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SCIENCE

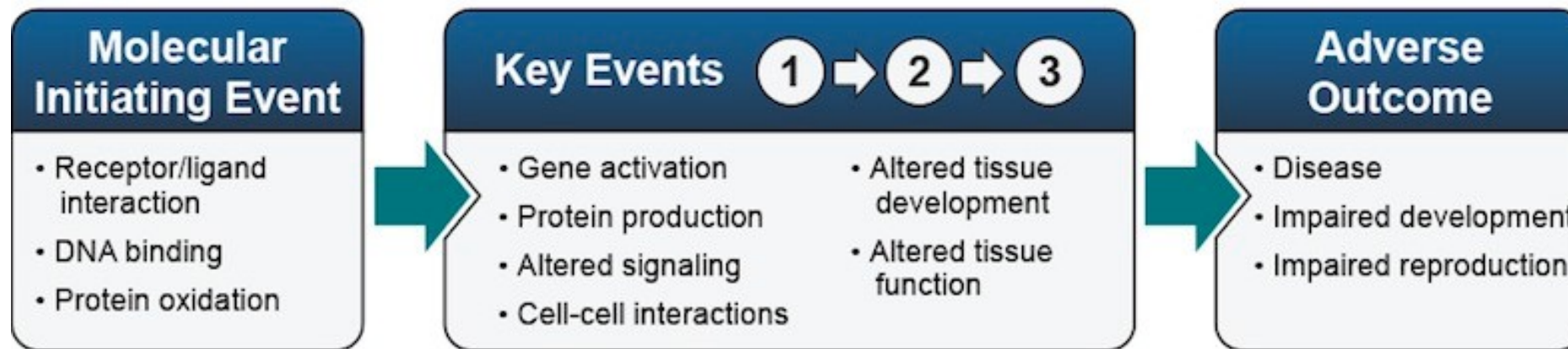
In vitro exposures to whole smoke & aerosols: standard & novel (3D) *in vitro* models

New Approach Methods (NAMs) Symposium-II: (CORESTA 2023)

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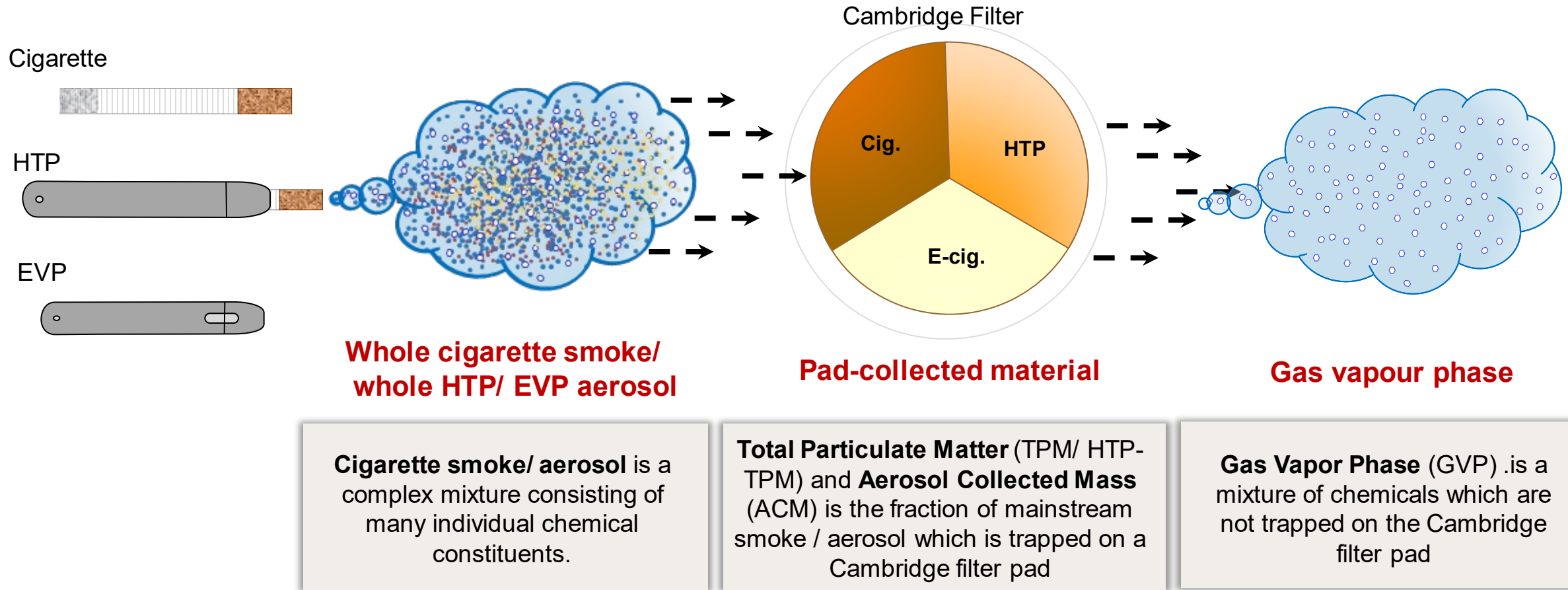
TALK OUTLINE:

- What is whole smoke?
 - The use of whole smoke/aerosol in regulatory assays (Examples IVM and NRU)
 - The use of a 3D human Bronchial model (NAM) to assess HTP and EVP vs cigarette
 - How data collected in the 3D model can be used to populate data in an Adverse Outcome Pathway (AOP)
- An adverse outcome pathway (AOP) is a model identifying the sequence of molecular and cellular events required to produce a toxic effect when an organism is exposed to a substance *



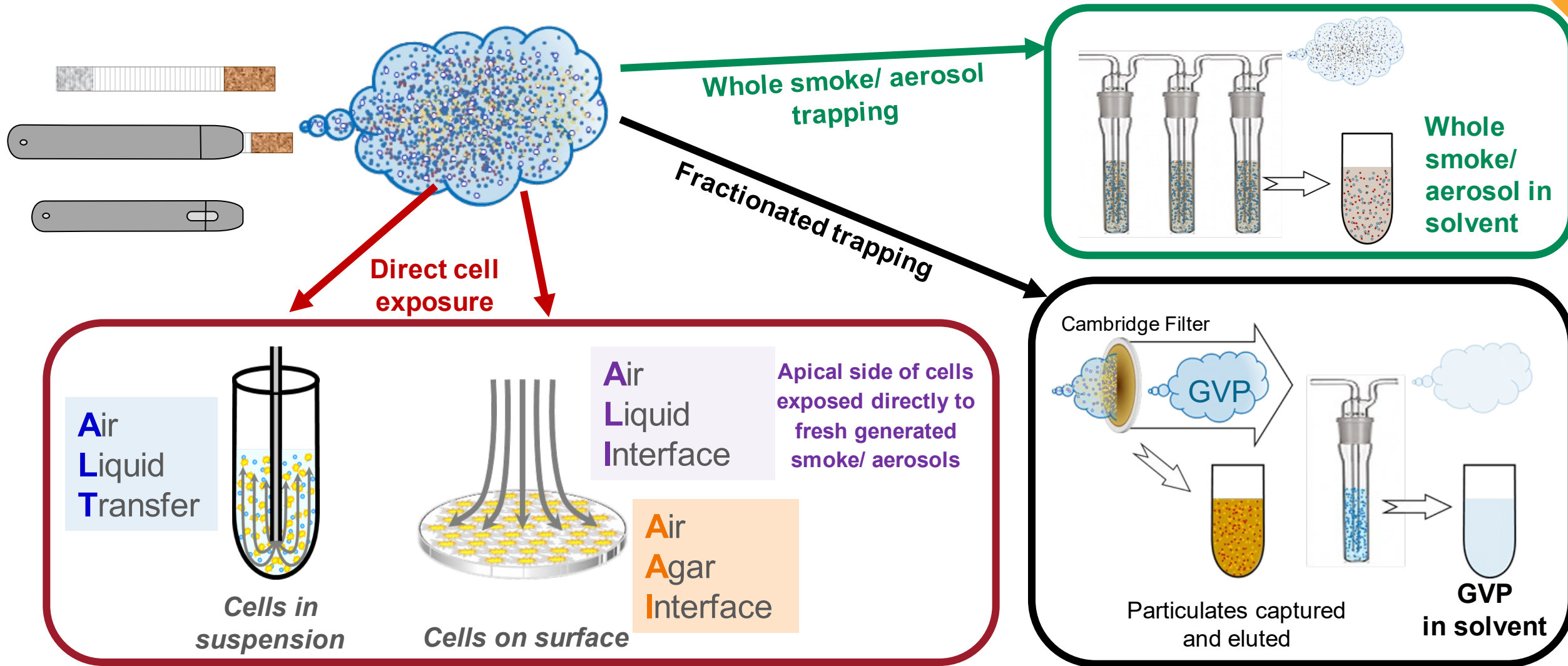
*<https://ntp.niehs.nih.gov/whatwestudy/niceatm/comptox/ct-aop/aop>

Whole smoke and aerosol collection – available methods



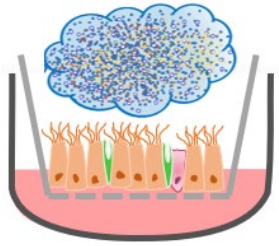
All components of whole smoke/ aerosol, TPM/ ACM and GVP are relevant for toxicological evaluation

Toxicity testing of whole smoke and aerosol – techniques



All components of whole smoke/ aerosol, TPM/ ACM and GVP are relevant for toxicological evaluation

Direct exposure of cells - techniques



Air-liquid interface

Air
Liquid
Interface

Apical side of cells exposed directly to fresh generated smoke/aerosols. Cells can be maintained on various surfaces

IVM test with V79 cells

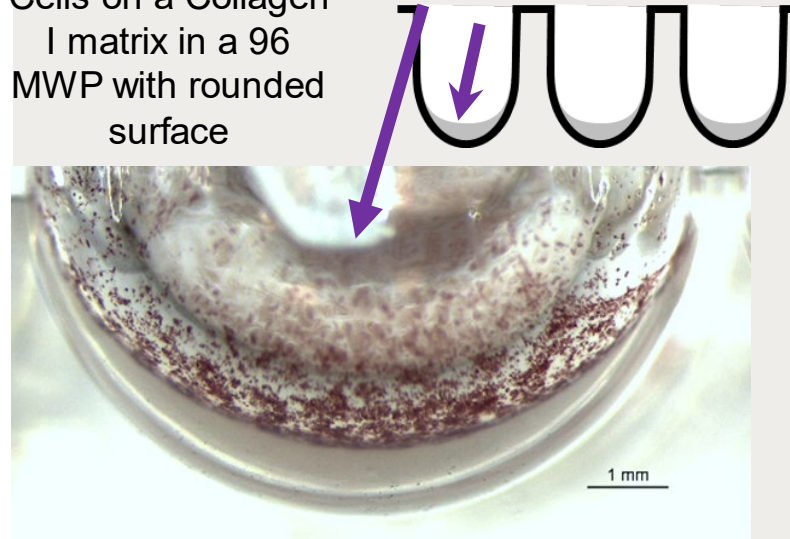
Cells in transwells in a 24 MWP
Basal medium supply



V79 cells on a membrane

NRU test with human bronchial cells (BEAS-2B)

Cells on a Collagen I matrix in a 96 MWP with rounded surface



3D tissue models in transwells or inserts



Transwell

Insert

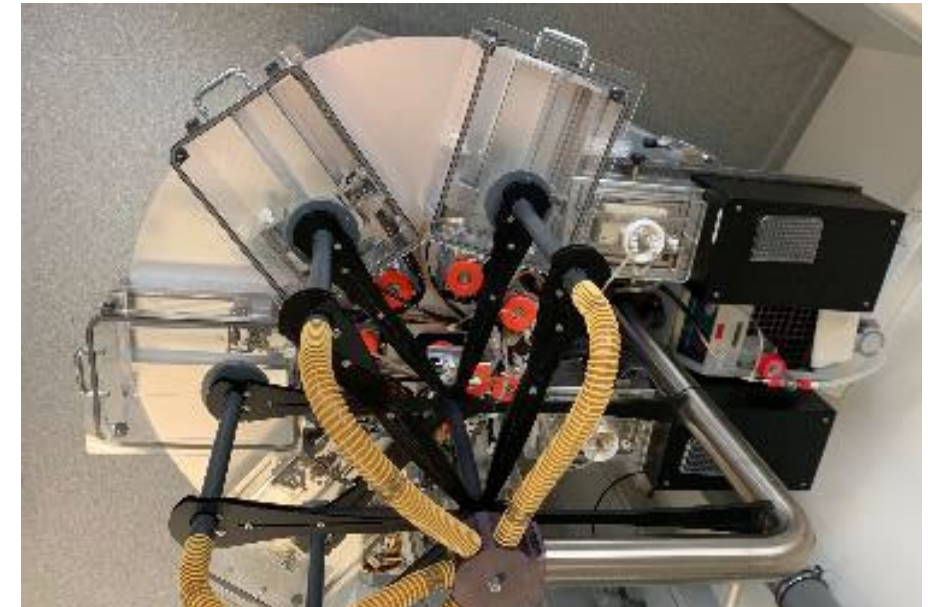
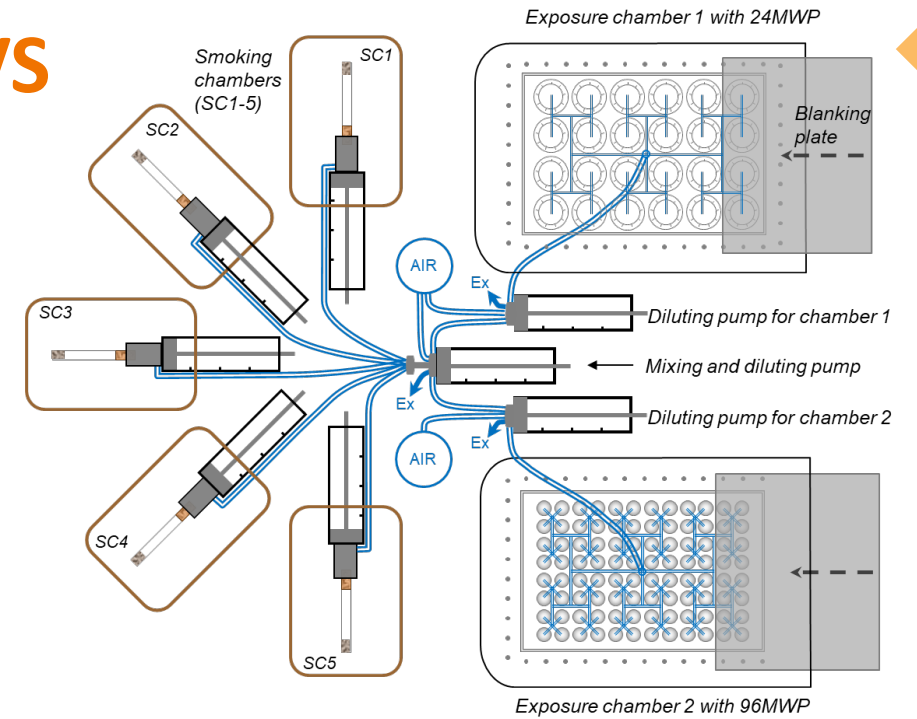


3D cell reconstructed mucociliary epithelial model on a membrane stained with Alcian blue

Direct exposure of cells at the ALI – SAEIVS

Smoke and Aerosol Exposure *in vitro* System

- Exposure at ALI requires specialised equipment
- Delivers smoke/ aerosol to cells in under 10 sec
- Only 20% loss of particles
- Blanking plate to cover rows of wells allows exposure in a puff-dependent manner



Reduced cytotoxicity and mutagenicity for HTP and EVP– NRU & IVM example results

Methods:



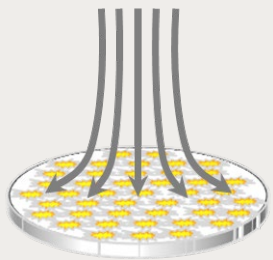
Reference Cigarette (1R6F/3R4F)



HTP with Tobacco stick



EVP, Tobacco flavour, 2.4% nicotine



Air
Liquid
Interface

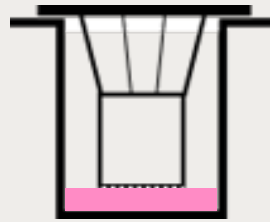
NRU

BEAS-2B cells on a Collagen I matrix in a 96 MWP with round bottom



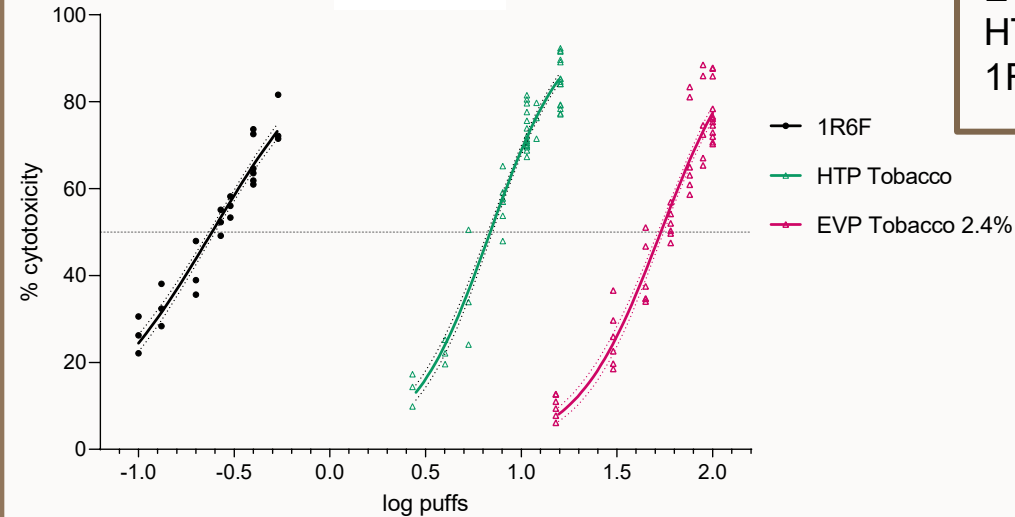
IVM

V79 Cells in transwells in a 24 MWP



Results:

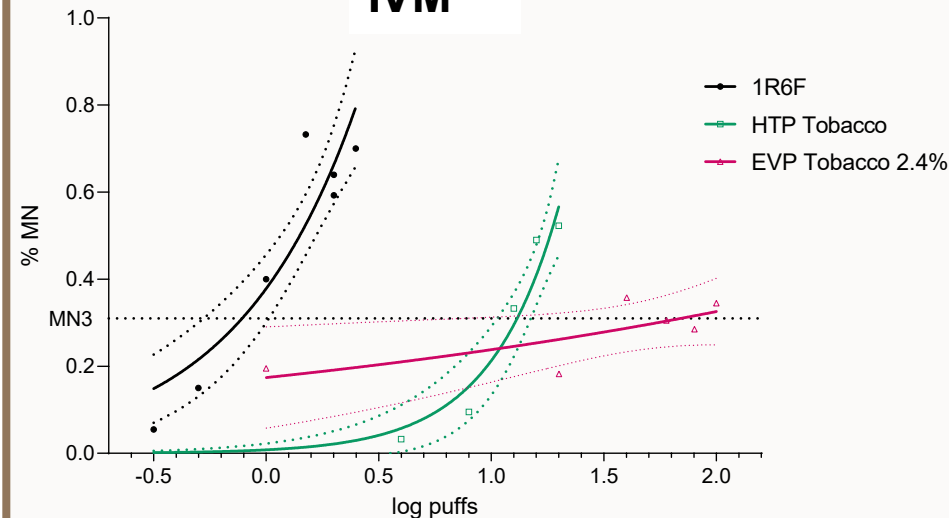
NRU



NRU: EC 50 values [puffs]:

EVP Tobacco 2.4%	53.60
HTP Tobacco	6.88
1R6F	0.242

IVM



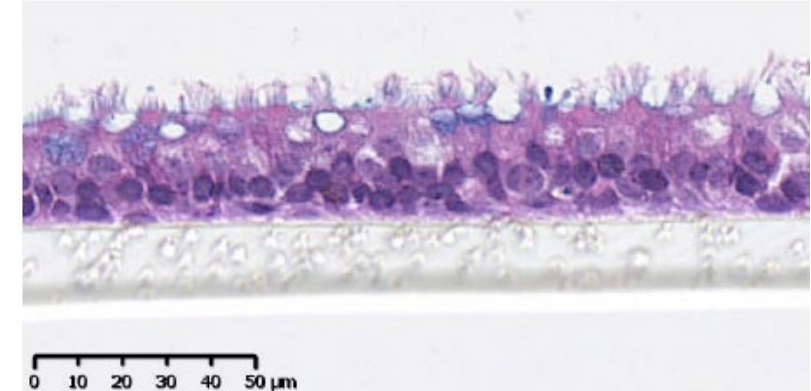
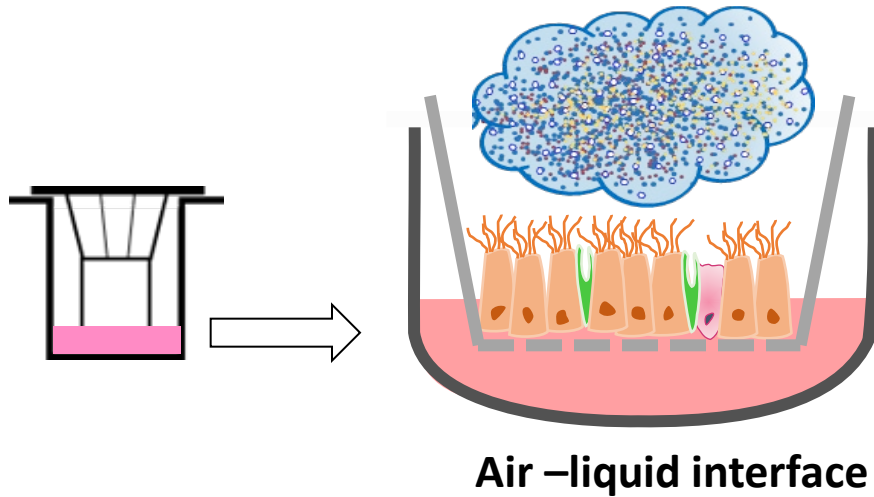
IVM: Genotoxicity:

EVP Tobacco 2.4%	No
HTP Tobacco	Yes
1R6F	Yes

ECMN3 [puffs]:

HTP Tobacco	13.03
1R6F	0.782

3D bronchial tissues can be used to elucidate early key events



Advantages of a 3D culture :

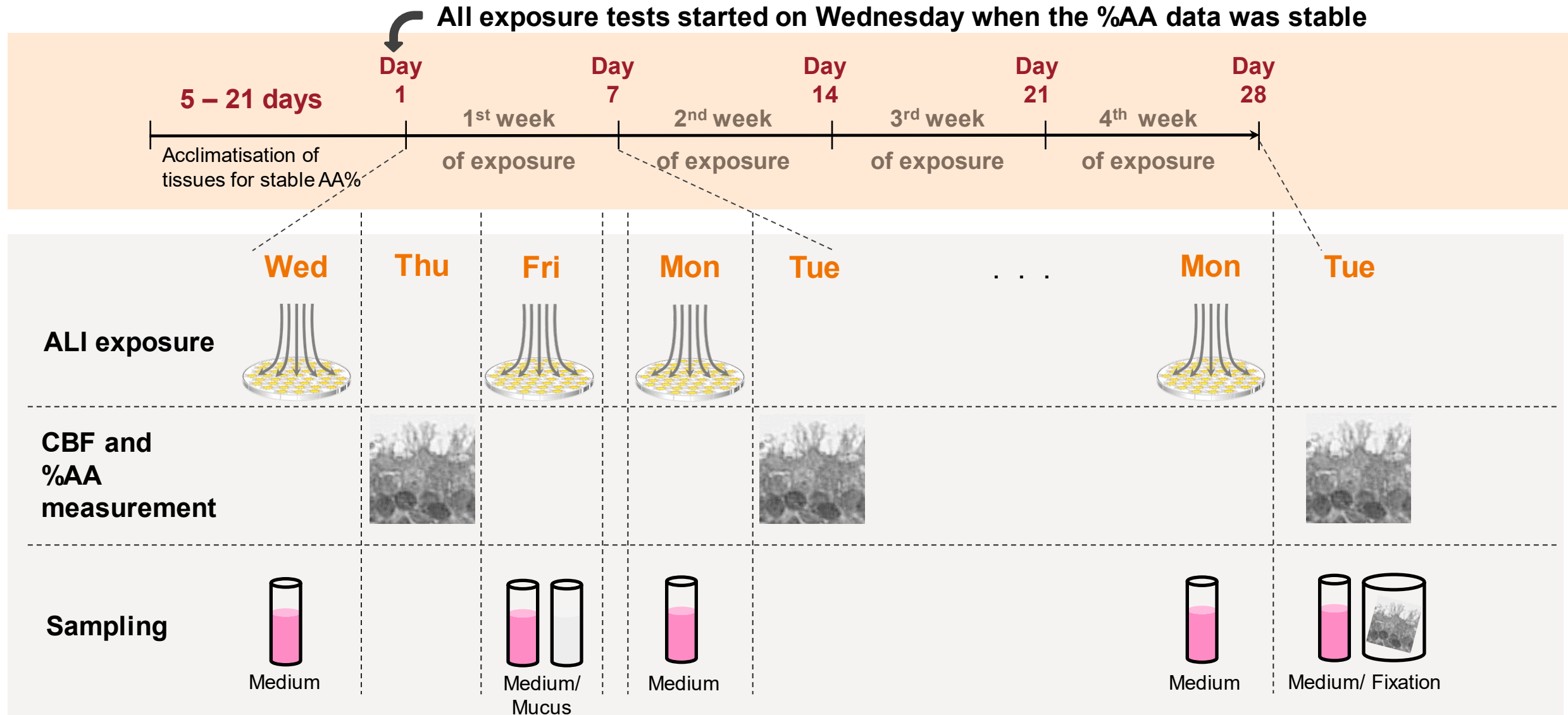
- Cells grown and exposed at ALI
- Contains multiple cell types
- Physiologically relevant (beating cilia, mucus, tight junctions, metabolic competence, inflammatory mediators)
- Stable for up to 1 year

Repeated exposure to fresh smoke/ aerosol

- Increased consumer relevance
- Development of disease phenotypes occurs following a period of time/ is likely the effect of more than a single exposure or cellular/molecular event

In vitro 3D differentiated epithelium of the human upper respiratory tract (MucilAir™ - Epithelix, Switzerland, Alcian blue)

Workflow – 28 days repeated exposure of cells (3 days/week)



Marked toxicity of 1R6F in 28-Day repeated exposure compared to HTP



HTP with Tobacco stick

1R6F Reference Cigarette

Puffs	Dilution	Exposure time [min]
16 / 32 / 48	1/2 (50% aerosol + 50% air)	8 / 16 / 24
16 / 32 / 48	1/14	~ 5

Puffing regime ISO 20778 55ml puff; 2 seconds duration; 30s puff interval

Fig A

Average nicotine concentration
in basal medium

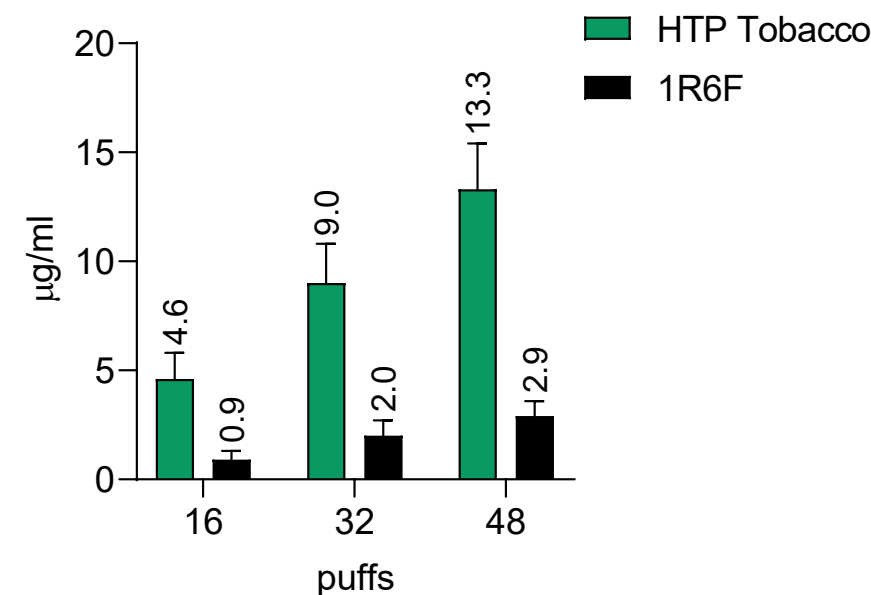
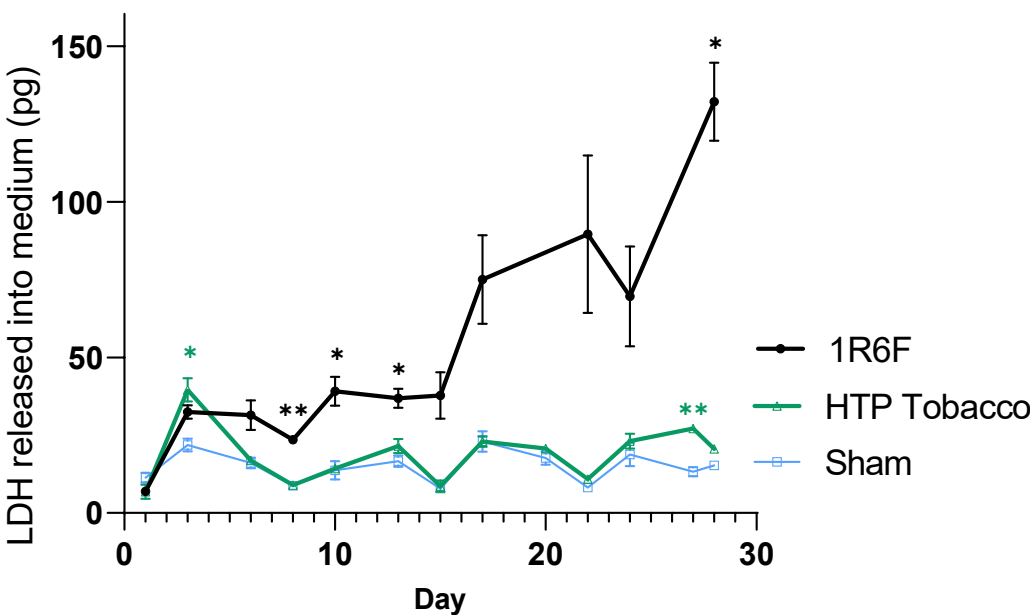


Fig B

48 puffs



Marked reduction in cell active area for 1R6F compared to HTP in 28-Day repeated exposure study

Fig A (diluted 1:2)

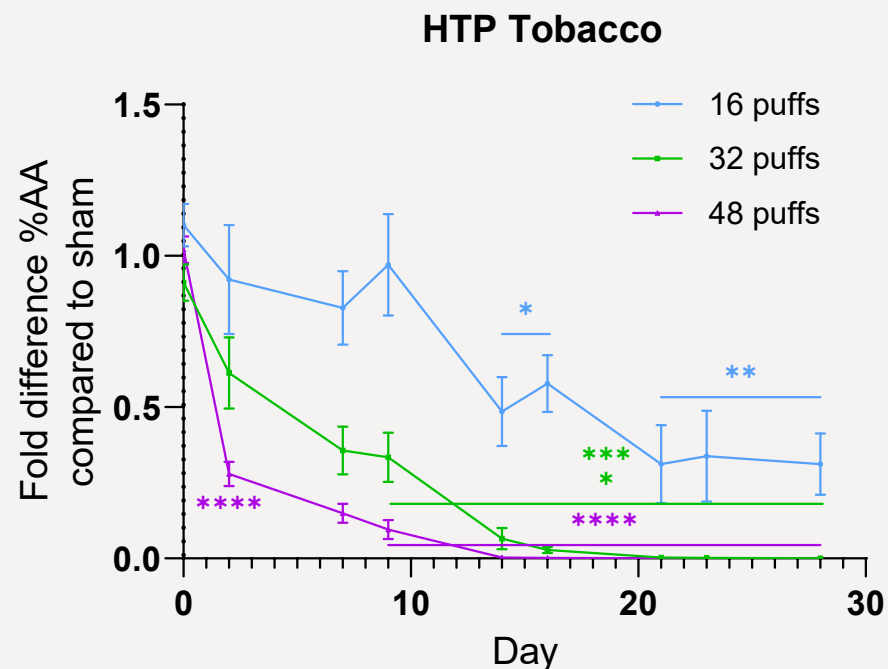
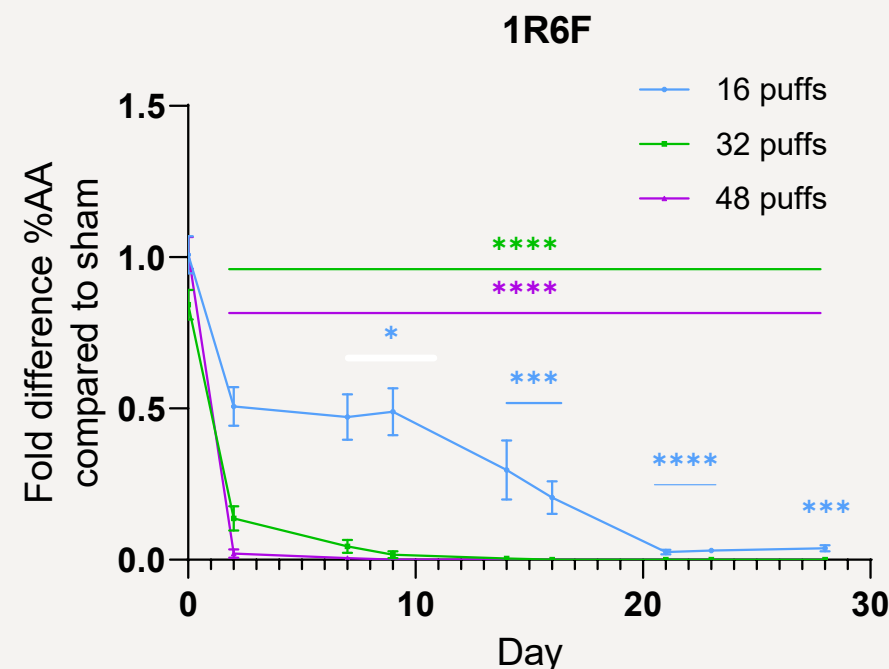


Fig B (diluted 1:14)



Active Area (AA) and Cilia beating frequency (CBF, not shown) was assessed with Sisson-Ammons Video Analysis (SAVA; Ammons Engineering, Clio, MI, USA)

Chapman, F., et al., (2023). Twenty-eight day repeated exposure of human 3D bronchial epithelial model to heated tobacco aerosols indicates decreased toxicological responses compared to cigarette smoke. *Frontiers in Toxicology*, 5, p.1076752.

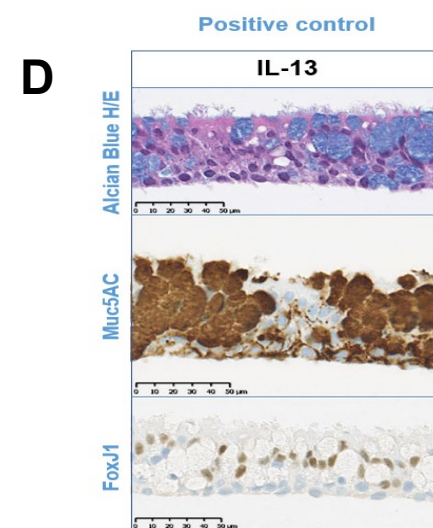
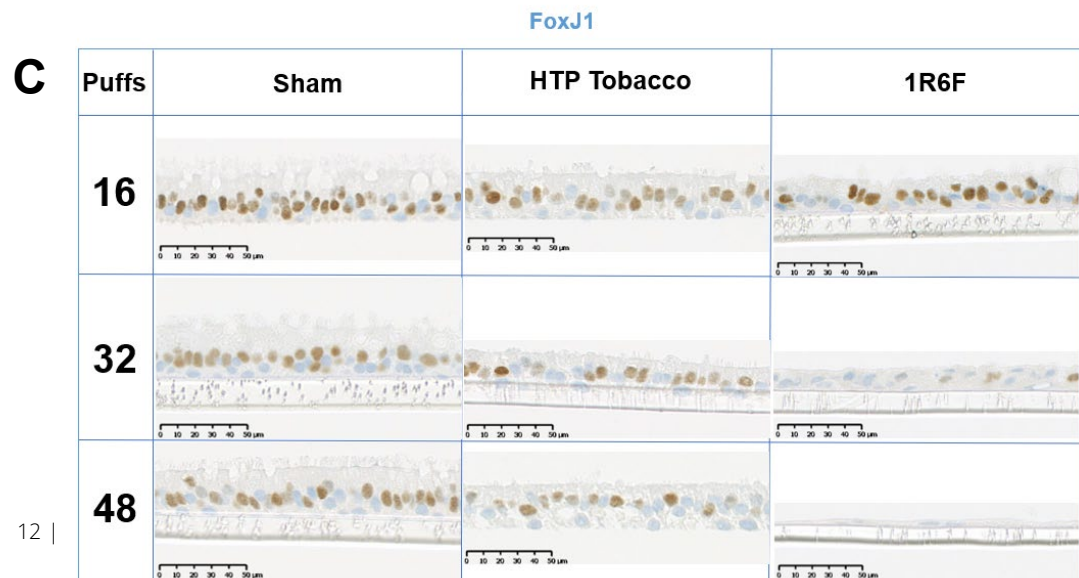
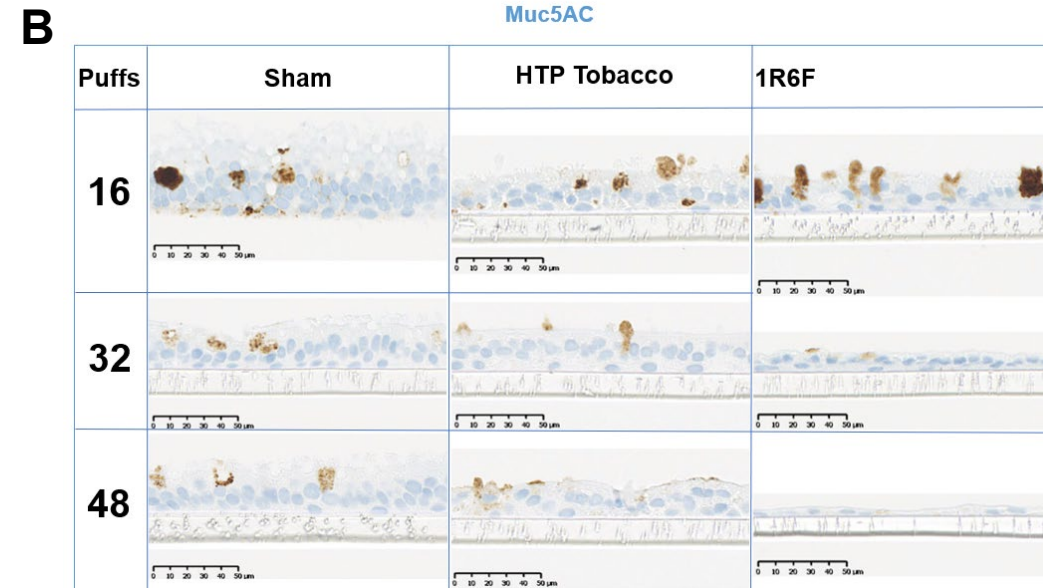
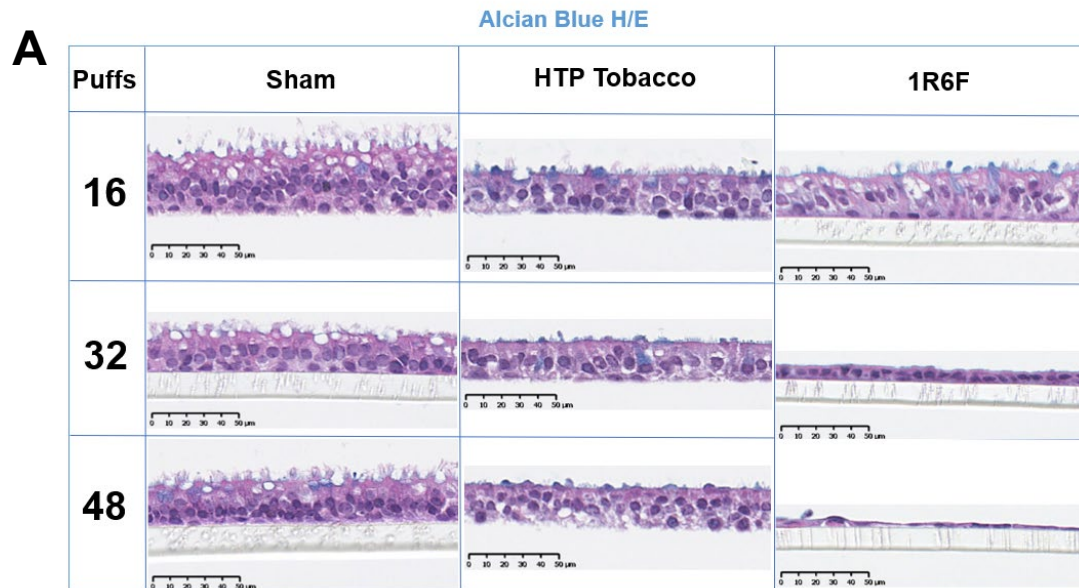
Behrsing, H.P., et al., (2022). Ciliary beat frequency: Proceedings and recommendations from a multi-laboratory ring trial using 3-D reconstituted human airway epithelium to model mucociliary clearance. *Alternatives to Laboratory Animals*, 50(4), pp.293-309.



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Marked histological effects for 1R6F seen vs HTP after repeated exposures for 28 days





Muc5AC = is the main mucin produced by the goblet cells in the tracheobronchial surface epithelium

FoxJ1 = the regulator of motile ciliogenesis

IL13 stimulates goblet cell hyperplasia increasing Muc5AC

EVP delivers higher nicotine and virtually non cytotoxic compared to combustible cigarettes

	EVP with Tobacco flavour and 2.4% nicotine	Puffs	Dilution	Exposure time [min]
	3R4F Reference Cigarette	30 / 60 / 90	1/1 (undiluted)	15 / 30 / 45
		30 / 60 / 90	1/17	~ 5

Puffing regime ISO 20778 (1R6F)/20768 (EVP) 55ml puff; 2/3 seconds duration; 30s puff interval

Fig A

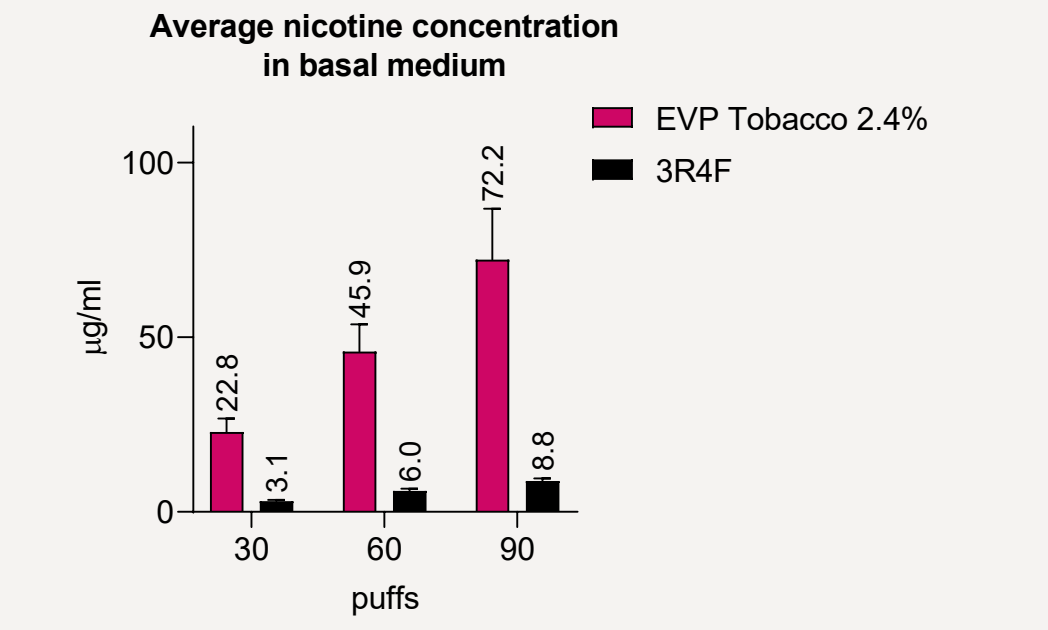
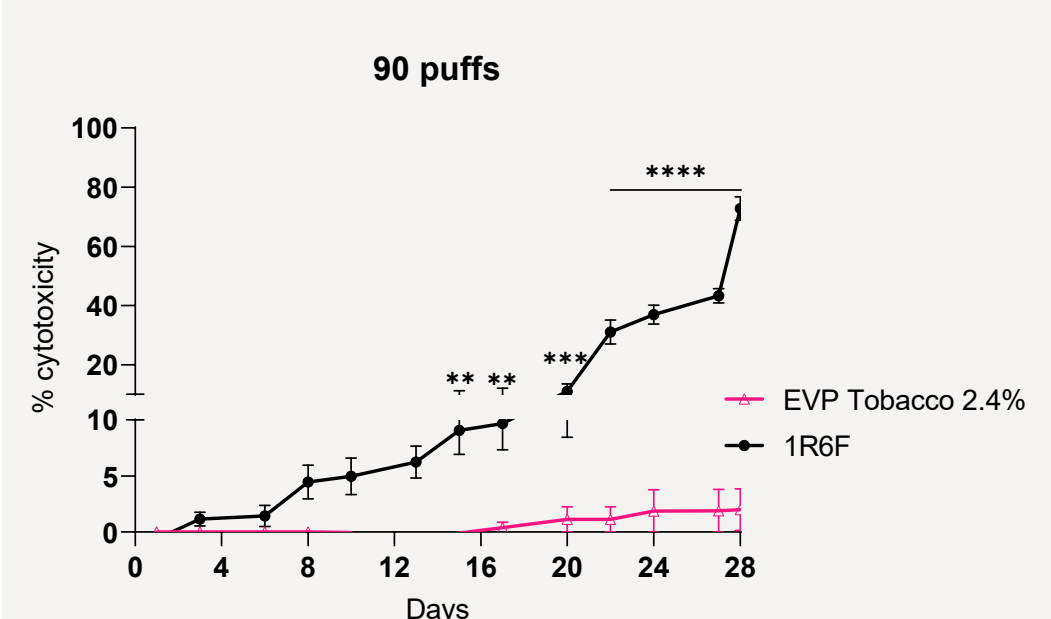


Fig B



Active area, 28-Day repeated exposure results for EVP and combustible cigarettes

Fig A EVP AA (undiluted)

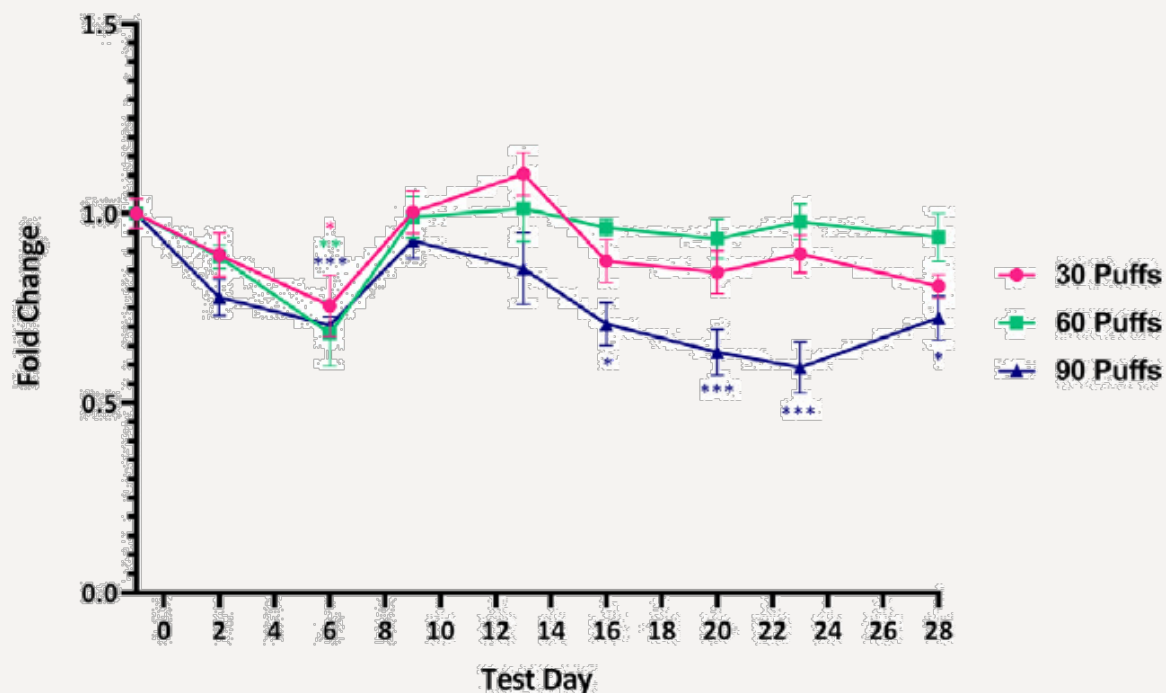
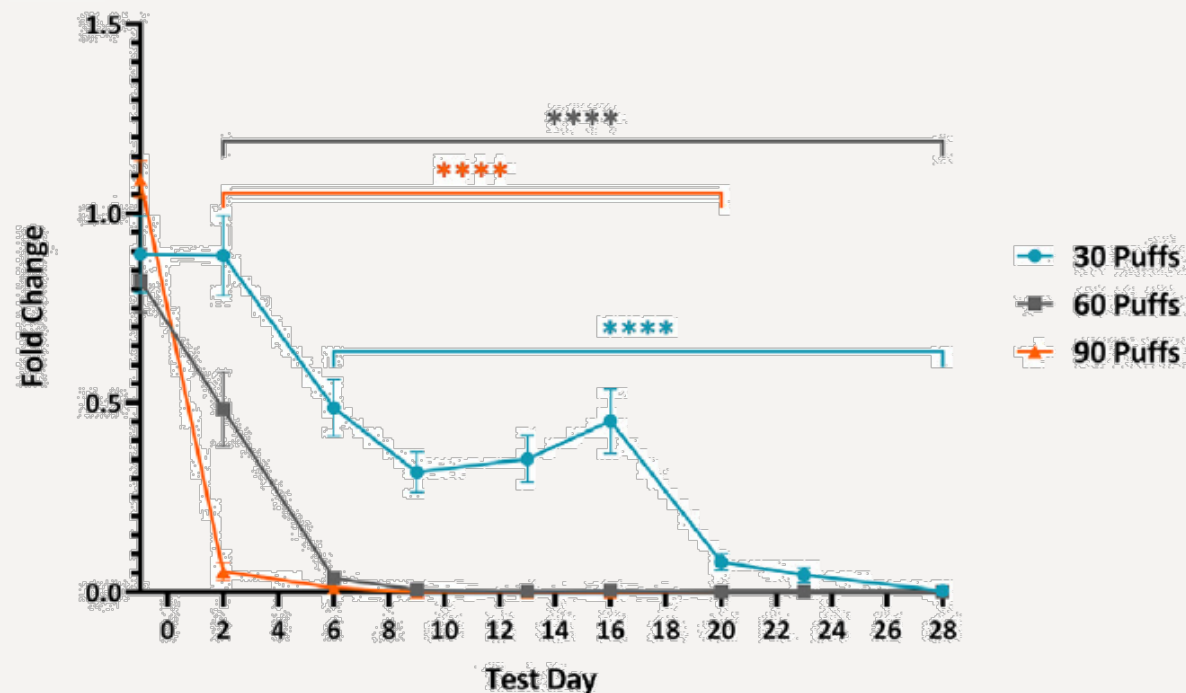
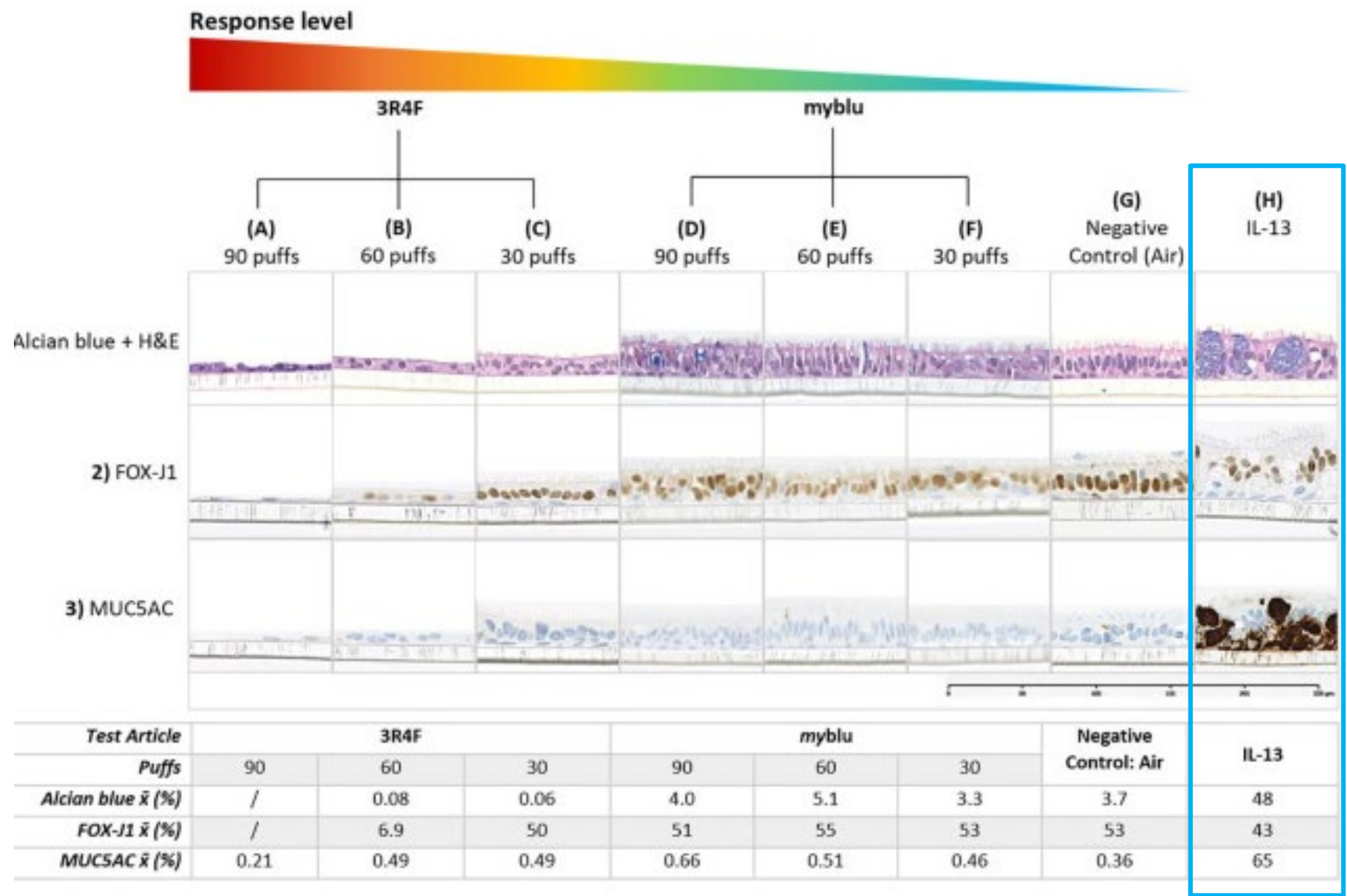


Fig B 3R4F AA (diluted 1:17)

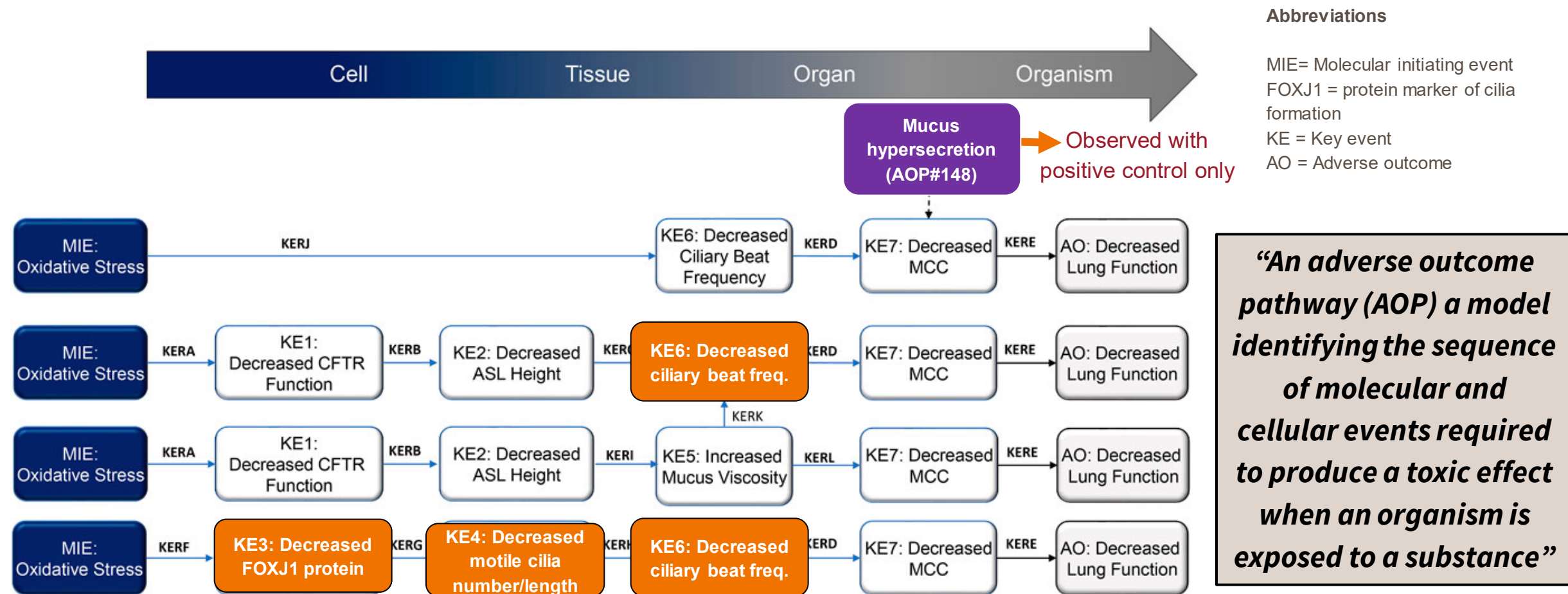


Marked histological effects of 3R4F following repeated exposures to 3D tissues, compared to EVP with minimal differences to the air control



How do NAMs support the standard battery?

Moving towards additional tests that are more predictive of human health and based on human cells allowing us to study selected Key Events of AOPs as outlined below in the repeated 3D studies (Luettich *et al.*, 2021)



Summary

- All components of whole smoke/ aerosol are relevant for toxicological evaluation
- Direct exposure of cells with fresh smoke/ aerosol at the ALI has the highest human relevance
- The relevance of the 3D models may be further increased using additional cells such as (e.g. endothelial cells and macrophages) as a source of IL13
- The use of AOPs helps organise biological data, following a proposed causal chain of events
- Further work could include the use of human pooled donor samples and co-cultures lung-liver for example

ACKNOWLEDGMENTS

- Biological Toxicology Laboratory, Epithelix for conducting the *in vitro* studies and cell staining
- Co-authors on the various manuscripts cited in this presentation

