human respiratory tract





Upper respiratory tract: MucilAir™-HF



Lower respiratory tract: EpiAlveolar™

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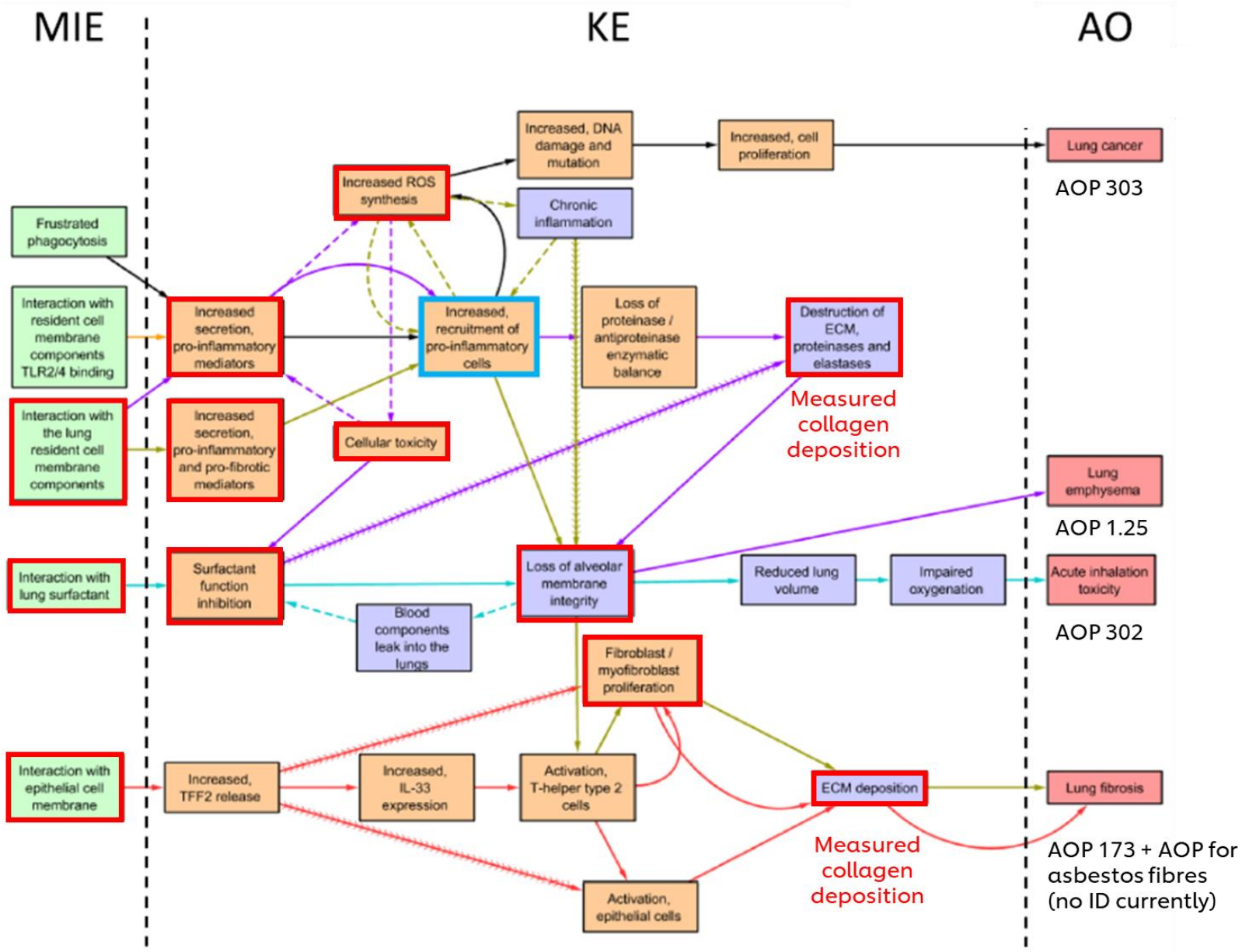
A mechanistic approach:

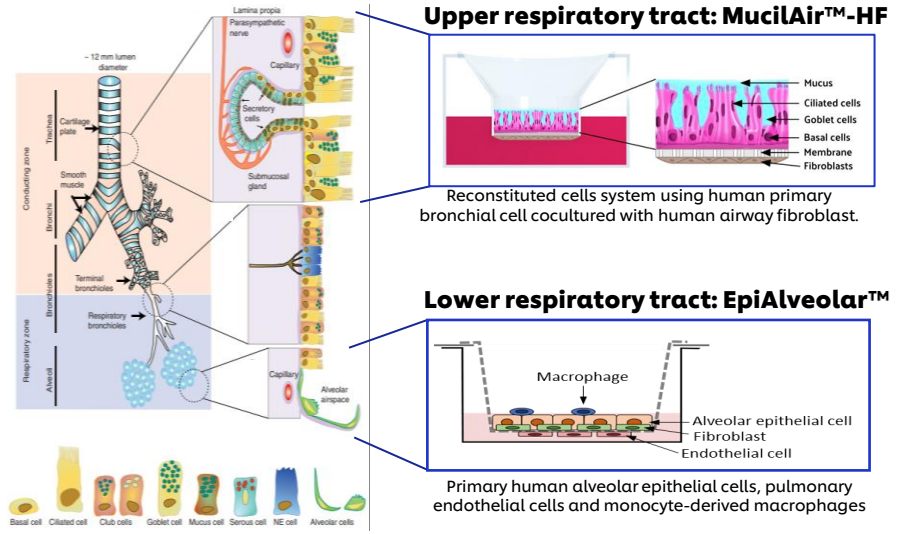
From a mechanistic point of view our strategy covers multiple molecular initiating event (MIE) and key events (KEs)

MIEs/KEs covered by current toolbox



Halappavar et al. (2020)
DOI: 10.1186/s12989-020-00344-4

Human-relevant strategy for selecting NAMs for lung toxicity NGRA

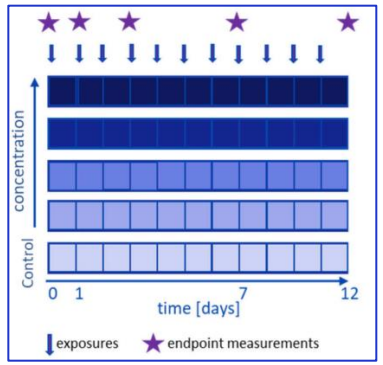


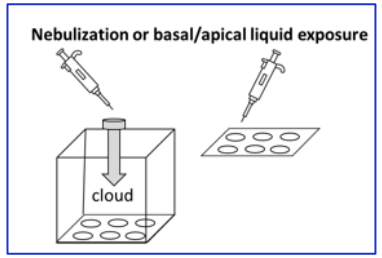
modified after Bustamante-Marin et al. (2017)
DOI: 10.1101/cshperspect.a028241

Selection Criteria:

- *In vivo*-like exposure to pulmonary toxicants: air liquid interface (ALI) exposure
- 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 of cells including immune competent cells/macrophages and fibroblast (AOP 173,1.25, 303,302)

12-day exposure scheme:

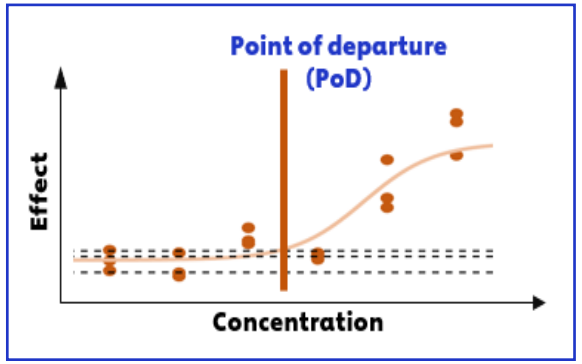




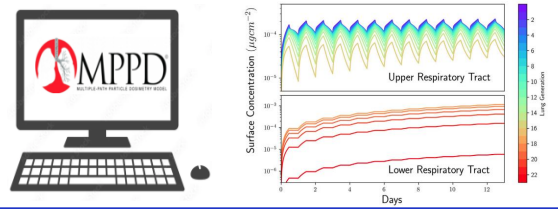
Human-relevant strategy for selecting NAMs for lung toxicity NGRA

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

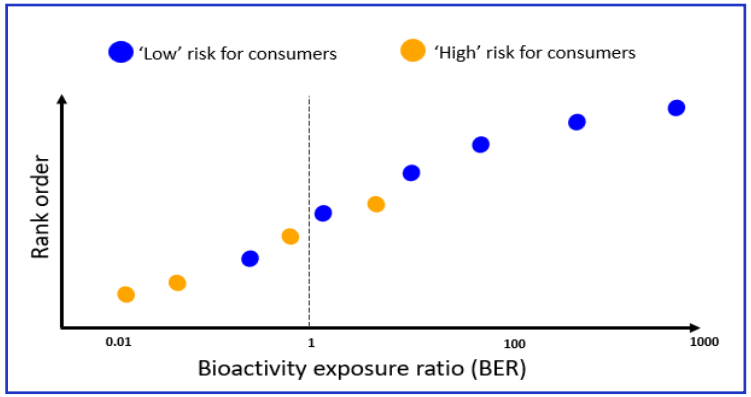
	Effects	Readouts	Acute toxicity	Chronic effect	Laboratories
Upper respiratory tract MucilAir™-HF model	Tissue functionality changes	Mucus secretion, cilia beating (CBF), mucociliary clearance (MCC)	Irritation, enhanced chance of airway infection	Goblet cell hyperplasia, asthma, chronic obstructive pulmonary disease (COPD)	Epithelix Sàrl (Geneva, Switzerland)
	Cytotoxicity, barrier integrity, inflammatory and transcriptomic modulation	Trans-epithelial electrical resistance (TEER), cytokine/ chemokine modulation	Local cytotoxicity, irritation, inflammation	Airway remodelling, Asthma, COPD, lung fibrosis	
Lower respiratory tract EpiAlveolar™ model	Barrier integrity, inflammatory and transcriptomic modulation	TEER, cytokine/ chemokine modulation, transcriptomics analysis	Local cytotoxicity, inflammation, wound healing	Airway remodelling/ scarring, lung fibrosis	Institute for In Vitro Sciences, Inc. (Gaithersburg, MD, USA) and Charles River Laboratories Edinburgh Ltd (Tranent, UK)



Exposure data: inhalation particle deposition



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



Evaluation of the NAM toolbox: selection of test substances

- Two groups of test substances that may reach the respiratory tract and induce lung toxicity:

1) Reference materials: may trigger lung toxicity via different mechanisms, including inflammation, oxidative stress, cilia beating frequency (CBF), and mucus production/viscosity changes

No.	Reference Material	Reason for testing
Positive reference materials		
1	Acrolein	Induces CBF and mucin secretion increase
2	$CFTR_{inh}$-172	mimics the cystic fibrosis inflammatory process
3	Chlorocresol	Induces CBF decrease
4	Isoproterenol hydrochloride	induces CBF increase
5	Lipopolysaccharide (LPS)	respiratory tract inflammation agent
6	Nicotine	oxidative stress agent
7	TNF-α	induce inflammation and evidence of inducing CBF increase
Negative reference material		
8	Sulforaphane	anti-inflammatory agent

- Some well-document **biological responses to these materials were mild or absent in both *in vitro* human lung systems**

The example of LPS:

✓ LPS did not trigger intense inflammatory cytokine secretion in EpiAveolar model

✓ LPS induced mild inflammatory response in MucilAir™-HF tissues exposed to TNF-α and LPS

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Evaluation of the NAM toolbox: selection of test substances

- Two groups of test substances that may reach the respiratory tract and induce lung toxicity:

2) Benchmark chemicals: exposure scenarios that are associated either with no effects in humans or have been reported to cause adverse respiratory effects

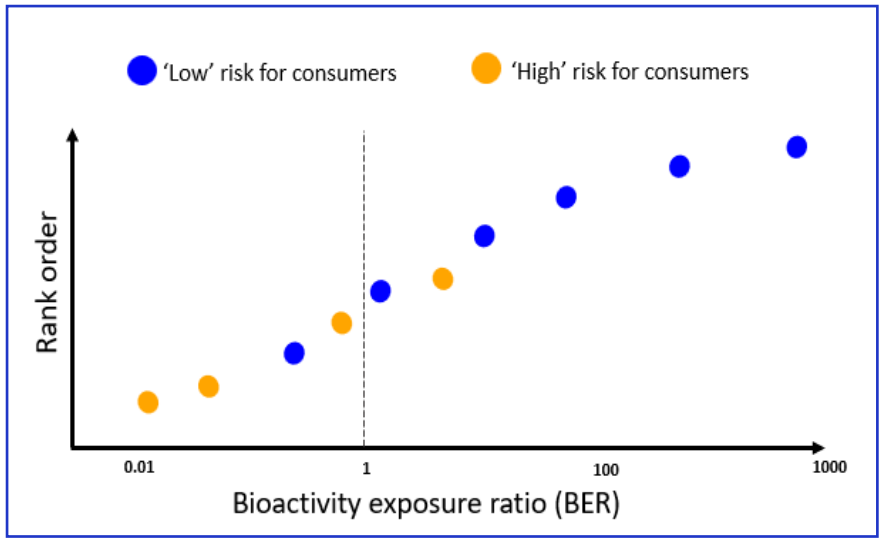
Criteria selection:

a) a human exposure can be defined, e.g., inclusion level of a chemical in a given product type, and how it is used;

b) existing toxicological information (animal, human, *in vitro*);

c) evidence to support the high- or low-risk classification for each chemical-exposure scenario pair based on existing safety assessment and/or regulatory limits.

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



Evaluation of the NAM toolbox: selection of test substances

2) 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	**BE PVM/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 sprays/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
10	**Doxorubicin**	High	Interstitial lung disease in cancer patients	Therapeutic dose
11	**Amiodarone**	High	Alveolar/interstitial pneumonitis with a subacute onset	Therapeutic dose

Tested in MucilAir™-HF only – Tested in EpiAlveolar™ only - Tested in both tissue models

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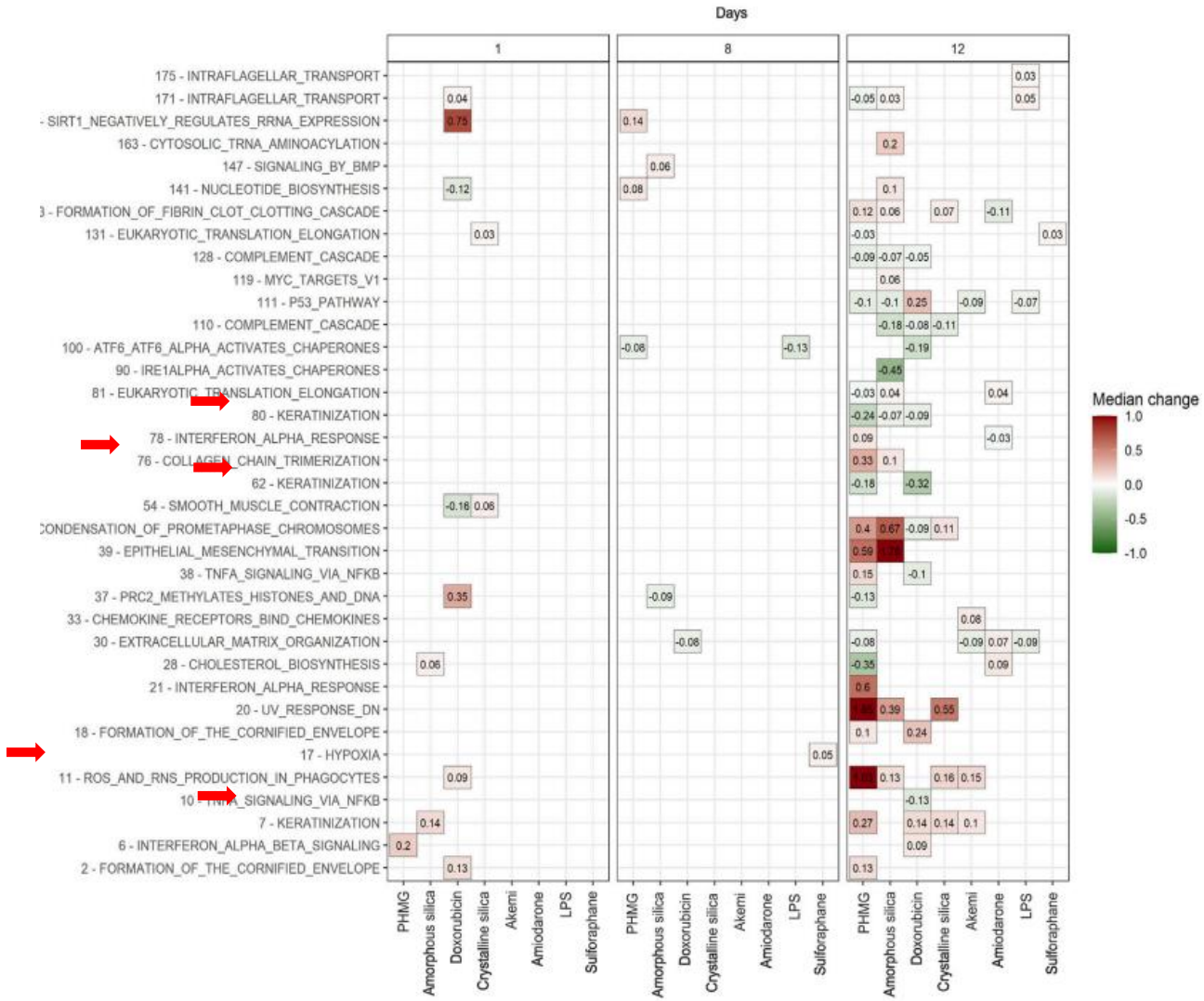
Transcriptomics is useful to elucidate mechanism of toxicity in the EpiAlveolar model

- **Pathway-level information extractor (PLIER) method[1]:**
  - ✓ Calculation of a transcriptomics POD
  - ✓ identifying patterns of co-regulated genes associated with biological knowledge (latent variables (LVs))
- Most of the LVs modulated by **PHMG, Amorphous silica, and Doxorubicin** captured **biological activity corresponding to the key factors leading to pulmonary fibrosis**:
  - ✓ inflammation, oxidative stress, epithelial mesenchymal transition which ultimately leads to excessive deposition of extracellular matrix.



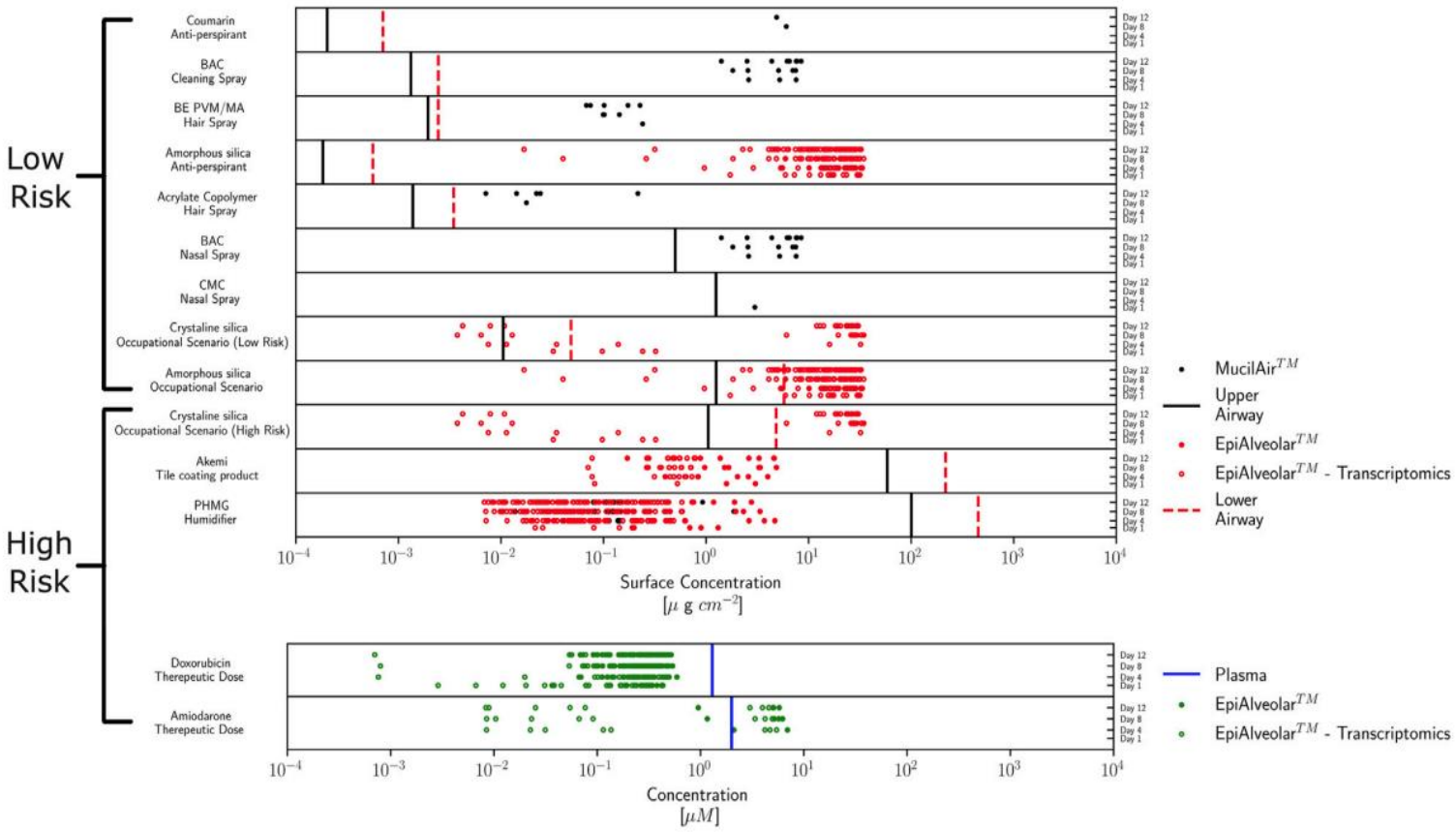
In a risk assessment context this information would suggest that these chemicals could cause **pulmonary fibrosis *in vivo*** and would warrant further investigation

1. Basili D et al., 2022. DOI: 10.1021/acs.chemrestox.1c00444

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

- 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)

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



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

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

  *Uncertainty safety factor of 3 applied in the chlorothalonil acute inhalation risk assessment[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. https://www.regulations.gov/document/EPA-HQ-OPP-2018-0517-0006.2018

Concluding remarks

- The **upper MucilAir™-HF and lower EpiAlveolar™ human lung models** are *in vitro* systems within a NAM toolbox able to identify **the bioactivity (i.e., *in vitro* PoD derivation) and therefore associated risk of materials** that may reach the respiratory tract and induce lung toxicity
- **Bioactivity readouts are useful to obtain *in vitro* PoDs through a 12-day repeated exposure protocol:** tissue barrier integrity loss, tissue functionality, modulation of cytokines/chemokines, and/or transcriptomics
- **Transcriptomics** has utility for establishing a PoD but also for **gaining mechanistic insights to generate hypotheses** within the context of a risk assessment framework
- **BER approach** - obtained *in vitro* PoDs combined with computational exposure estimates: the performance of this approach is determined by its ability to differentiate between chemical/exposure scenarios of low and high risk to humans based on the size of the BER
- For the **11 benchmark chemicals investigated in 14 human exposure scenarios**, it was possible to correctly separate their risk classification by using **the lowest BER per readout and lung model**

Inhibition of lung surfactant function by surfactants and polymers.

An in vitro method anchored in AOP302 for decreased lung function

Experimental work done using the Constrained Drop Surfactometer, (BioSurface Instruments) by PhD student Sreyoshee Sengupta and senior researcher Jorid B. Sørli, NFA, Copenhagen, Denmark, jbs@nfa.dk

Rheology and modelling by Hugh Barlow, Unilever

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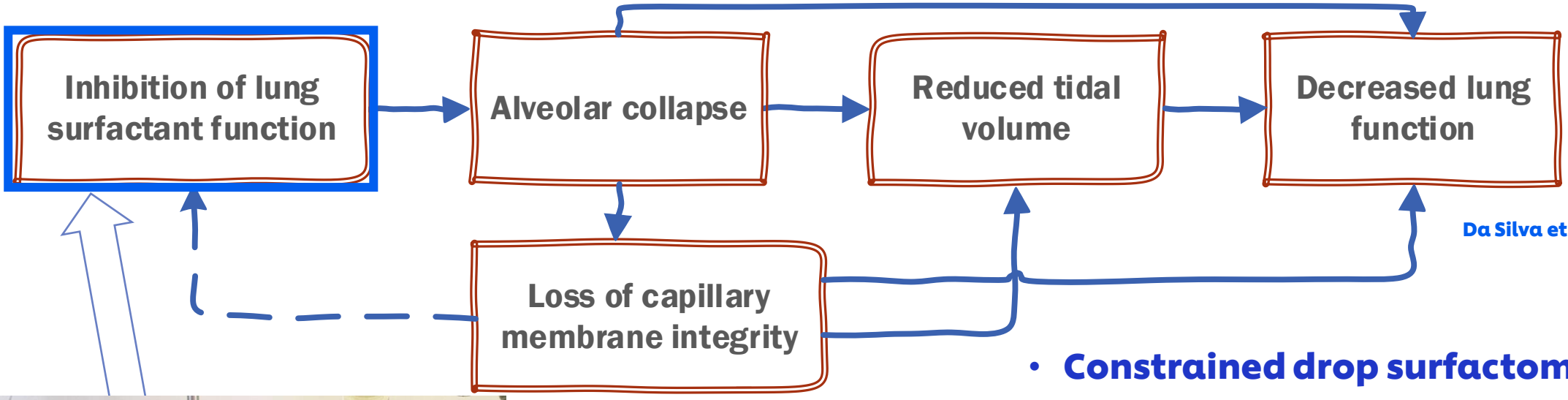
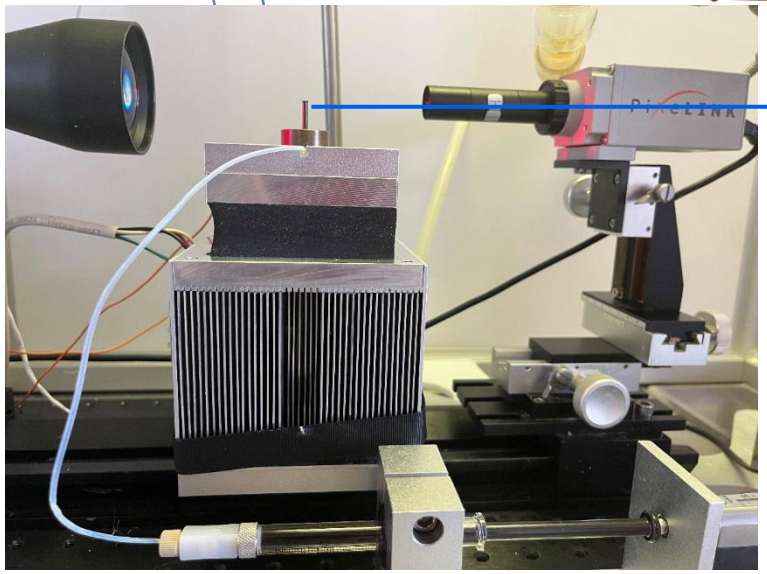
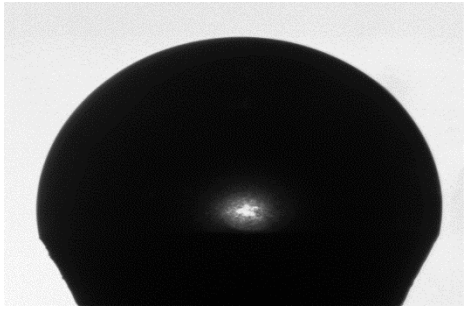
AOP 302: Lung surfactant function inhibition leading to reduced lung function



Da Silva et al 2021 CrTox

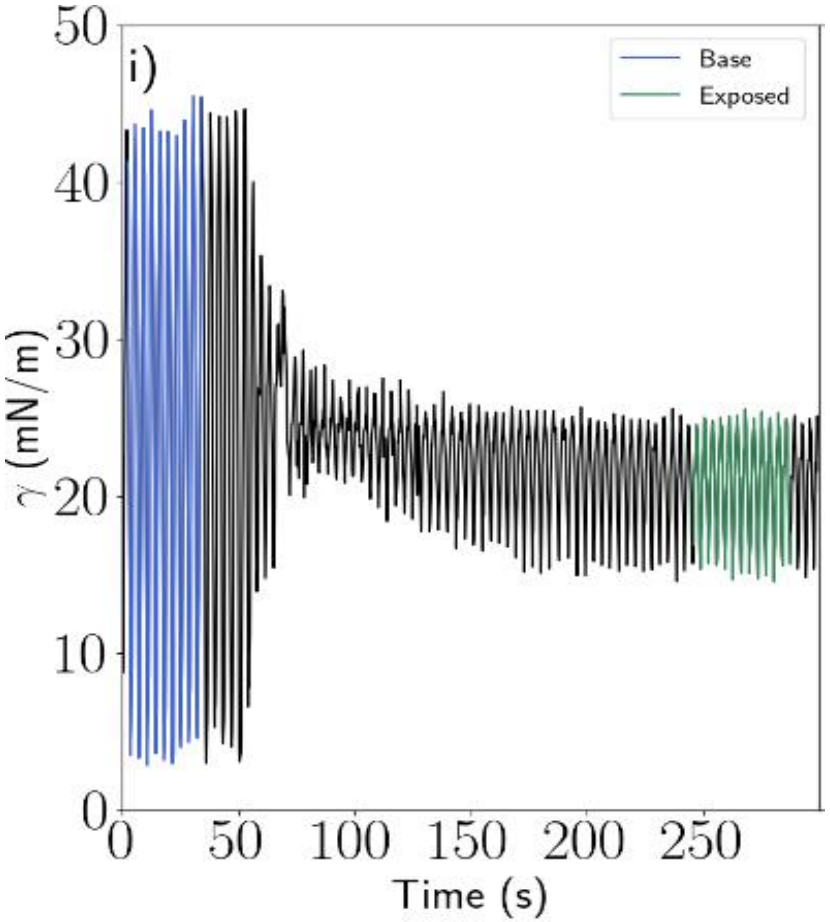




- **Constrained drop surfactometer**
  - Changes the volume, and thus surface area of the drop to mimic breathing, 20-30% reduction and cycles of 3 seconds
  - Pictures of the drop analysed using ADSA software to determine area and surface tension
  - Expose lung surfactant to test substance as aerosols

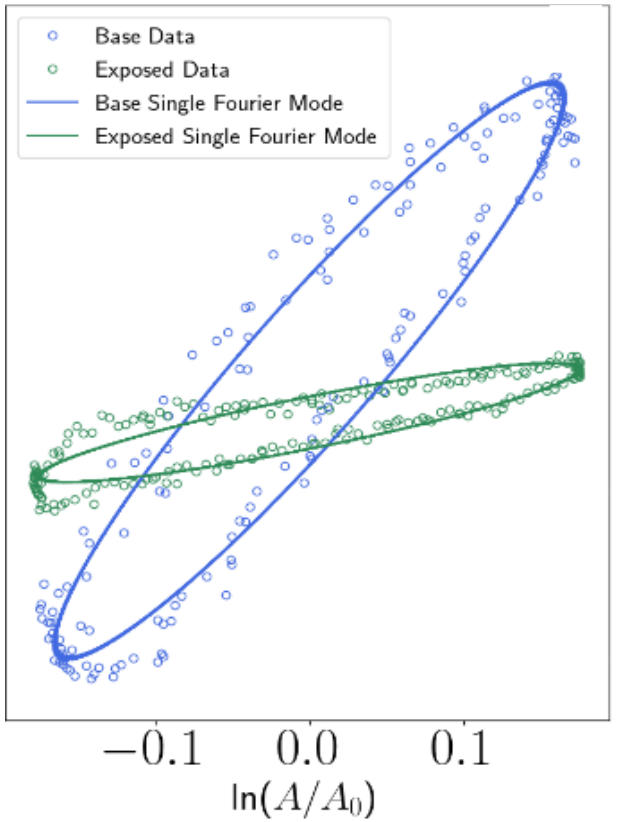
Hypothesis

- **Interaction of surfactants and polymers with the surface active components modifies the visco-elastic properties of the lung surfactant film**



- **Elastic (E') = change in surface tension /change in area**
- **Viscous (E'') = Molecular motion at the human breathing frequency**
- **Change in visco-elastic response**

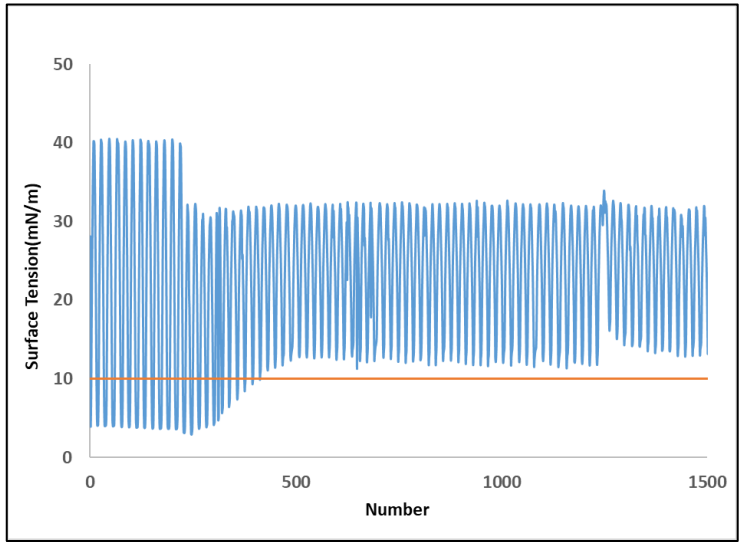
$$E^* = E' + E''$$



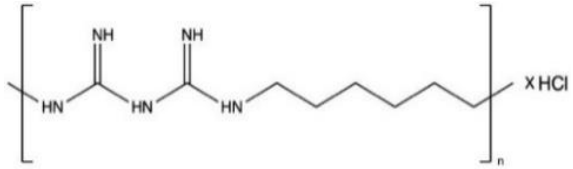
Effect of surfactants and polymers

Sodium Dodecyl Sulphate (SDS)

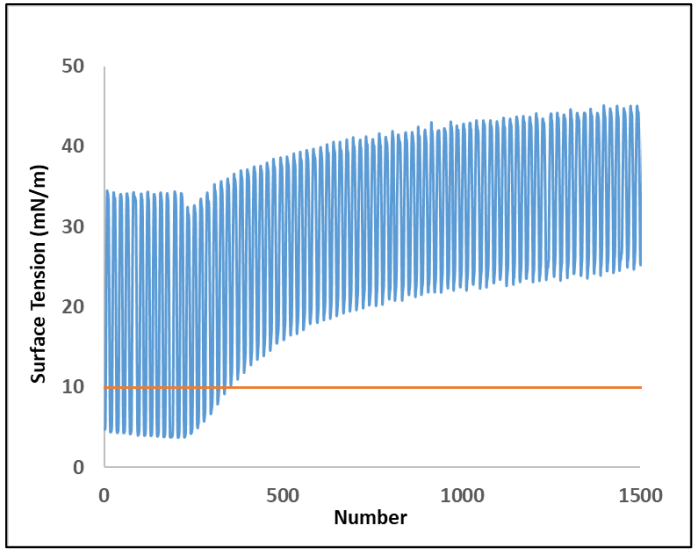
- Cleaning agents, seldom in spray products
- Causes acute respiratory toxicity



Polyhexamethylenebiguanidine hydrogen chloride (PHMB)



- Antimicrobial activity
- Not advisable in spray products in the EU



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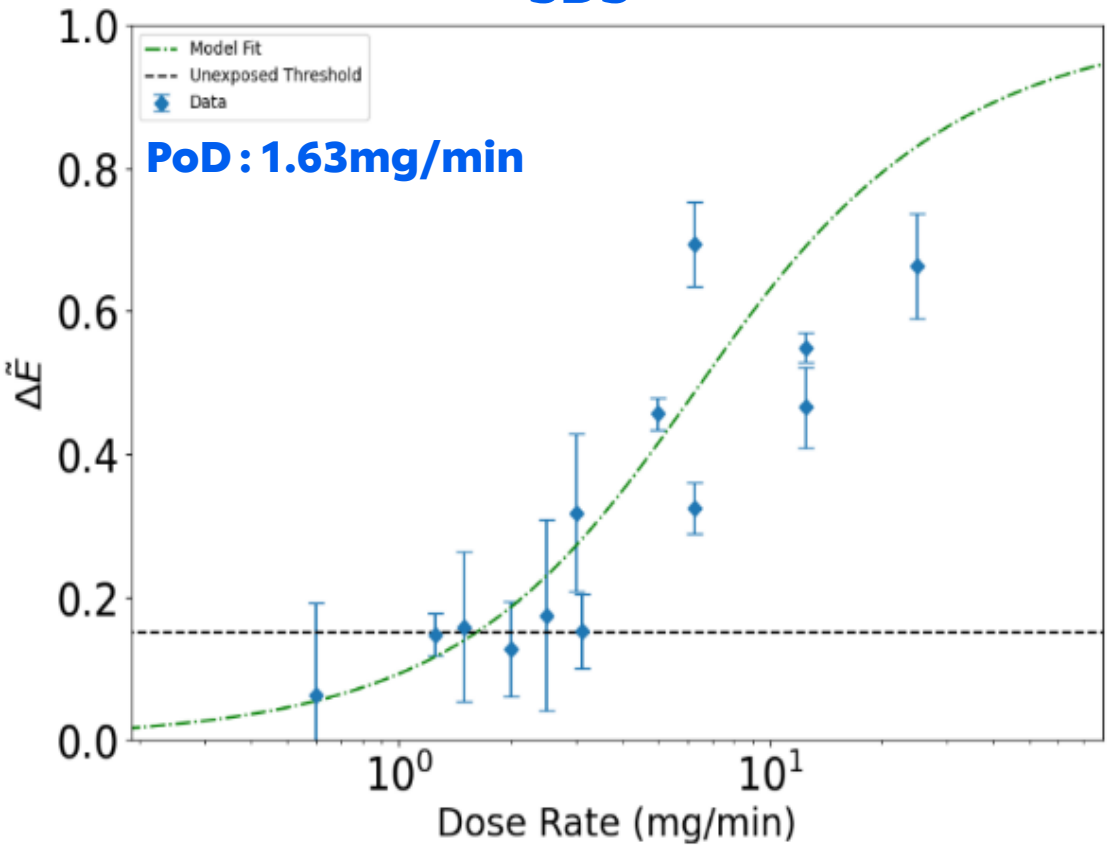
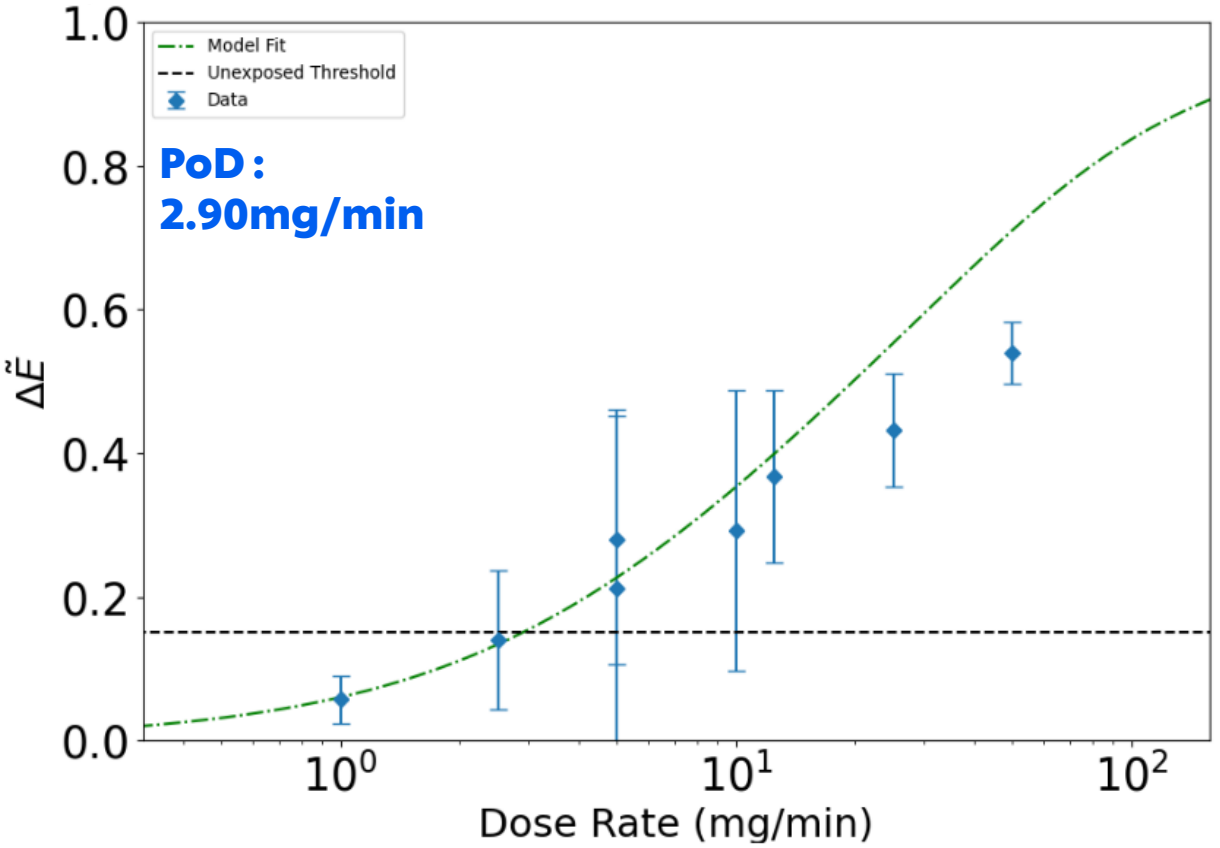
Determination of Point of Departure

SDS



PHMB



Papers:
Barlow et al 2024 (model) preprint: https://doi.org/10.1101/2024.10.16.618442 and Sengupta et al 2024 (data on all tested chemicals) in Biorxiv https://www.biorxiv.org/content/10.1101/2024.10.18.618437v1

Concluding remarks

- Strategy of selecting **non-animal NAMs informed by AOPs** associated with pulmonary toxicity can provide relevant biological coverage
- Further evaluation of **the performance of NAM toolboxes for a wider substance dataset** with varied mechanisms of action, uses, and balanced low and high-risk benchmarks to build confidence in the protectiveness of the approach
- There is a need to **establish scientific confidence** by improving the reproducibility, standardization of protocols, and *in vitro* culture methodologies
- **Benchmarking decision outcomes provides an alternative to the traditional validation of NAMs:**

  apical effects in rodent studies *vs.* NAMs in the context of making protective safety decisions

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- Ouarda Saib
- Patrik Engi
- Tymoteusz Pietrenko
- Matthew Dent
- Carl Westmoreland
- Zoë Deag
- Claire Peart
- Mark Liddell
- Beate Nicol

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