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Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology

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ENDS US Conference, Arlington, VA, USA

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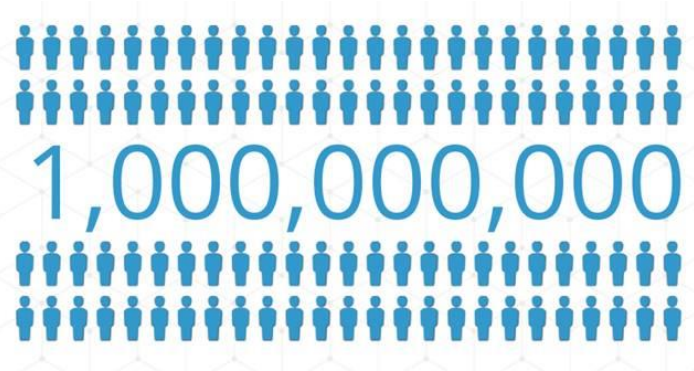
The research described in this presentation was sponsored by Philip Morris International.

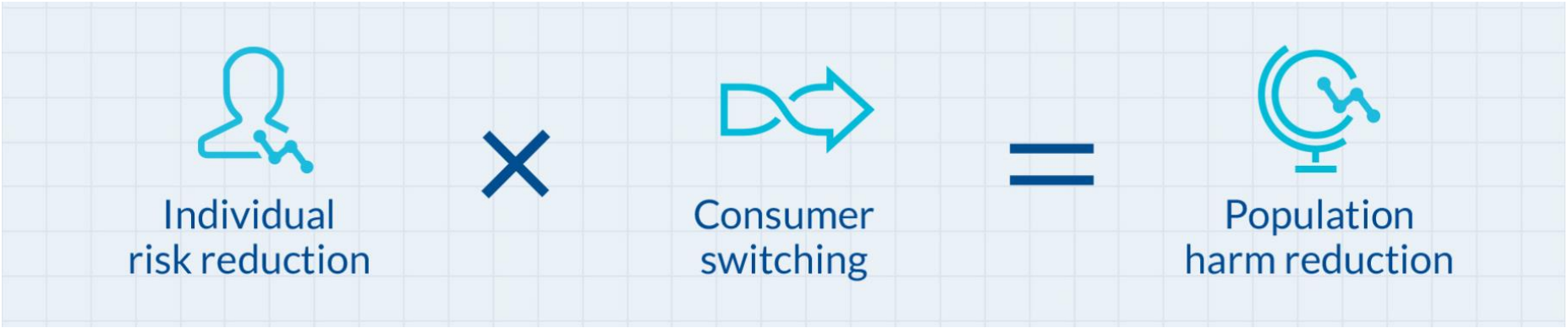
Biomedical Research at PMI

- Smoking causes serious diseases such as cardiovascular diseases, lung cancer, and chronic obstructive pulmonary disease.
- Philip Morris International is developing smoke-free products with the potential to reduce individual risk and population harm in comparison with smoking cigarettes.
- To determine whether such products have the potential to reduce individual risk, we are conducting extensive and rigorous scientific studies comparing their biological impact with that of a reference cigarette (3R4F) on a mechanism-by-mechanism basis.

What Is the Objective of Harm Reduction?

- Smoking is addictive and causes a number of serious diseases
- Worldwide, it is estimated that more than 1 billion people will continue to smoke in the foreseeable future*
- Offering smoke-free alternatives to adult smokers is a sensible, complementary addition to existing tobacco control strategies



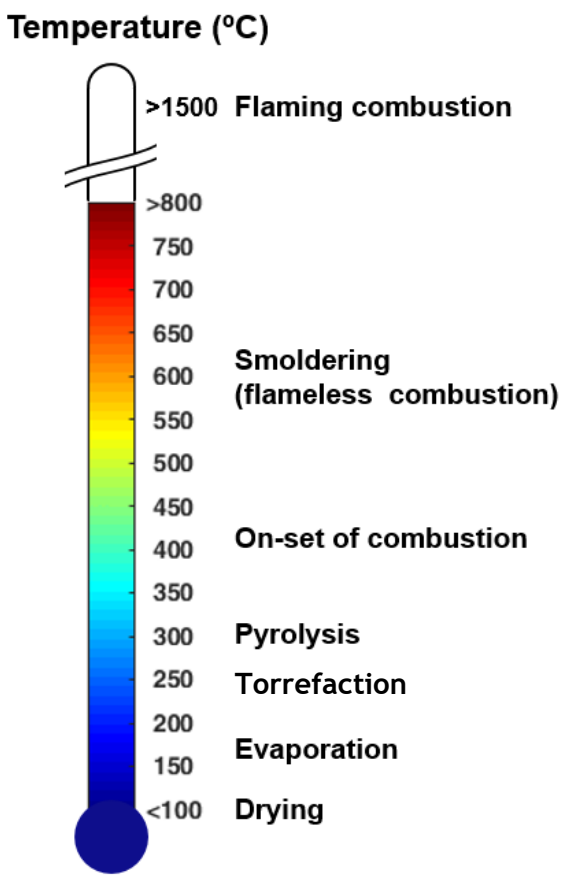


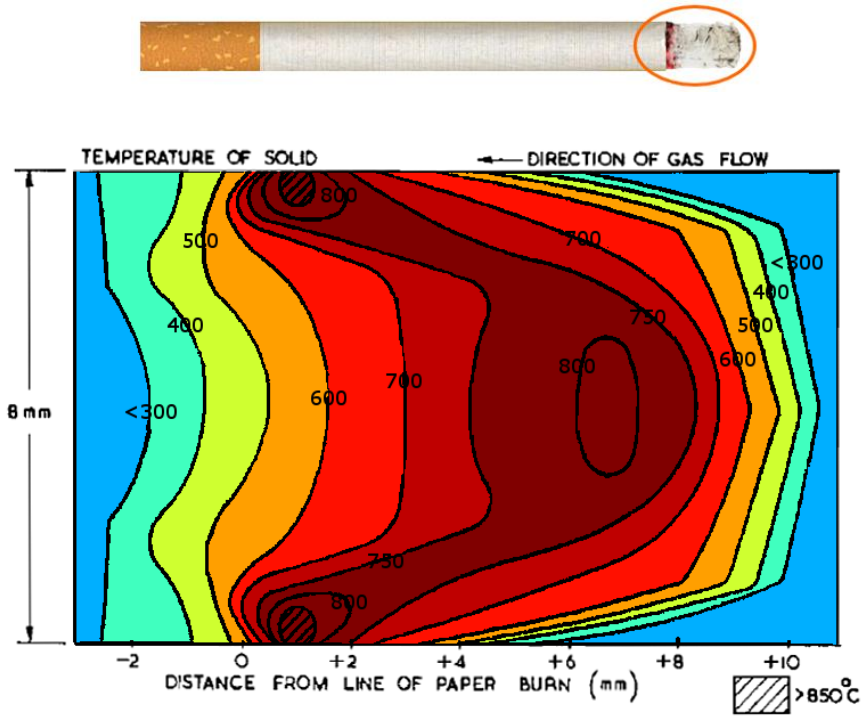
Successful harm reduction requires that current adult smokers be offered a range of Reduced-Risk Products (RRP) they can fully switch to, should they decide not to quit.

* http://www.who.int/tobacco/publications/surveillance/reportontrendstobaccosmoking/en/index4.html
Figure adapted from Clive Bates presentation to E-Cigarette Summit (19 Nov 2013)
Note: Reduced Risk Products ("RRP") is the term PMI uses to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switched to these products versus continued smoking.

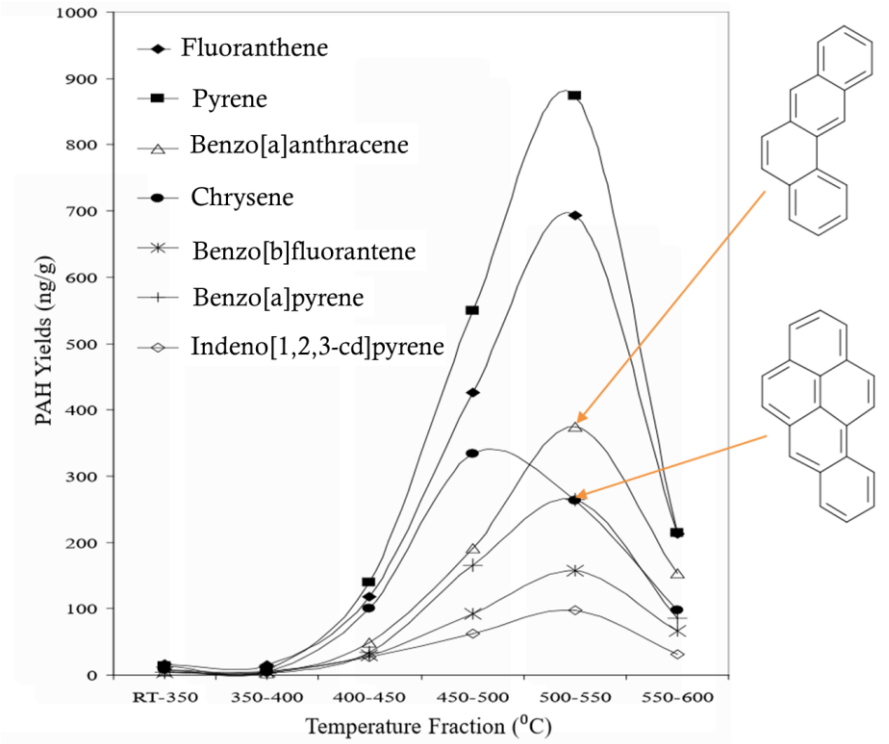
Elimination of Combustion Is Key

Scientific studies have shown that, as the temperature of tobacco increases, the levels of harmful chemicals formed increase





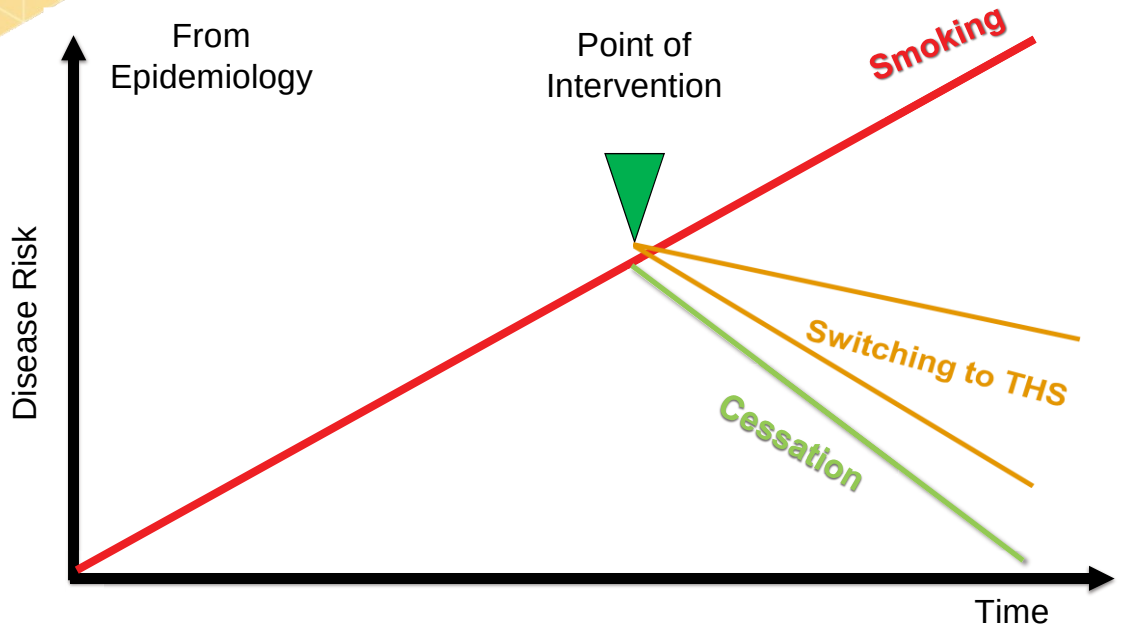
Source: Baker R. R., 1975, Temperature variation within a cigarette combustion coal during the smoking cycle, High Temp. Sci., 7, 236-247. Coloration by PMI.



Source: McGrath, T.E., Wooten, J.B., Chan W.G. and Hajaligol, M.R., 2007, Formation of polycyclic Aromatic Hydrocarbons from Tobacco: the "Link" between Low Temperature Residual Solid and PAH Formation, Food and Chemical Toxicology, 45,6,1039-1050

PMI's Scientific Assessment Approach

Assessment Framework



The assessment framework integrates what is known about cigarette (CC) smoking and incorporates both epidemiological and mechanistic evidence to define the assessment approach.

These assessment steps are designed to provide five levels of evidence as the assessment program is completed.

Source: Smith, M.R., et al., Evaluation of the Tobacco Heating System 2.2. Part 1: Description of the system and the scientific assessment program. *Regulatory Toxicology and Pharmacology* (2016). http://dx.doi.org/10.1016/j.yrtph.2016.07.006

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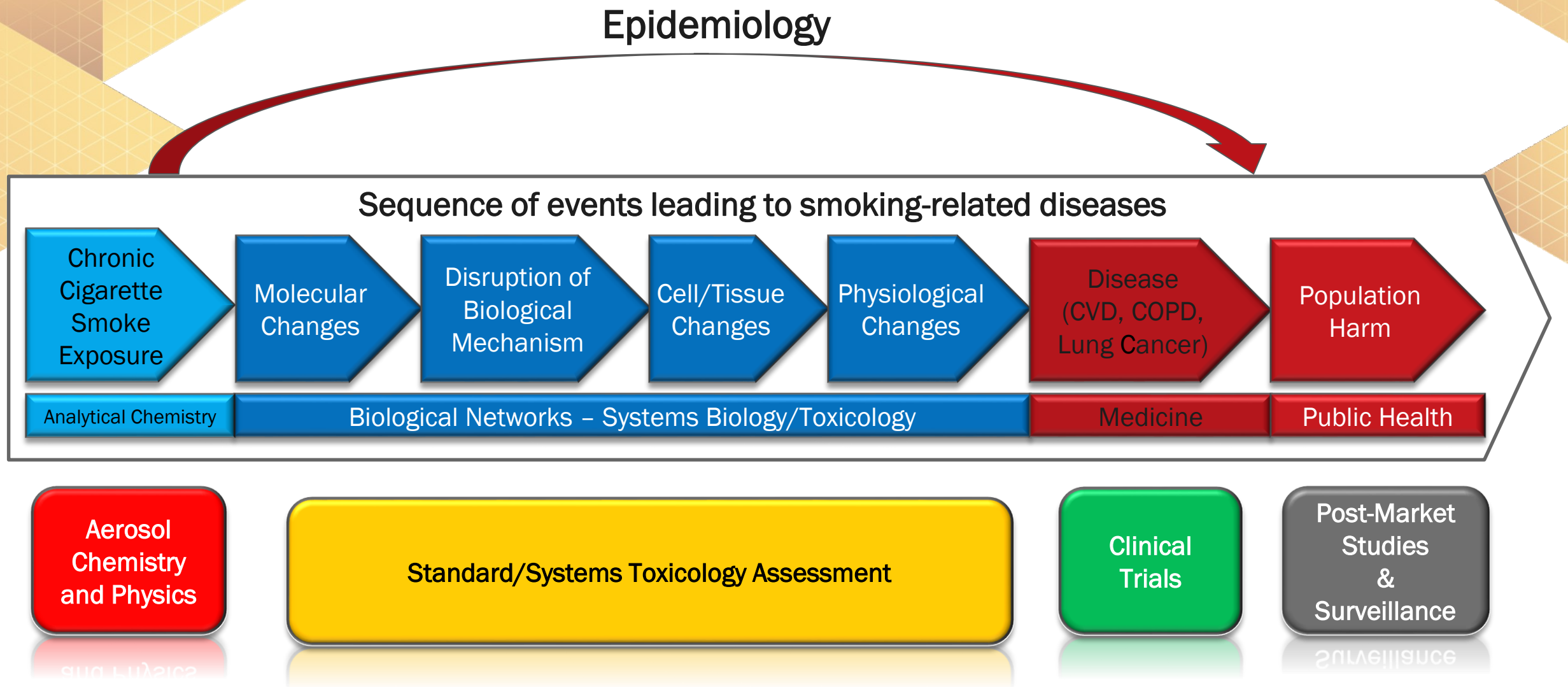
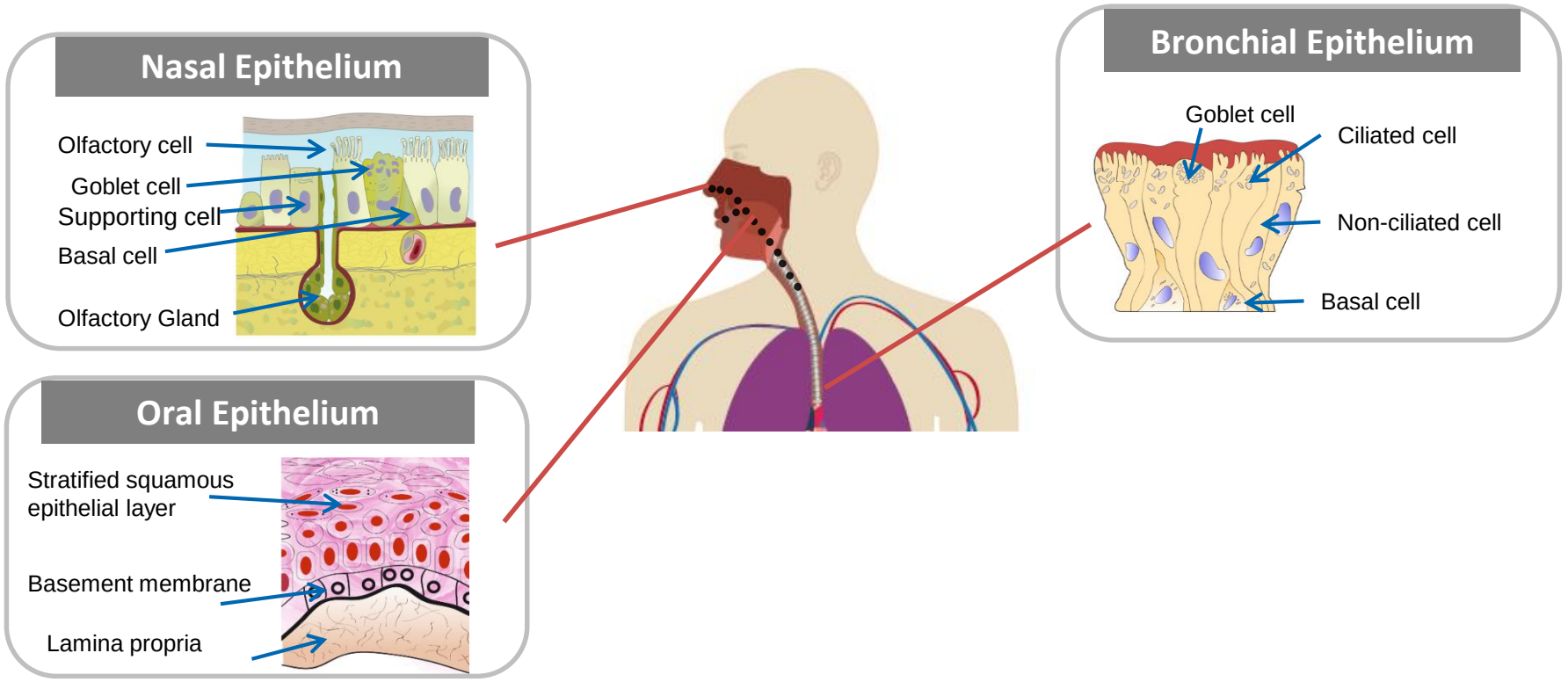
From Chronic Exposure to Population Harm: A Causal Chain of Events



Smith, M. R., et al. (2016). "Evaluation of the Tobacco Heating System 2.2. Part 1: description of the system and the scientific assessment program." Regulatory Toxicology and Pharmacology 81: S17-S26.

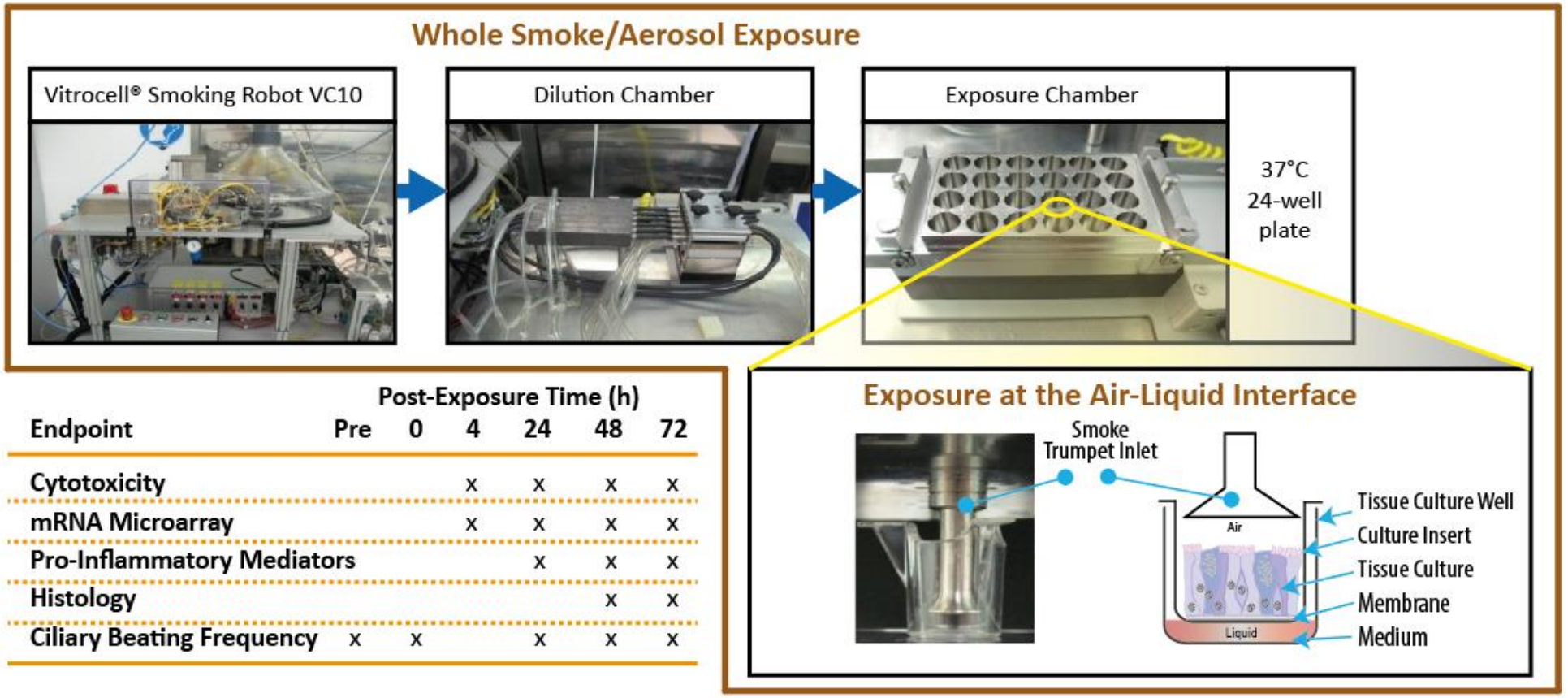
In Vitro Toxicology: Relevant Test Systems

- An EU directive "on the protection of animals used for scientific purposes" (EU Directive 2010/63/EU) strongly promotes the use of alternative animal test methods.
- 3R (Replace, Reduce, and Refine), a strategy for alternative methods to animal testing, was described in 1959 by Russell and Burch. One significant strategy to avoid animal testing is the use of cell systems where cells from different animals and tissues are grown and tested in plates or wells (in vitro).
- Both cell lines and primary cell cultures have limitations, and the results might be difficult to interpret or cannot be extrapolated to help elucidate possible in vivo effects. Relevant biological test systems facilitate identification of biomarkers of exposure response and disease.



Sridhar S, et al. (2008) Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. BMC genomics 9: 259

Relevant Exposure Modes for Assessment of Aerosols

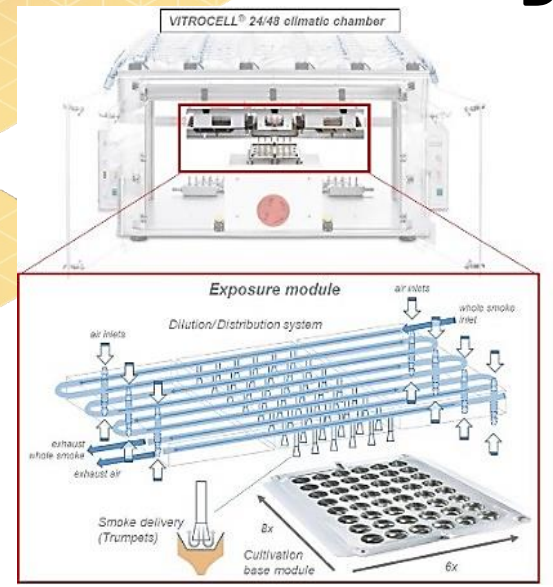


Experimental Data Workflow

Upper panel: Organotypic cultures of human primary respiratory epithelial cells can be exposed directly to 3R4F smoke or electronic nicotine delivery system (ENDS) aerosols by using the Vitrocell® system.
Lower panel: The cells were exposed to smoke/aerosols during different exposure times. Then, various endpoints were captured after different post-exposure times.

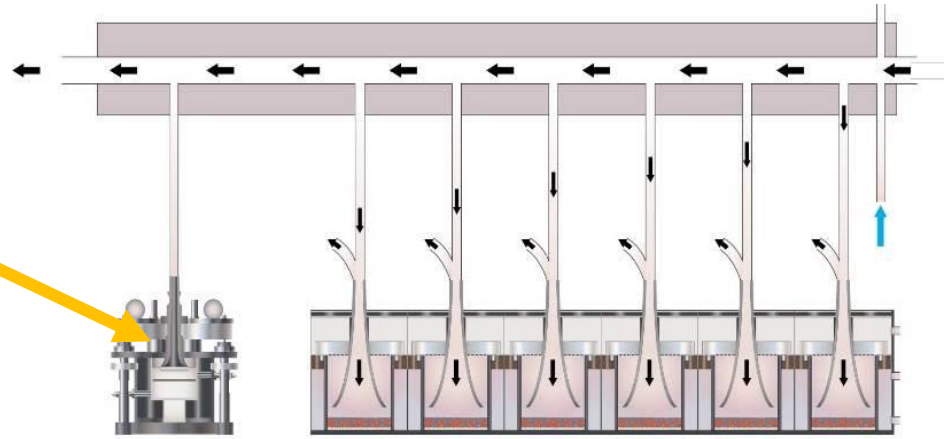
Majeed, S., et al. (2014). "Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke." Chemistry Central Journal 8(1): 62.
Thorne, D., et al. (2013). "Characterisation of a Vitrocell(R) VC 10 in vitro smoke exposure system using dose tools and biological analysis." Chemistry Central Journal 7(1): 146

Schematic Representation of the Vitrocell® 24/48 Exposure System Facilitating Aerosol Delivery to Air–Liquid Interface Cultures

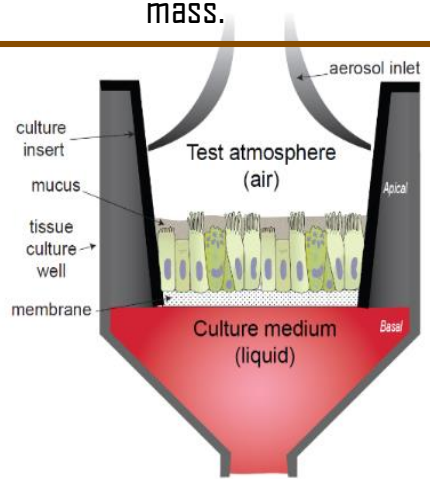


A climatic chamber with an exposure module

Up to 48 wells can be exposed simultaneously.
The base module has a format of 8 rows x 6 columns.



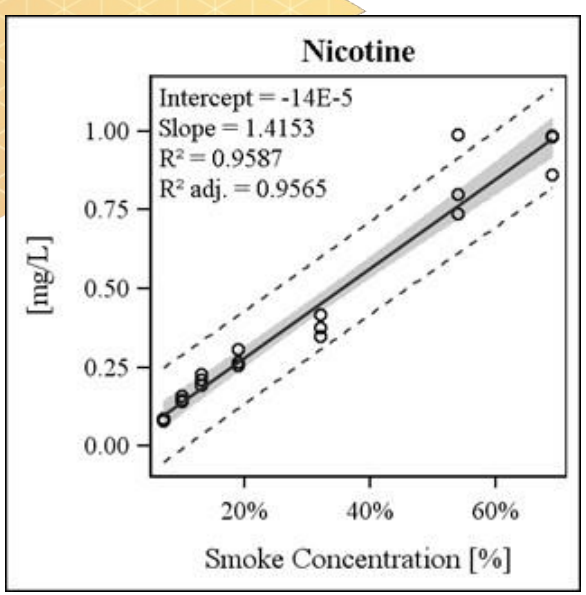
Particles depositing on the quartz crystal microbalance are detected by a change in its resonance frequency, which is proportional to the deposited mass.

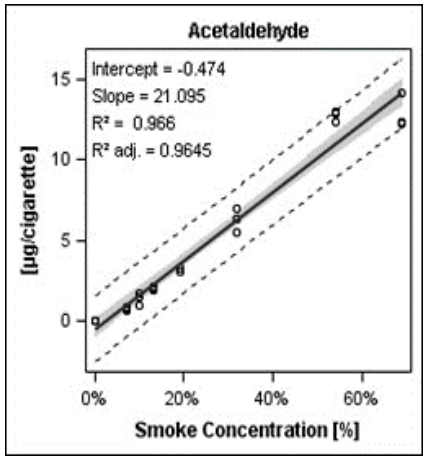


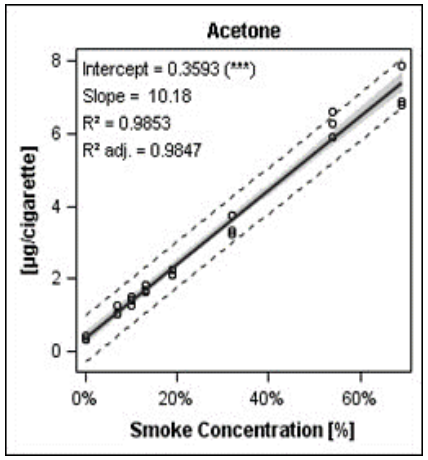
Cells are grown at the air–liquid interface on porous membranes and supplied with medium from their basal side.

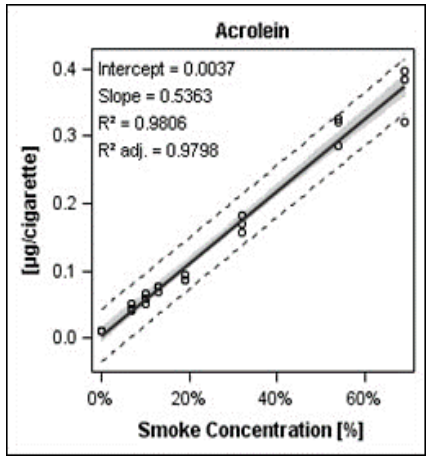
Majeed, S., et al. (2014). "Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke." Chemistry Central Journal 8(1): 62.

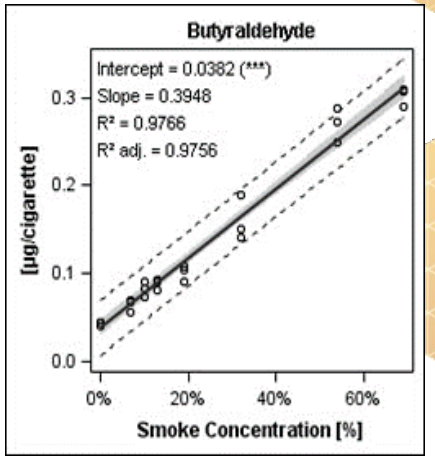
Linear Correlation Between the Concentrations of Trapped Nicotine and Carbonyl and the Concentration of cigarette smoke (CS) Inside the Vitrocell® 24/48 System

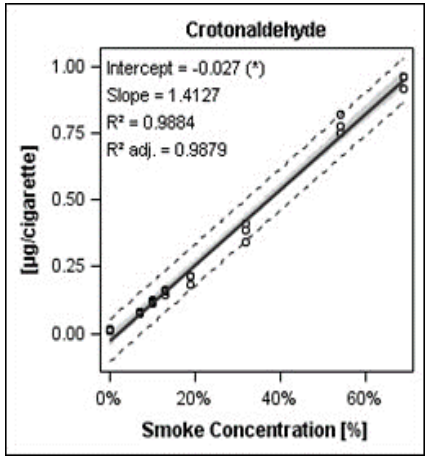


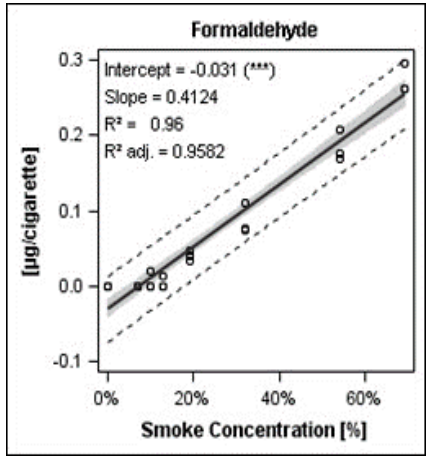


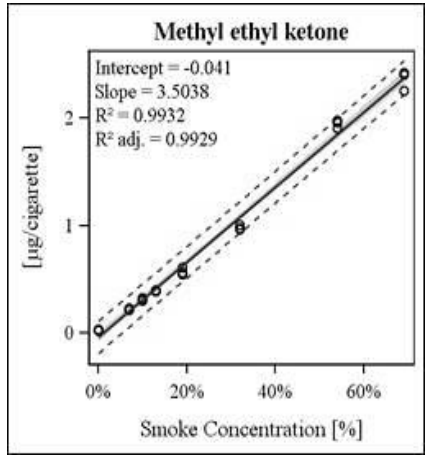


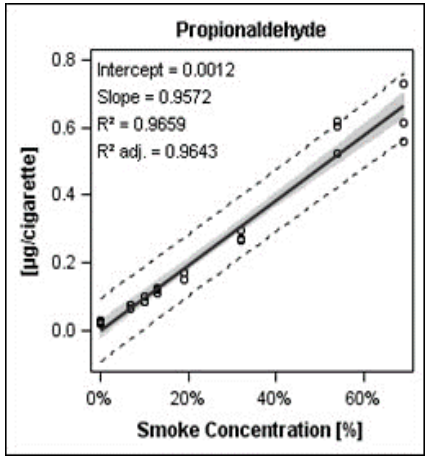












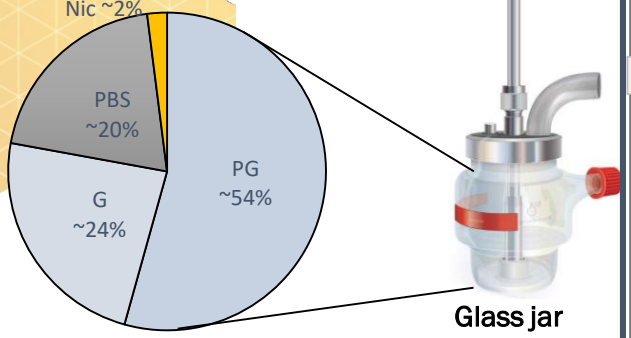
Whole-smoke application (multi-dilution) and quantification of nicotine and 8 different carbonyls inside the VITROCELL® 24/48 exposure chamber over a concentration range of 7–69% cigarette smoke.

Majeed, S., et al. 2014. Characterization of the Vitrocell(R) 24/48 in vitro aerosol exposure system using mainstream cigarette smoke. Chemistry Central journal, 8, 62.

Aerosol Characterization in In Vitro Exposure Studies

Compressed air heated up to 37°C

Nebulizer

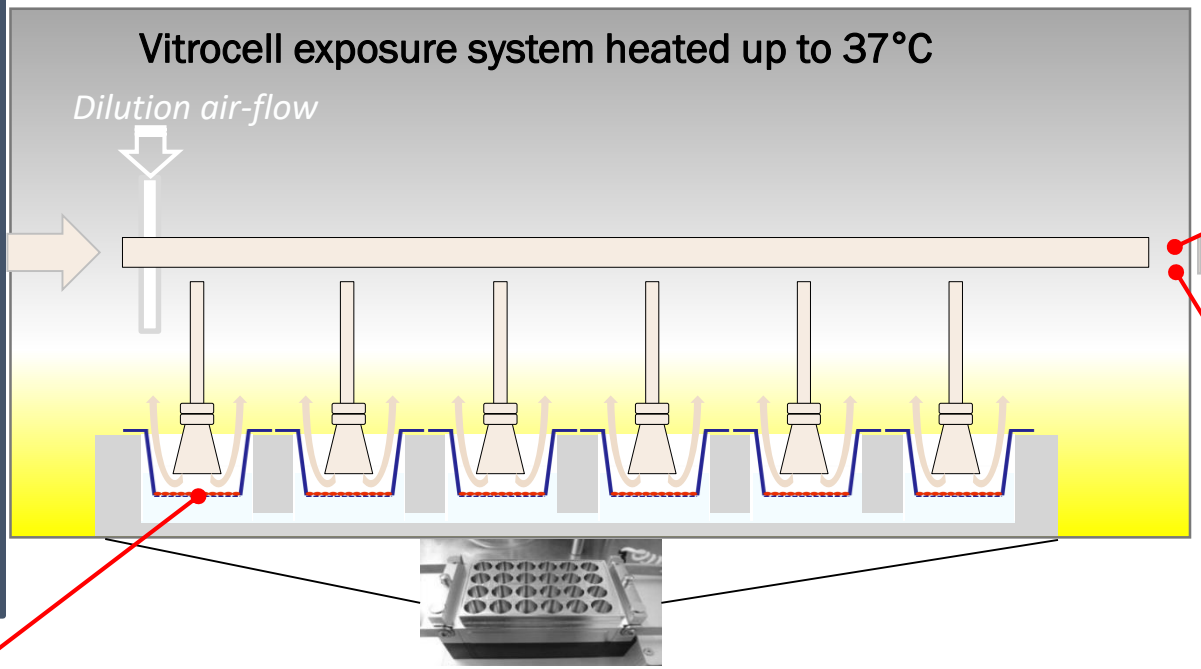


E-liquid mixture composition

Glass jar containing e-liquid heated up to 37 °C

Vitrocell exposure system heated up to 37°C

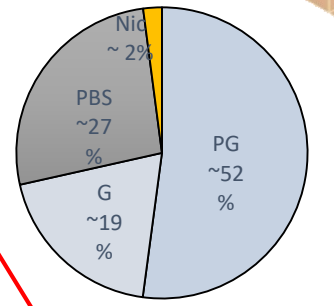
Dilution air-flow



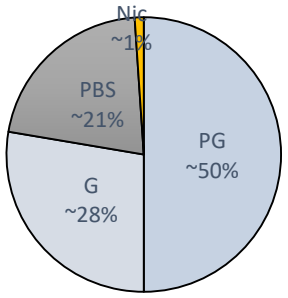
Aerosol trapping on a Cambridge filter pad

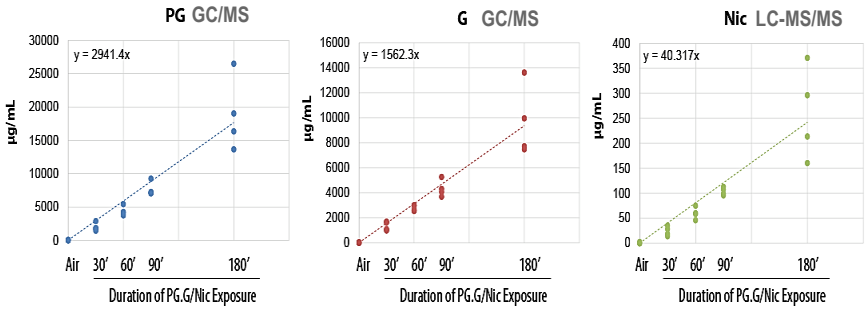
Filter holder

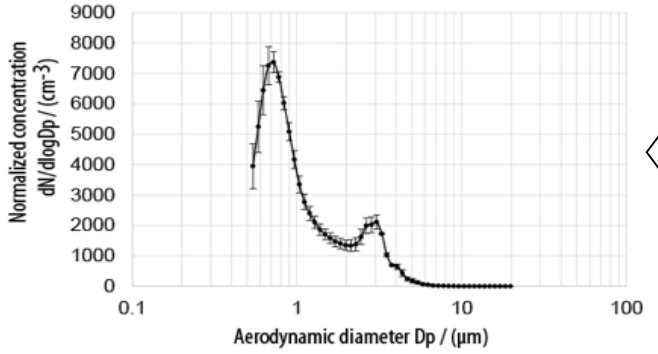
GC-FID (flame ionization detector)



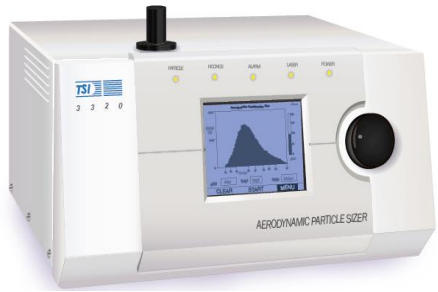
PG, G, and Nic deposition in phosphate-buffered saline





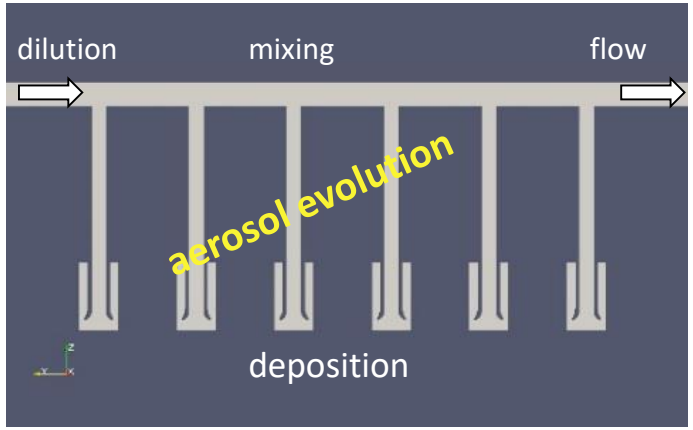


Aerodynamic particle sizer

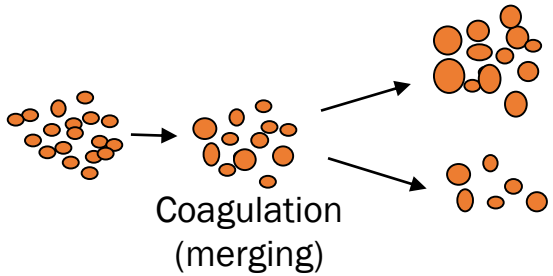


Modeling Transport and Evolution of Aerosols for Computing Deposition in Air–Liquid Interface Experiments

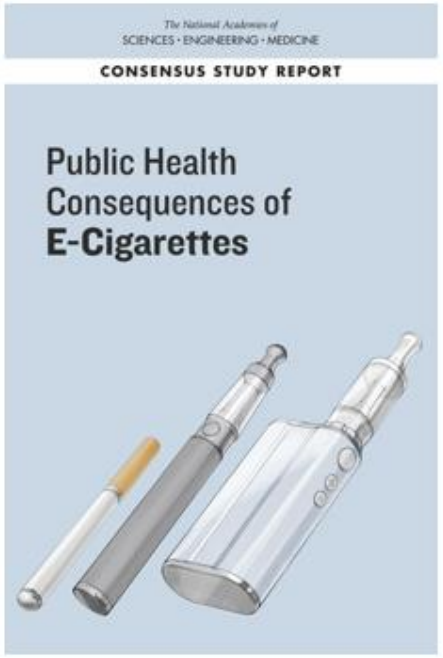
- Computational fluid dynamics efforts concerning physical aerosol characterization facilitate accurate computation of deposition rate
  - Droplet size
  - Droplet number density
- Mixing efficiency of the dilution system
  - Investigation of the required residence time for the aerosol to reach a uniform particle number concentration in the exposure system
- Stability of aerosol in the dilution system
  - Assessment of the physical characteristics of aerosol undergoing dilution and transport in the exposure system
- Influence of operating conditions and physical mechanisms on aerosol deposition
  - Flow speed, temperature, relative humidity
  - Inertia, gravitational settling



Sketch of one-row flow path in a multi-well of the Vitrocell® exposure system



Aerosol Evolution/Aging



"There is substantial evidence that except for nicotine, under typical conditions of use, exposure to potentially toxic substances from e-cigarettes is significantly lower compared with combustible tobacco cigarettes" NAS, 2018

JAMA Network | Open

Original Investigation | Public Health

Comparison of Nicotine and Toxicant Exposure in Users of Electronic Cigarettes and Combustible Cigarettes

Maciej L. Goniewicz, PharmD, PhD; Danielle M. Smith, MPH; Kathryn C. Edwards, PhD; Benjamin C. Blount, PhD; Kathleen L. Caldwell, PhD; Jun Feng, PhD; Lanqing Wang, PhD; Carol Christensen, PhD; Bridget Ambrose, PhD; Nicolette Borek, PhD; Dana van Bemmel, PhD; Karen Konkel, PhD; Gladys Erives, PhD; Cassandra A. Stanton, PhD; Elizabeth Lambert, MSc; Heather L. Kimmel, PhD; Dorothy Hatsukami, PhD; Stephen S. Hecht, PhD; Raymond S. Niaura, PhD; Mark Travers, PhD; Charles Lawrence, PhD; Andrew J. Hyland, PhD

…"[The] Findings suggest exclusive e-cigarette use results in measurable exposure to tobacco-related constituents; however, compared with cigarette smoking, biomarker concentrations of nicotine and toxicants among e-cigarette–only users were much lower"
Goniewicz et al., 2019

Challenges in Toxicity Assessment of Electronic Cigarettes

Lack of Standards for Selection of Chemicals to Be Monitored

- The list of HPHCs—established for cigarettes—is not applicable to ENDS

Lack of Standards for Analytical Methods

- Increase sensitivity and reproducibility
- Allow comparison among studies

Lack of Standards for Testing Potential Toxicity of Inhaled Flavors

- "Generally recognized as safe" as currently used for food ingredients is informative but might not be applicable for inhalation

Lack of Standards for Aerosol Generation

- Puffing regimen and coil temperature impact chemical generation (i.e., carbonyls)
- Vaping topography is heterogeneous
- CORESTA recommendation (recently developed – Method No. 81) https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf

FARSALINOS, K. E. & LE HOUEZEC, J. 2015. Regulation in the face of uncertainty: the evidence on electronic nicotine delivery systems (e-cigarettes). Risk Manag Healthc Policy, 8, 157-67.FLORA, J. W., MERUVA, N., HUANG, C. B., WILKINSON, C. T., BALLENTINE, R., SMITH, D. C., WERLEY, M. S. & MCKINNEY, W. J. 2016. Characterization of potential impurities and degradation products in electronic cigarette formulations and aerosols. Regulatory Toxicology and Pharmacology, 74, 1-11.
DAVIS, B., DANG, M., KIM, J. & TALBOT, P. 2015. Nicotine concentrations in electronic cigarette refill and do-it-yourself fluids. Nicotine Tob Res, 17, 134-41.
TIERNEY, P. A., KARPINSKI, C. D., BROWN, J. E., LUO, W. & PANKOW, J. F. 2015. Flavour chemicals in electronic cigarette fluids. Tob Control.

Various Types of Electronic Cigarettes

There are 8000 flavors now available and around 242 new flavors added every month.

Generic Combustible Tobacco Cigarette	First-Generation Electronic Cigarette	Second-Generation Electronic Cigarette	Third-Generation Electronic Cigarette

Electronic Cigarettes

Shown to demonstrate approximate scale (size).

Taken from the "Public Health Consequences of E-Cigarettes." The National Academies Press. 2018.

The illustrations are intended to be generic representation of a device within each category. They are not meant to represent any specific product.

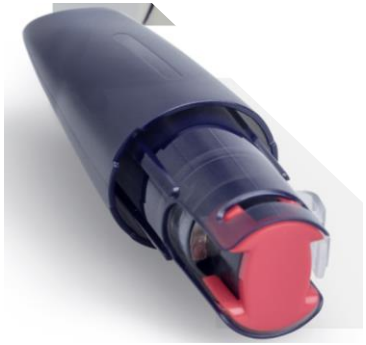
BALS, R., BOYD, J., ESPOSITO, S., FORONJY, R. & HIEMSTRA, P. S. 2019. Electronic cigarettes: a task force report from the European Respiratory Society. 53.

TIERNEY, P. A., KARPINSKI, C. D., BROWN, J. E., LUO, W. & PANKOW, J. F. 2016. Flavour chemicals in electronic cigarette fluids. *Tobacco Control,* 25, e10-e15.

NAS 2018. Public health consequences of e-cigarettes. Washington, DC: The National Academies Press. doi: https://doi.org/10.17226/24952.

A Novel Electronic Cigarette with MESH™ Technology

- The IQOS MESH uses closed-system e-liquid caps to prevent tampering and liquid leakage
- A new heating element and mouth piece are built into each replaceable cap to maintain product hygiene
- The current-generating wick is eliminated, and the coil is replaced with a metal mesh
- The temperature of the heater is controlled and maintained between 200–220°C rather than varying depending on the puff strength
- A low-liquid detection system will cut off the power supplied to the mesh heater once the level of the liquid has dropped below a certain level, eliminating dry puffs



Cap containing e-liquids

In Vitro Multilayer Assessment with a Systems Toxicology Approach by using 2D and 3D Airway Epithelial Cultures

First Layer Assessment

2D NHBE (Normal human bronchial epithelial) cells

24 h

- Incubation with *MESH* Classic Tobacco liquid (containing PG, G, nicotine and flavors)
- Incubation with Base liquid (containing PG, G, and nicotine without flavors)
- Incubation with the total particulate matter (TPM) of 3R4F reference cigarette smoke

ENDPOINTS

Cytotoxicity measurement using real-time cell analysis (RTCA)

Second Layer Assessment

2D NHBE

4 h

24 h

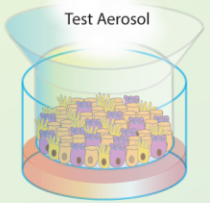
- Incubation with *MESH* Classic Tobacco liquid (containing PG, G, nicotine and flavors)
- Incubation with Base liquid (containing PG, G, and nicotine without flavors)
- Incubation with the TPM of 3R4F reference cigarette smoke

ENDPOINTS

High content screening assays:
- Cell membrane permeability
- Cytochrome c release
- DNA damage (pH2AX)
- Glutathione content
- Oxidative stress (ROS)
- Stress kinase (c-Jun)

(NHBE: Normal human bronchial epithelial) cells

Third Layer Assessment



3D Human Organotypic Epithelial Cultures (Buccal and Small Airway)

Various Doses*

- Exposure to *MESH* Classic Tobacco aerosol
- Exposure to Base aerosol
- Exposure to cigarette smoke
- Exposure to air

ENDPOINTS
4 h Post-exposure
- mRNA microarray
- miRNA microarray

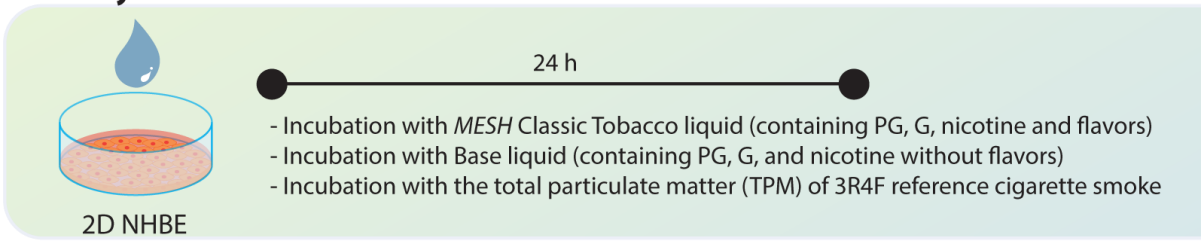
ENDPOINTS
24 h Post-exposure
- mRNA microarray
- miRNA microarray

ENDPOINTS
48 h Post-exposure
- Adenylate kinase measurement
- Histology analysis
- mRNA microarray analysis
- miRNA microarray analysis
- Targeted proteomics analysis (PRM)†
- Multi-analyte profiling (MAP) of secreted inflammatory mediators

ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

Cell Viability Was Impacted to a Greater Degree in 2D NHBE Cells Treated with 3R4F Total Particulate Matter (TPM) than in Cells Treated with MESH Classic Tobacco or Base E-Liquids

First Layer Assessment

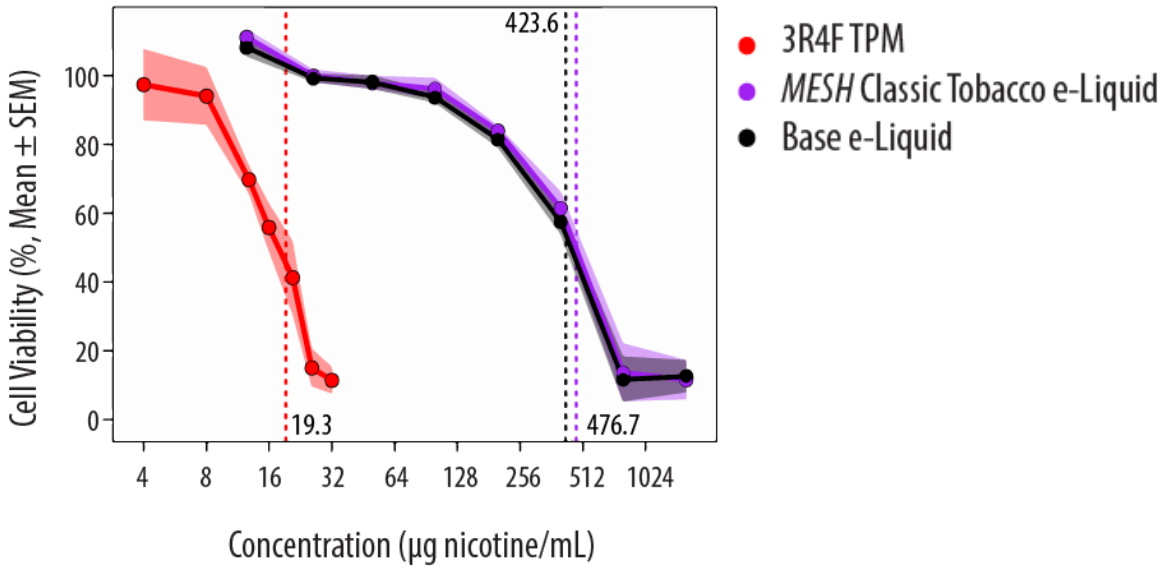


	PG (%)	VG (%)	Nic (%)	Flavors	Other (e.g., Water)
MESH Classic Tobacco Liquid*	39	39	1.8	✓	✓
Base Liquid	39	39	1.8	✓	✓

*The pH is around 8.5

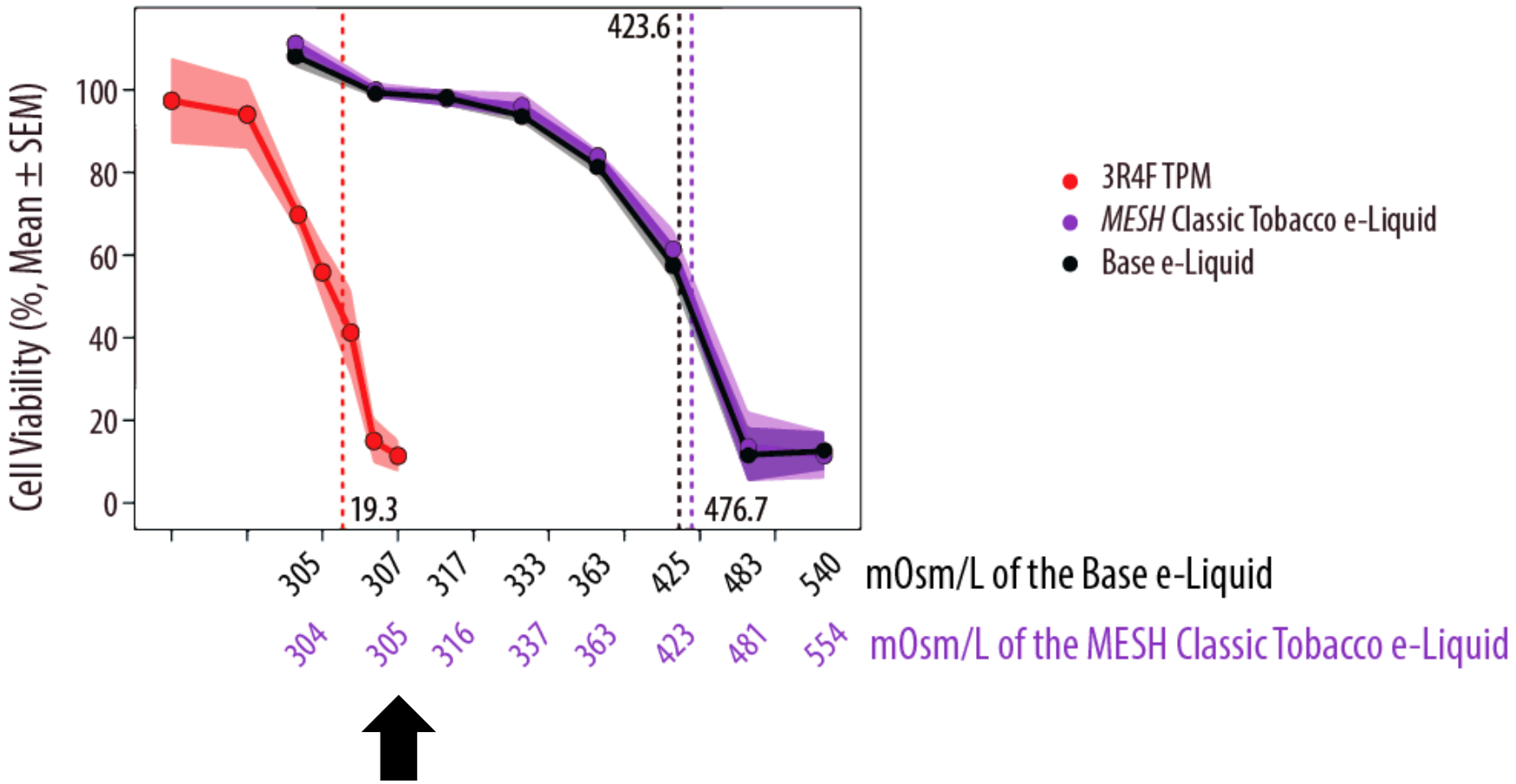
Cigarette smoke TPM extract impacted cell viability to a much greater degree than MESH Classic Tobacco and Base Liquids when compared relative to their nicotine concentrations.

Cytotoxicity Measurement Using RTCA



ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

Cytotoxicity of MESH Classic Tobacco and Base Liquids Was Generally Attributed to the Increasing Osmolarity of the Liquids

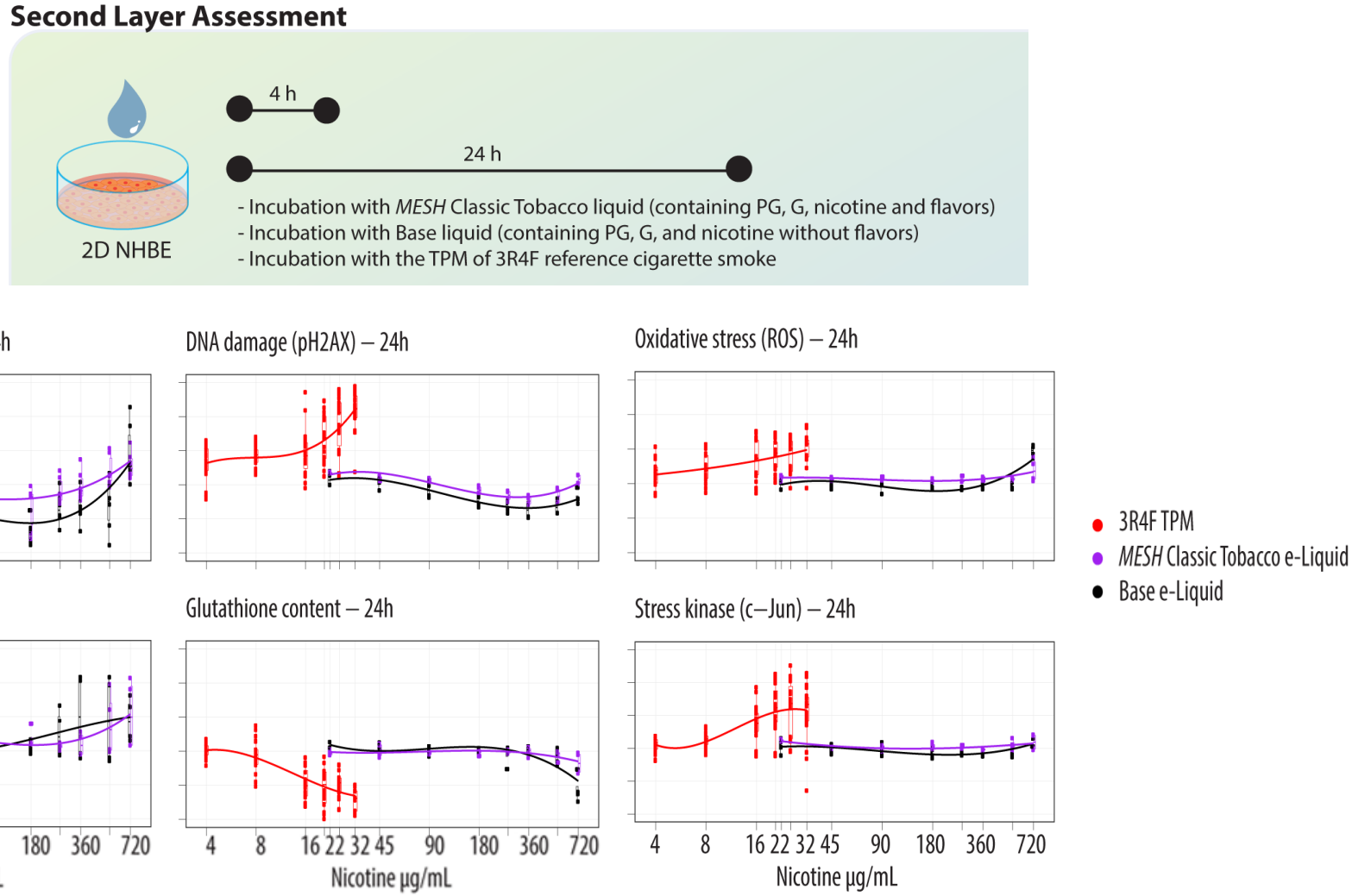


Isotonic fluids generally have an osmolarity of 270–310 mosm/L (Liu et al).

ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

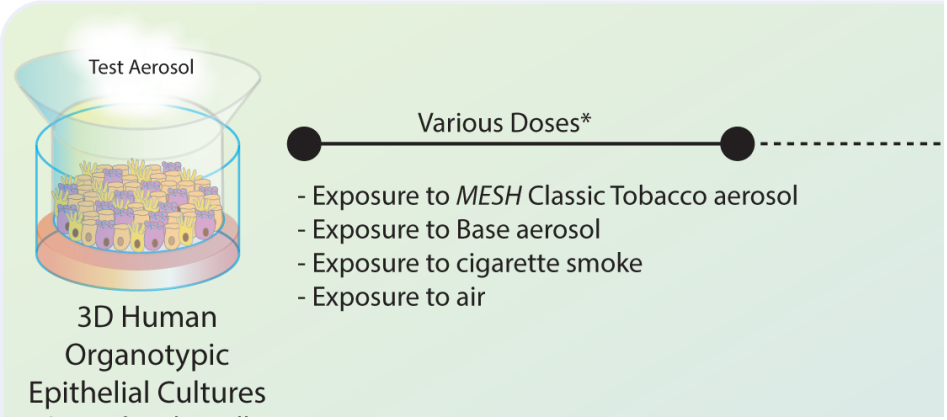
LIU, D. T. & SILVERSTEIN, D. C. 2015. Chapter 58 - Crystalloids, Colloids, And Hemoglobin-Based Oxygen-Carrying Solutions. In: SILVERSTEIN, D. C. & HOPPER, K. (eds.) Small Animal Critical Care Medicine (Second Edition). St. Louis: W.B. Saunders.

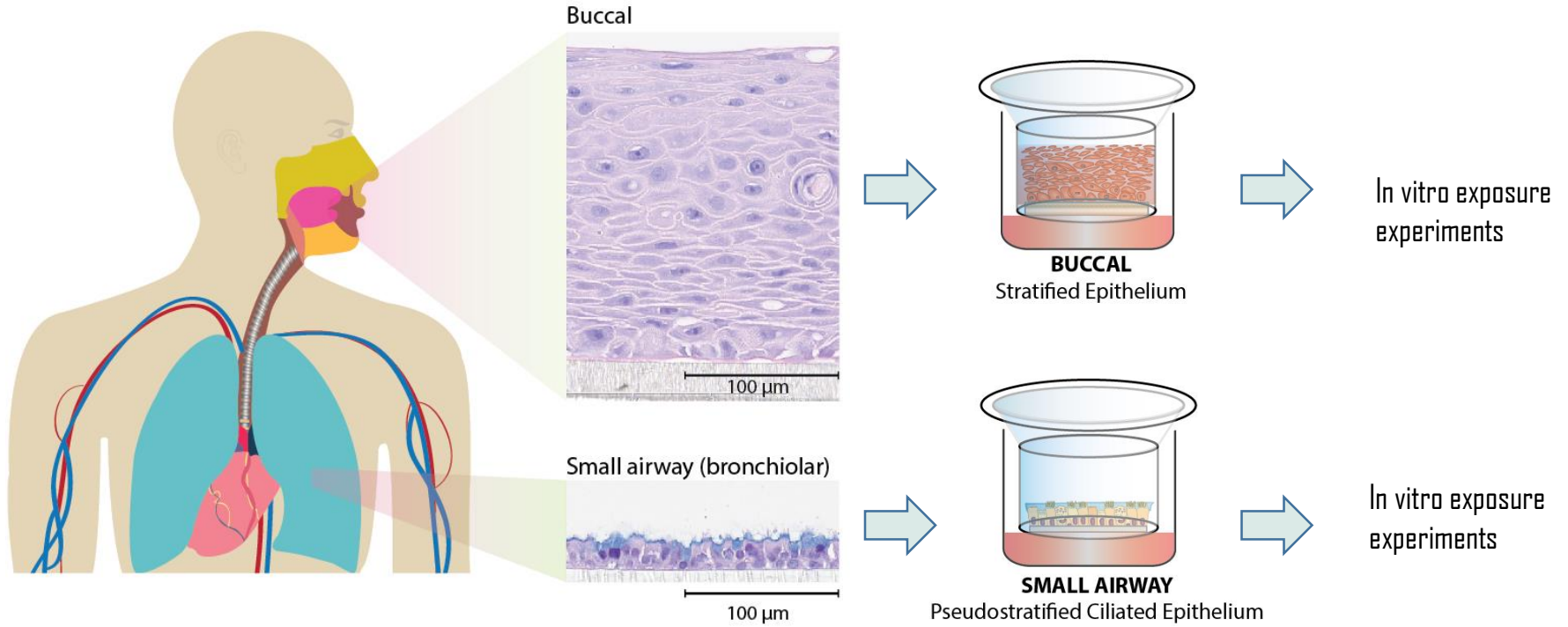
Changes in Cellular Markers Were Not Detected in 2D NHBE Cultures Treated with MESH Classic Tobacco and Base E-Liquids Unlike in Cultures Treated with 3R4F Total Particulate Matter (TPM)



ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

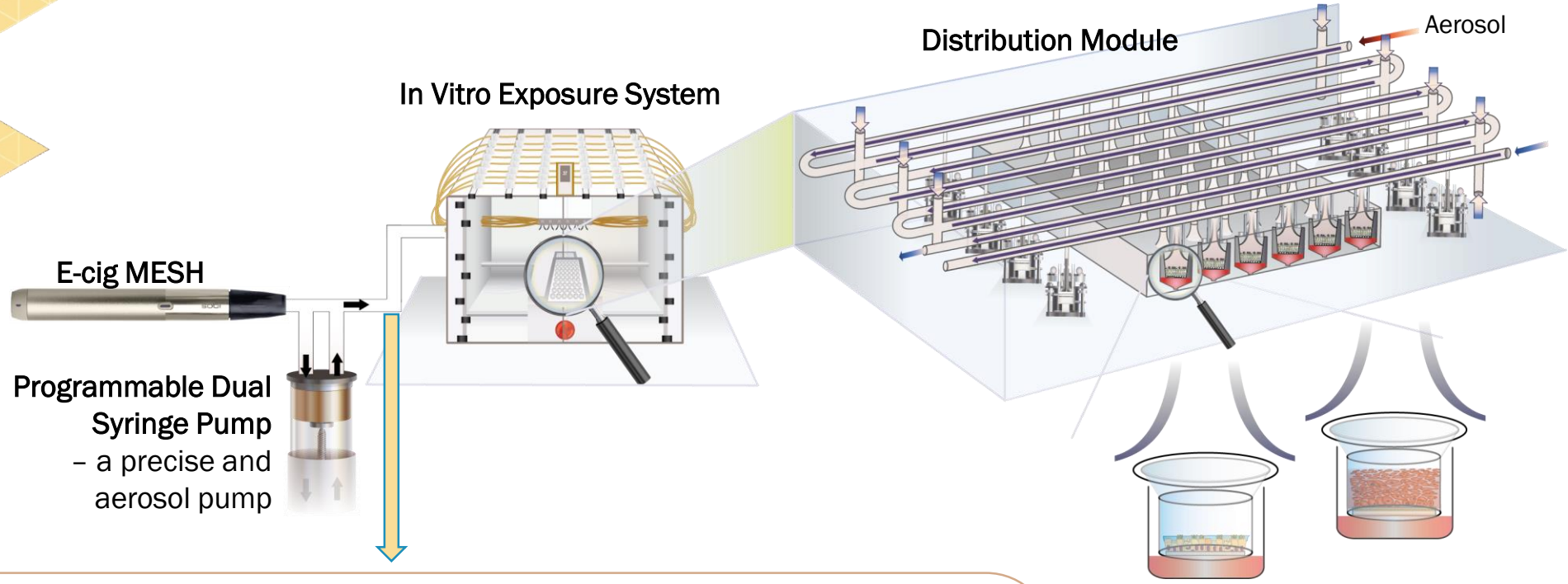
Third Layer Assessment





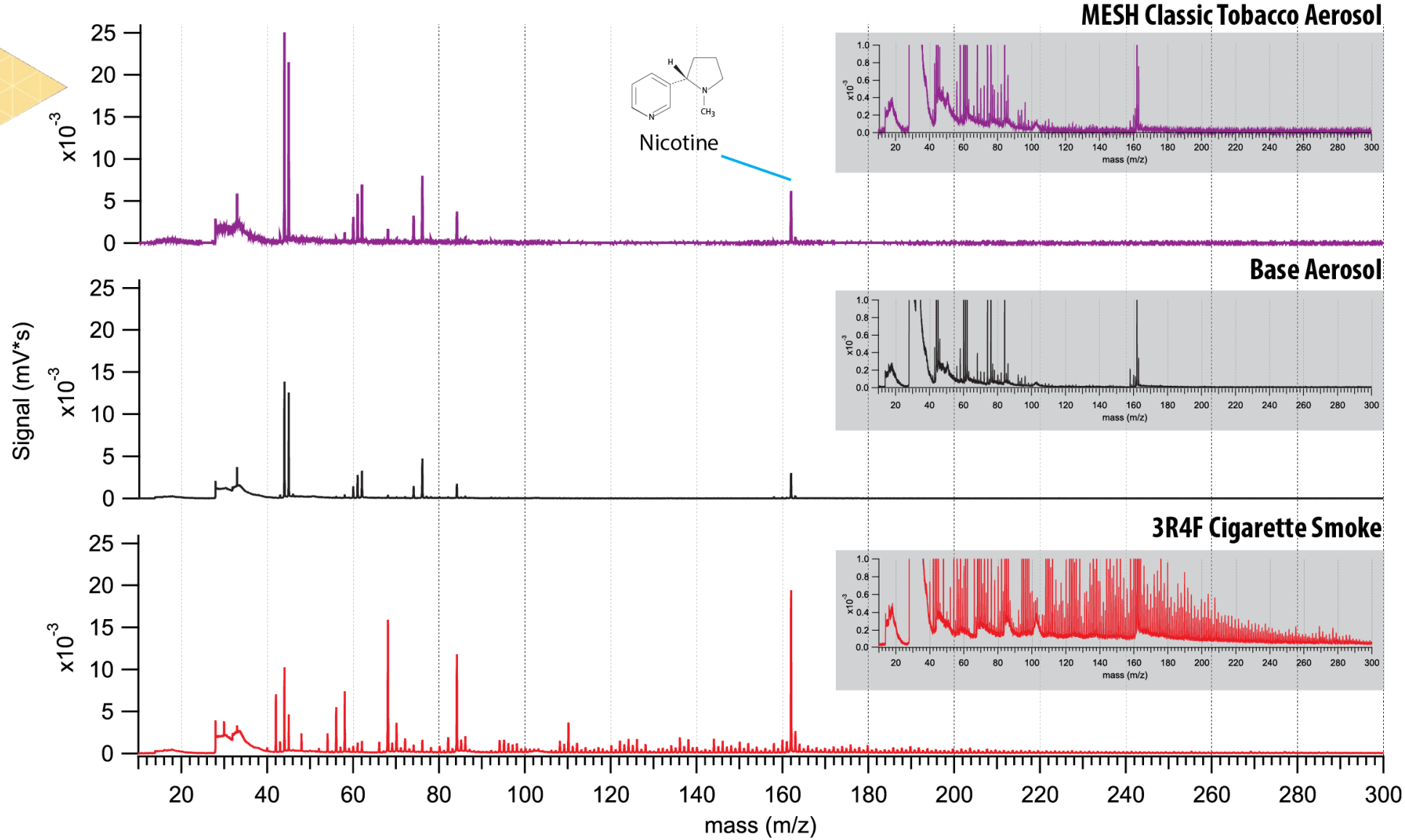
ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

Experimental Setup: In Vitro E-Cigarette Aerosol Exposure



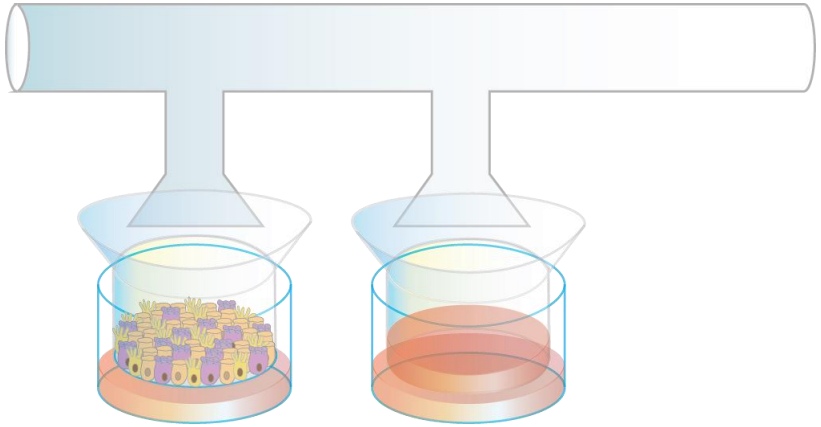
An on-line real-time measuring system for chemical gas analysis based on time-of-flight mass spectrometry

Untargeted Aerosol Monitoring By Single-Photon Ionization Mass Spectrometry: 3R4F Cigarette Generates a More Complex Aerosol than MESH Classic Tobacco and Base E-Liquids

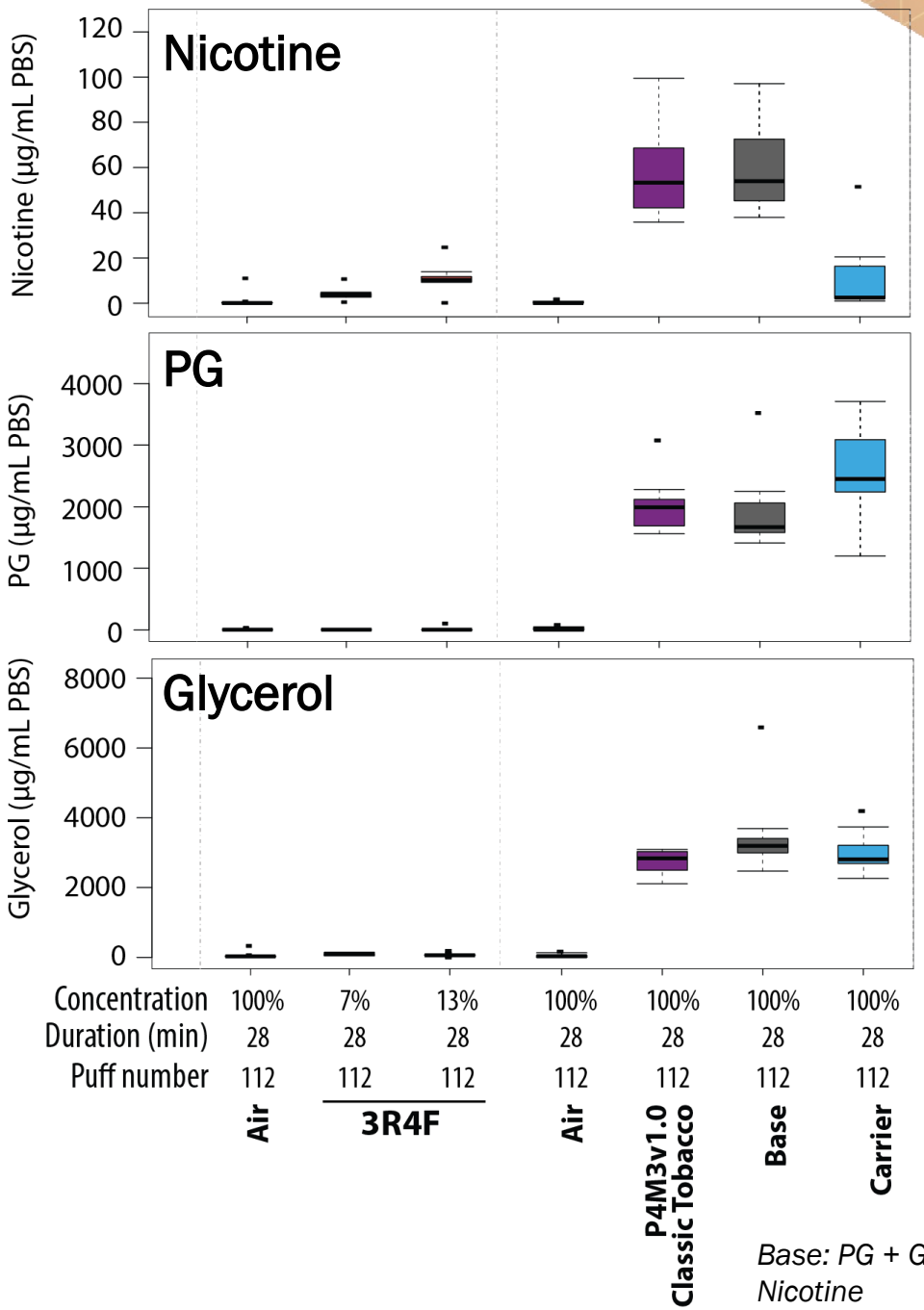


Monitoring of Compound Deposition in the Exposure Chamber During an Exposure Experiment: The Use of a Surrogate Test System (PBS)

In each exposure experiment, culture inserts were filled with phosphate-buffered saline (PBS) as a surrogate epithelial culture model.



Concentrations of nicotine in PBS were determined by liquid chromatography—tandem mass spectrometry (LC-MS/MS). Concentrations of propylene glycol and glycerol in PBS were determined by gas chromatography—mass spectrometry (GC-MS).



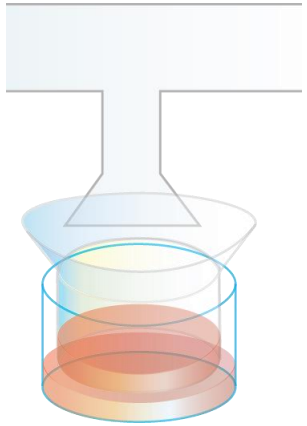
Base: PG + Glycerol + Nicotine
Carrier: PG + Glycerol only

Deposition Efficiency Can Be Estimated Based on the Concentrations of Compounds Deposited in the Exposure Chamber Relative to Those Present in the E-Liquids

	PG	Glycerol	Nicotine
MESH Classic Tobacco Liquid Composition (%, v/v)	**39**	**39**	**1.8**
Puff Number	112	112	112
Volume of Liquid Used per Puff (µL)	6.7	6.7	6.7
Total Volume of Compound Used for 112 Puffs (µL)	293	293	14
Total Mass of Compound Generated for 112 Puffs (µg)*	**304361**	**368747**	**13642**

**Calculated based on the density of PG, VG, and nicotine*

	PG	Glycerol	Nicotine
Deposition of Compound in the Exposure Chamber (µg/mL PBS)	1990	2840	53
Total Mass of Compound Deposited in 0.1 mL PBS (1 insert) (µg)	**199**	**284**	**5.3**
Deposition Efficiency**	**0.07%**	**0.08%**	**0.04%**

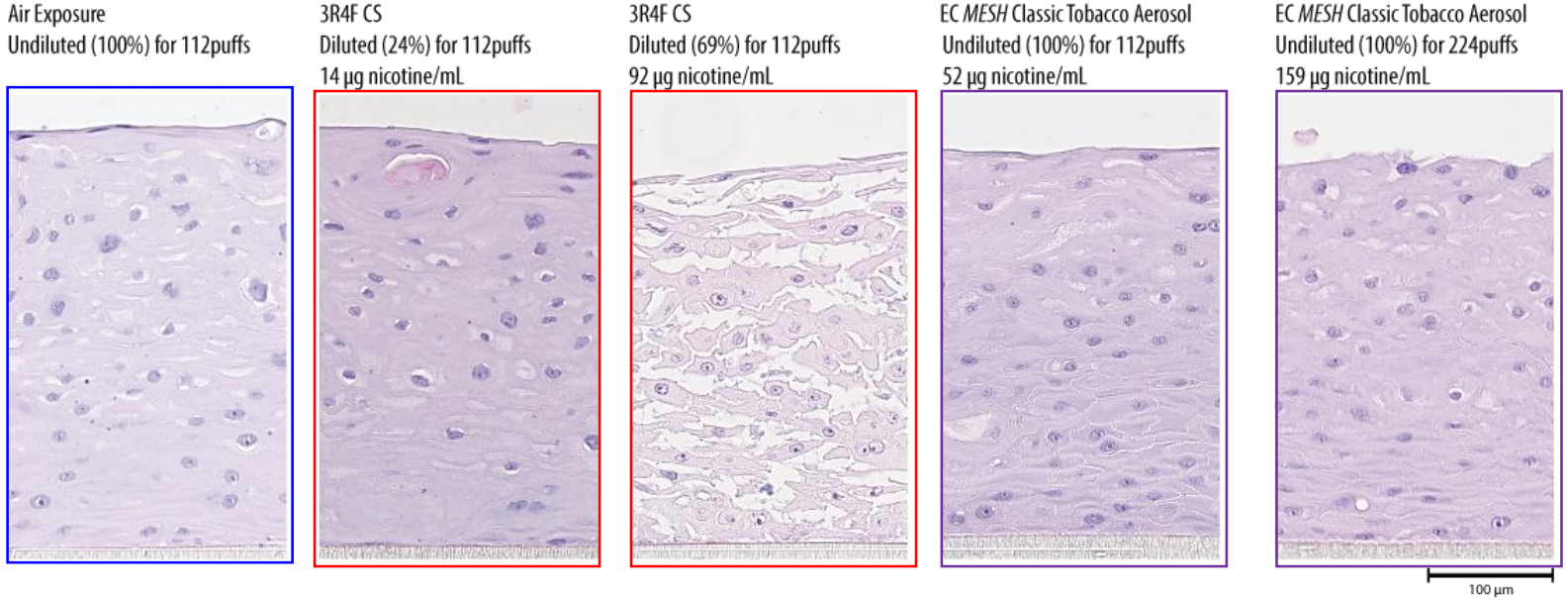


** $\frac{\text{Total Mass of Compound Deposited in 0.1 mL PBS (1 insert)}}{\text{Total Mass of Compound Generated for the 112 Puffs}}$ X 100%

3D Organotypic Cultures Exposed to e-cig MESH Classic Tobacco Aerosol Showed No Tissue Damage

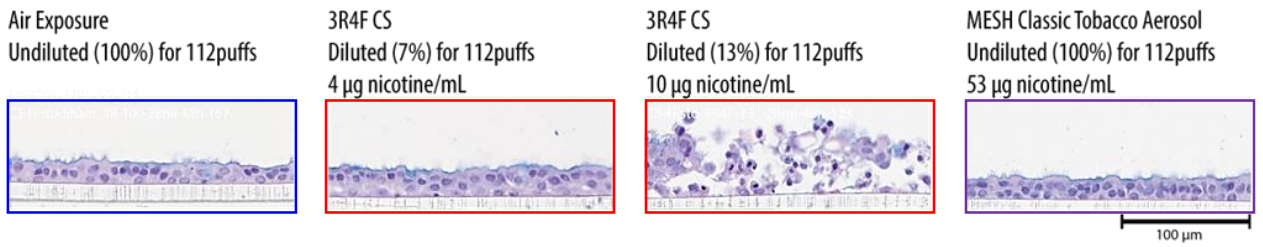
Hematoxylin and Eosin-stained Sections (Buccal Epithelial Cultures): Including Exposure to EC *MESH* Classic Tobacco Aerosol for 224 puffs

Buccal



Hematoxylin, Eosin- and Alcian Blue-stained Sections (Small Airway Epithelial Cultures)

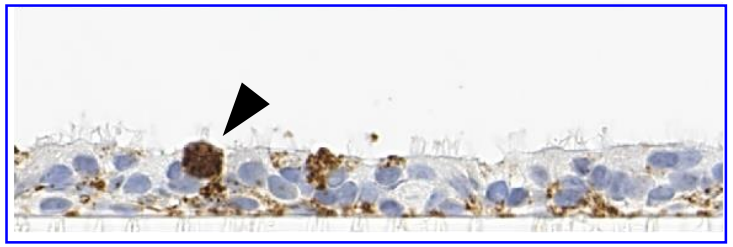
Small Airway



ISKANDAR, A. R., ZANETTI, F., MARESCOTTI, D., TITZ, B., SEWER, A., KONDYLIS, A., LEROY, P., BELCASTRO, V., TORRES, L. O., ACALI, S., MAJEED, S., STEINER, S., TRIVEDI, K., GUEDJ, E., MERG, C., SCHNEIDER, T., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2019. Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH™ technology. Archives of Toxicology.

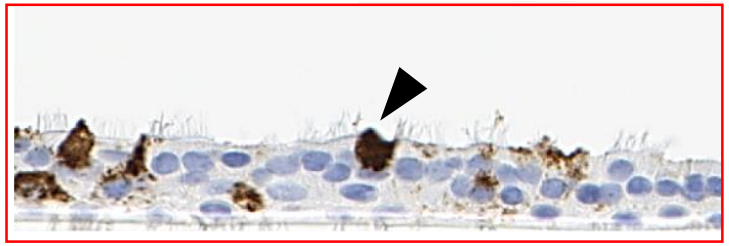
Muc5AC Expression Was Not Altered in Cultures Exposed to MESH Classic Tobacco Aerosol

Air Exposure
Undiluted (100%) for 112 puffs

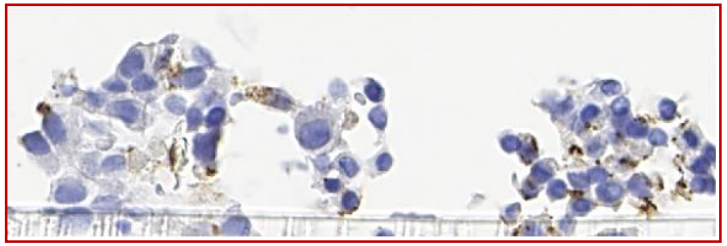


Muc5AC mucin, a major mucin secreted by tracheobronchial goblet cells, is found in the airway secretions of healthy individuals.

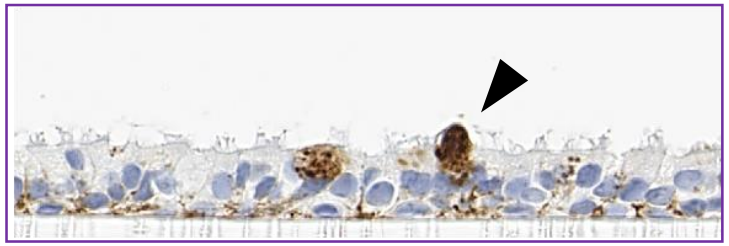
3R4F CS
Diluted (7%) for 112 puffs
4 µg nicotine/mL



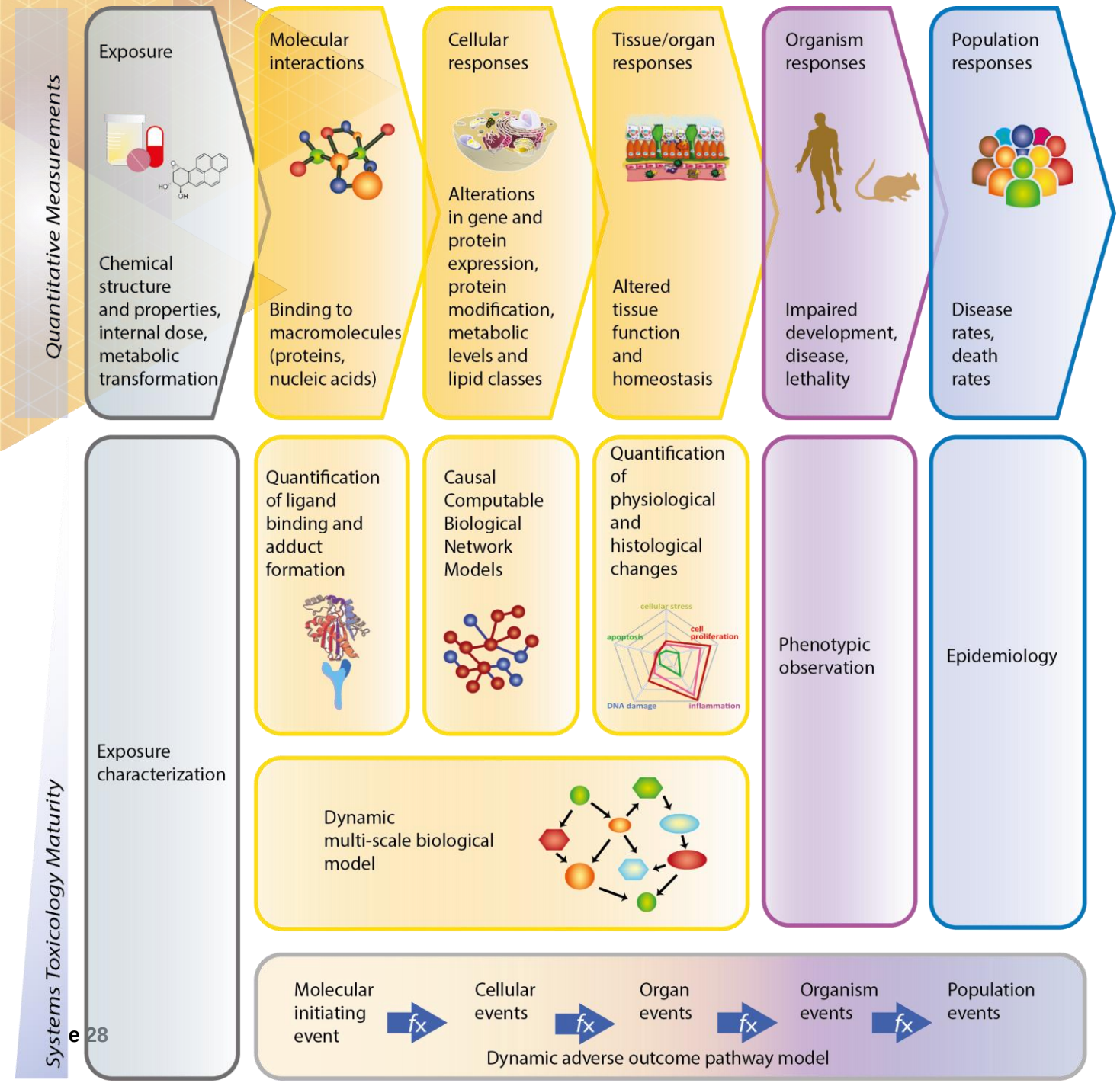
3R4F CS
Diluted (13%) for 112 puffs
10 µg nicotine/mL



MESH Classic Tobacco Aerosol
Undiluted (100%) for 112 puffs
53 µg nicotine/mL



Constant renewal of mucus by constitutive secretion from goblet cells facilitates the clearance function. Increased levels of Muc5AC have been reported in the airways of patients with asthma.



- **Electronic nicotine delivery systems evolve rapidly.**
- **Testing the potential toxic effects of exposure to tobacco product aerosols requires the use of relevant test systems and exposure modes as well as appropriate (physiologically relevant) doses.**
- **Systems biology approaches (omics) will uncover changes at cellular and molecular levels otherwise undetected in standard toxicity assays.**
- **Collaborative efforts between the scientific community, industry, and regulatory stakeholders are facilitating the adoption of 21st Century Toxicology approaches.**
- **The multilayer systems toxicology framework could be useful for assessing in vitro the potentially reduced impact of e-cigarettes relative to 3R4F cigarettes in a comprehensive manner and is a very insightful strategy for acquiring preliminary data that would be relevant for supporting potential clinical outcomes.**

The Utility of Omics Technology and In Vitro Human Organotypic Cultures in Tobacco Research: A Chronic Study

Integration of Omics-based Analysis with Pathophysiological Endpoints to Evaluate the Mode of Action for Cigarette Smoke Toxicity in an in vitro Human Airway Tissue Model

Rui Xiong[1], Qiangen Wu[2], Priya Tripathi[1], Levan Muskhelishvili[3], Kelly Davis[3], Matthew Bryant[2], Hans Rosenfeldt[4], Sheila M. Healy[4], Xuefei Cao[1]

[1]Division of Genetic and Molecular Toxicology, [2]Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, [3]Toxicologic Pathology Associates, Jefferson, AR 72079, [4]Division of Nonclinical Science, Center for Tobacco Products, U.S. Food and Drug Administration, Silver Spring, MD 20993

Proposed integrative approach for analyzing CS toxicity in a human air-liquid-interface (ALI) airway tissue model

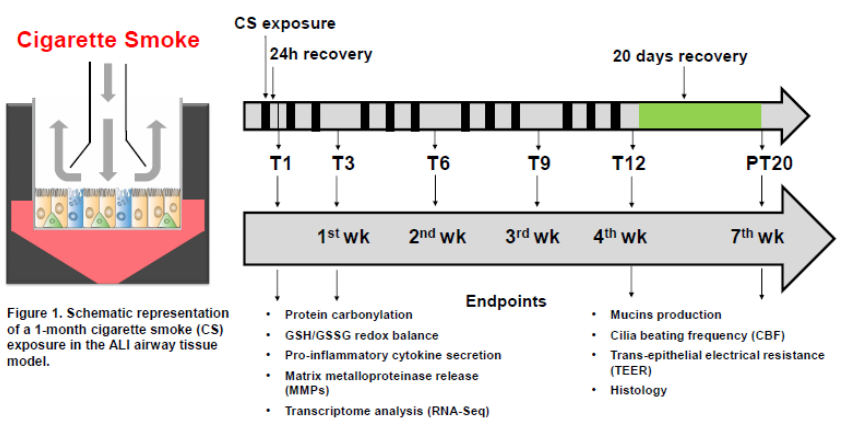
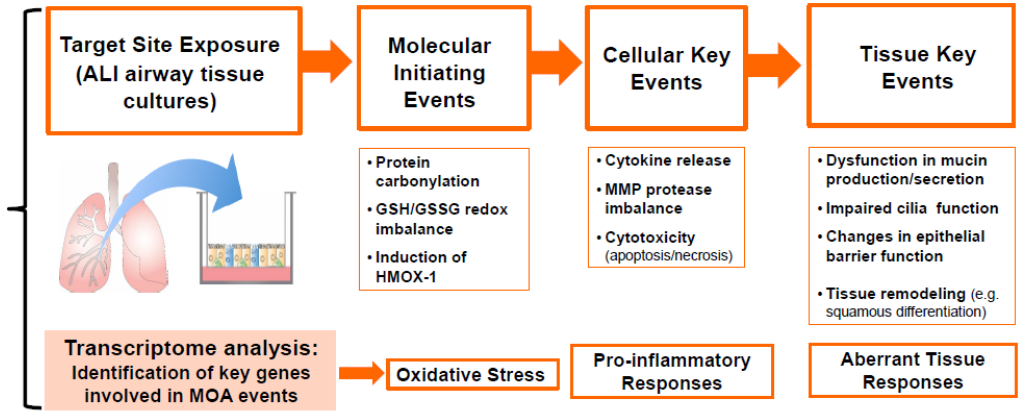
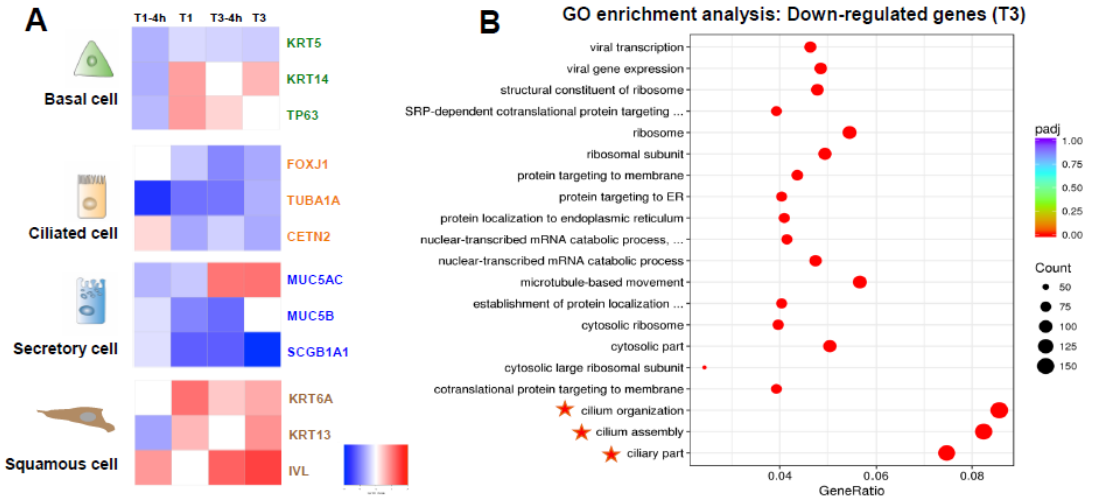


Figure 1. Schematic representation of a 1-month cigarette smoke (CS) exposure in the ALI airway tissue model.

Mode of Action Analysis



CS modulated genes involved in cell trans-differentiation

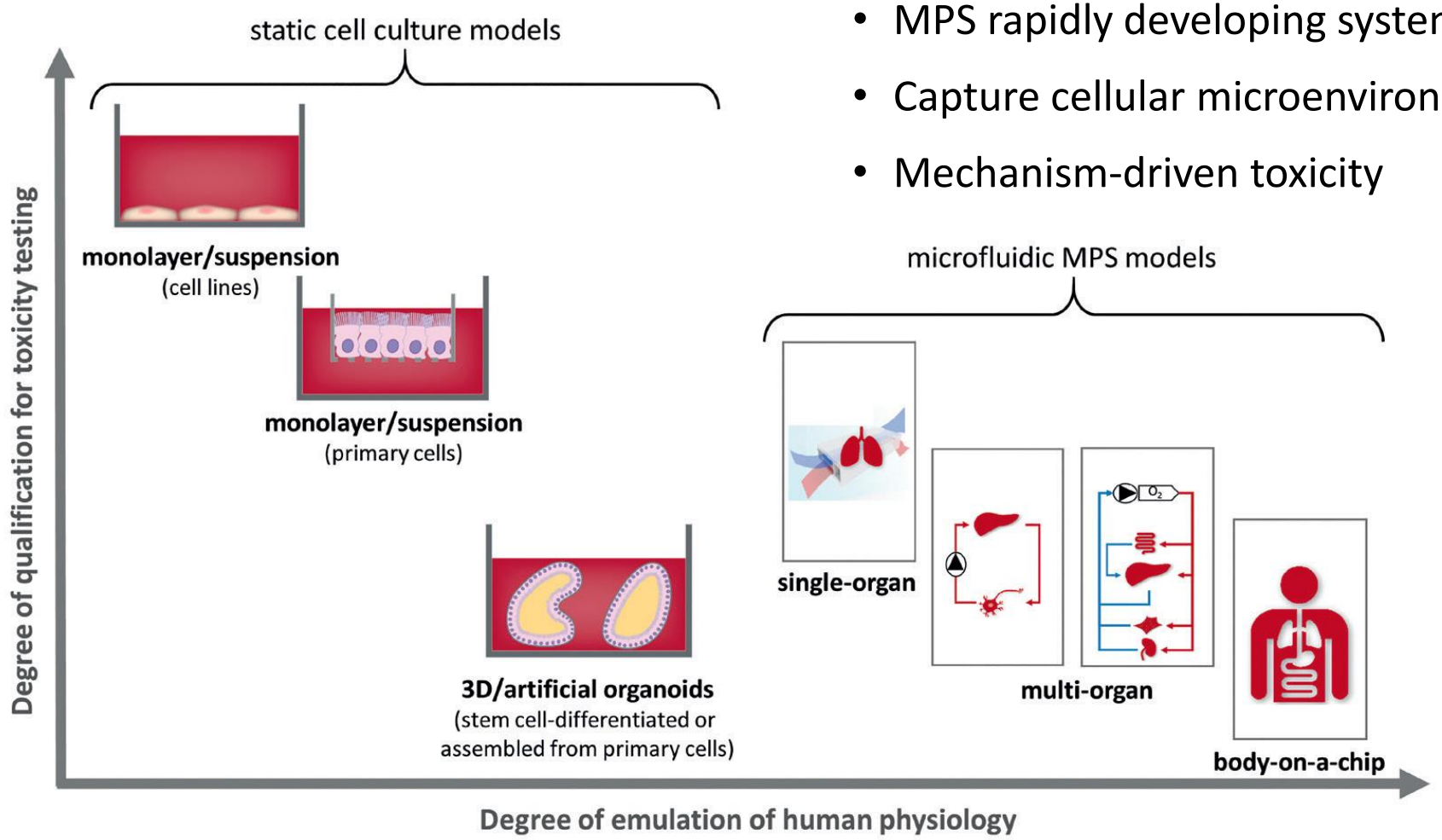


(A) The heatmap shows the changes in cell marker genes after cigarette smoke exposure

(B) The dot plot from GO enrichment analysis showing the biological networks and subnetworks (p-*adj* < 0.05) altered by cigarette smoke exposure

Poster was presented at the Society of Toxicology Conference 2019 (Baltimore, MD, USA)

Microphysiological Systems for Toxicity Testing



- MPS rapidly developing systems
- Capture cellular microenvironment of an organ
- Mechanism-driven toxicity

Beilmann, Optimizing Drug Discovery by Investigative toxicology: Current and future trends, ALTEX, 2019

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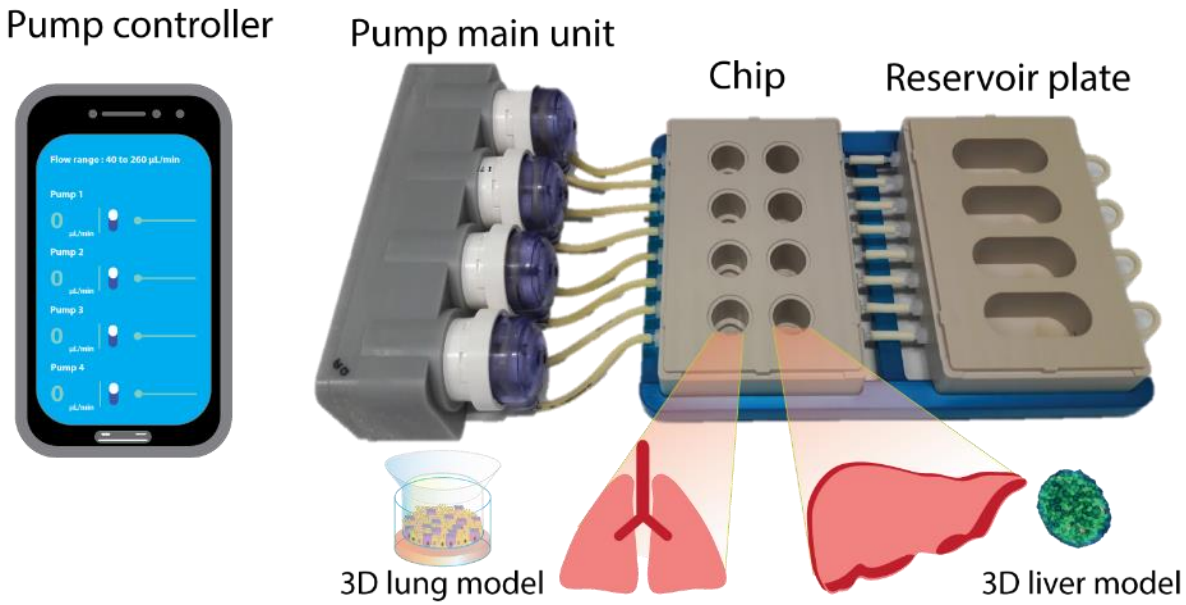
Lung–Liver on a chip

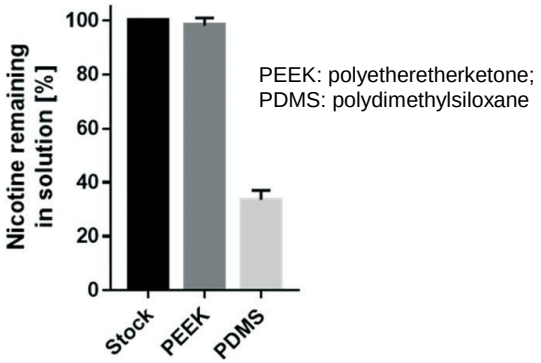


- ✓ **User friendly**
- ✓ **Biocompatible material**
- ✓ **Non-absorbent**
- ✓ **Reusable**
- ✓ **Modular**
- ✓ **24-well size**

Advantages

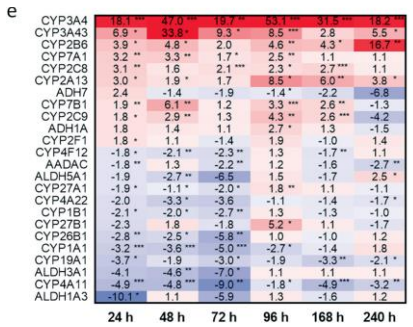
- Negligible binding to chip
- Functionally active phase-1 liver metabolism
- CBF stable in coculture

Compound Absorption

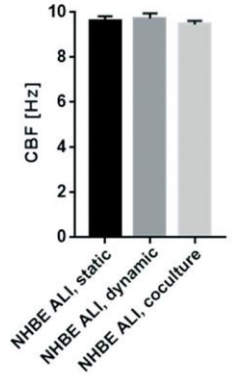


PEEK: polyetheretherketone;
PDMS: polydimethylsiloxane

Liver
Phase-1 Metabolism Genes



Lung
Cilia Beating Frequency



Challenges

- Buildup of metabolites and waste
- Next iteration will address clearance

Bovard, D., et al. "A lung/liver-on-a-chip platform for acute and chronic toxicity studies." Lab on a Chip 2018.

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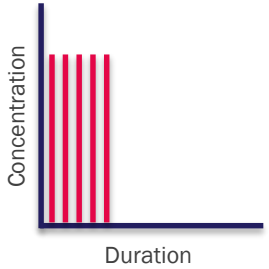
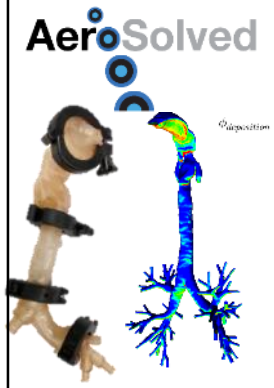
Strategy for Developing Hybrid CFD–PBPK Models

Aerosol Physicochemical Properties

Inhalation Topology

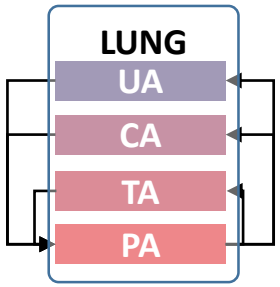


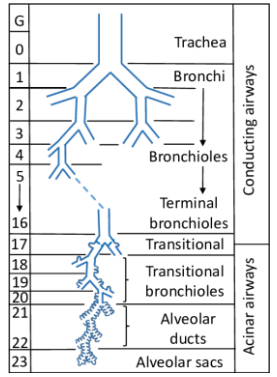
Computational Fluid Dynamics (CFD) based models to validate aerosol evolution and deposition



Identify key

- geometric changes
- number of generations - 2, 4, 24
- Aerosol evolution
- Deposition
- Heterogeneity

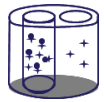




Weibel 1963, Raabe et al. 1976

Physiological Parameters

Physicochemical Properties



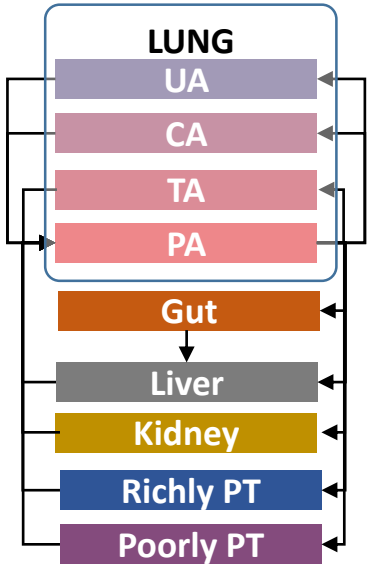
Protein Binding

Metabolism and Transport

Mucus velocities in airway generation

Human Respiratory Tract Wall Thickness

PBPK Model



Work in progress @ PMI R&D

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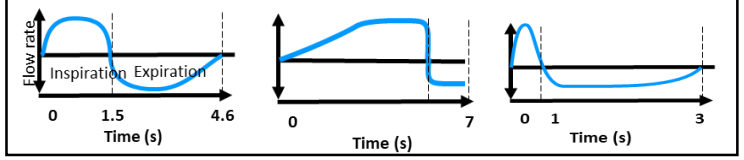
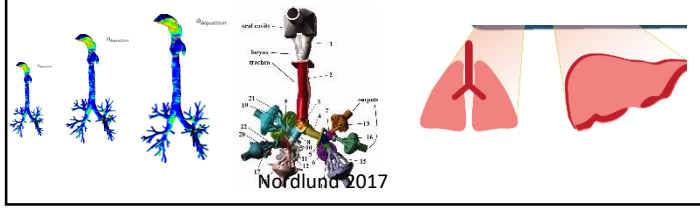
Vision: Population Safety Assessment for Next-Gen Inhaled RRPs

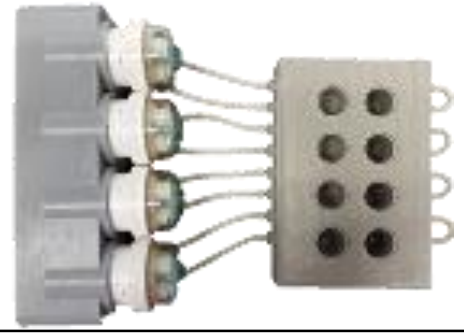
Exposure Patterns



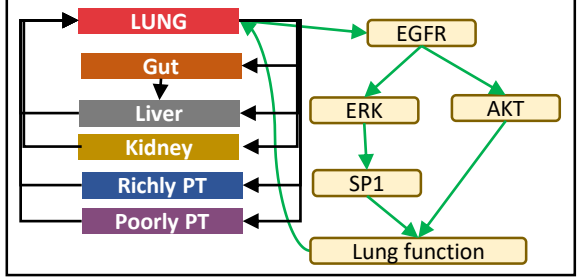
Aerosol Dosimetry & Scaled Clearance



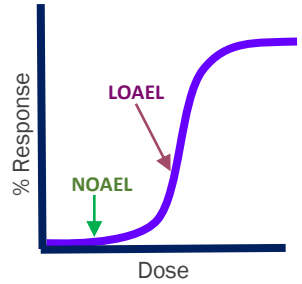
Microphysiological Systems



PBPK–AOP model

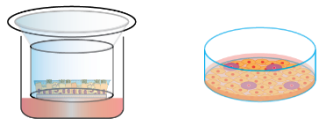


Safety Assessment

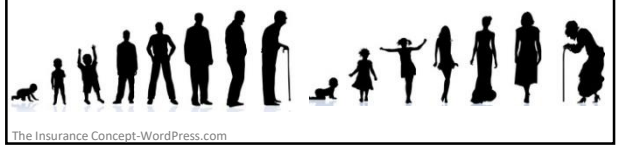


Donor Tissue

- Non-smoker
- Smoker
- Diseased
- Ethnicity



Age–Sex-Specific Physiology



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QIVIVE Challenges – In Vitro

Further Advancements in In Vitro Systems (MPS and Beyond) for Testing Inhaled RRPs

- **Clearance (Mucociliary and Kidney)**
- **Phagocytosis (Trapping)**
- **Spatial Differences in Lungs (Thickness, cell types)**

Validation

Regulation

- Robustness
- Reproducibility
- Relevance

Scope for Guidelines

OECD Guidelines

- Majority of In Vitro assays are NOT in OECD test guidelines
- Guidance document 211 (GD211) is not comprehensive

CAAT-Europe & other groups are working to towards

In Vitro Systems Standardization of Criteria and Protocols

- Cell/tissue experimental systems
  - Cell source, culture system (2D,3D)
  - Population, batch effects
  - Media composition, functionality, relevance
  - Lipid & protein content, volume of cells
- Free concentrations – parent and metabolite
- End points – metabolism, transport, & response
- Dynamic (in vivo-like) exposure testing
- Transferability and ease of use

QIVIVE

Improved Support for Predictive Modeling

Center for Alternatives to Animal Testing (CAAT)

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Collaborative efforts

NC 3Rs Innovation Platform
Exploiting 3Rs technologies

CRACK IT challenge 27
Dosing for Controlled Exposure (DoCE)

Aim: Develop methods to better account for in vitro bioavailability that reflect in vivo exposures for enabling robust QIVIVE.

Sponsor: Unilever and Shell

Phase 1: **In progress**
Phase 2: Not started

"Testing chemicals on cells in a way that mimics real-life exposure will lead to more human-relevant results and spare animals' lives."
- Amy Clippinger, president of the Science Consortium.

Replacing animal testing

speccomm | August 23, 2019

The PETA International Science Consortium, Imperial Brands, Altria Client Services (ALCS), British American Tobacco (BAT), and Philip Morris International have joined together to donate equipment that can help to replace the use of animals in respiratory testing with more human-relevant, non-animal test methods.

The equipment—worth $110,000 and manufactured by Germany-based Vitrocell Systems—was donated to the Institute for In Vitro Sciences (IIVS), a non-profit laboratory in Gaithersburg, Maryland, USA, that conducts animal-free testing. It will be used in the IIVS in vitro respiratory toxicology laboratory, which helps companies assess the effects of tobacco, nicotine and other aerosols on the human respiratory tract. Results from these tests will also help to show regulatory agencies, such as the U.S. Food and Drug Administration, that non-animal methods are accurate and effective and can be used instead of tests on animals.

- IIVS, a non-profit lab for assessing the effects of tobacco, nicotine, and other aerosols on the human respiratory tract.
- PETA, Altria, BAT, and PMI encourage these studies.

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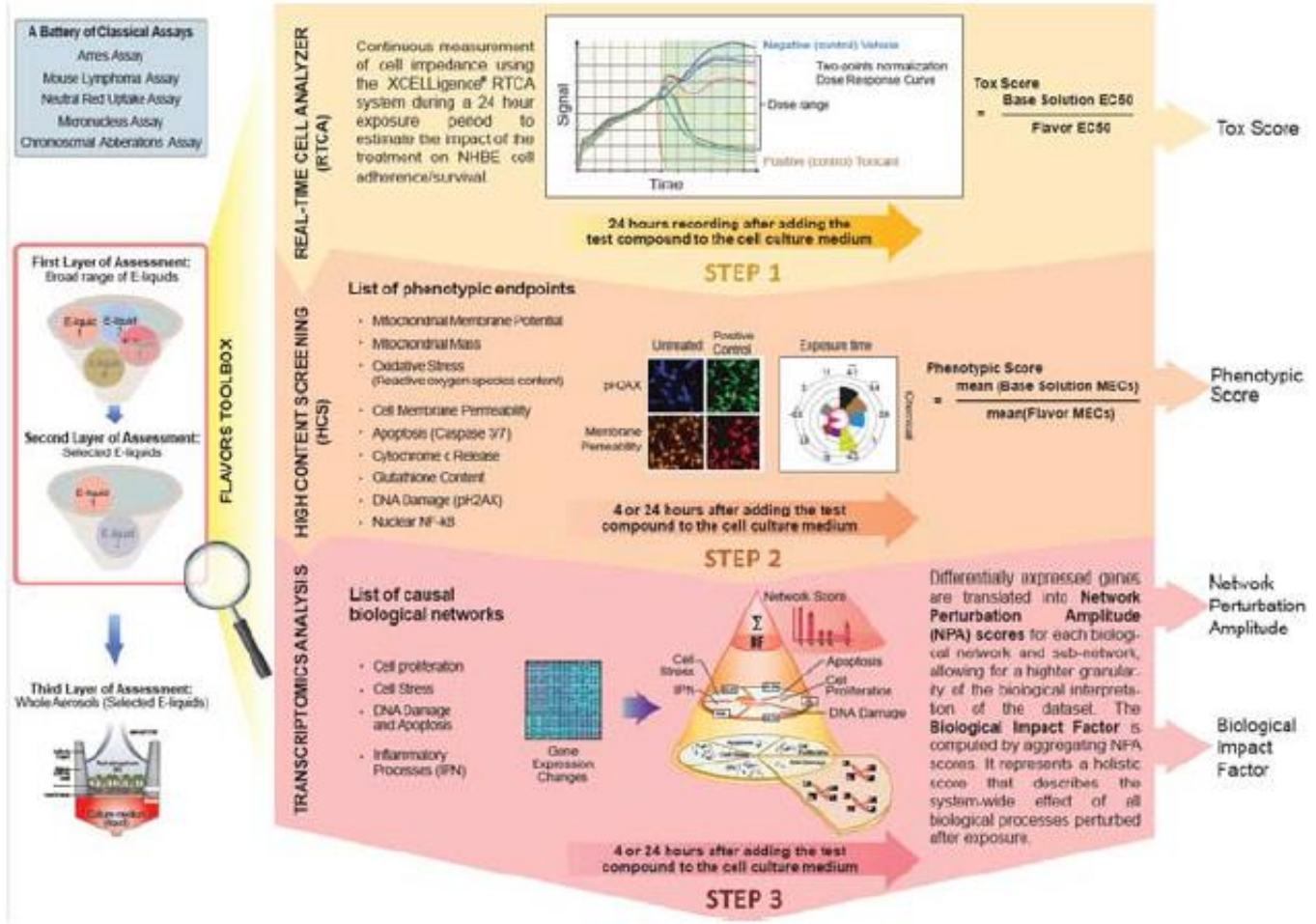
In Vitro Models

The Flavor Toolbox Approach

The Flavor Toolbox: A three-step workflow for assessing the toxicity of flavored solutions in NHBE cells. The Flavor Toolbox is a complementary approach to standard toxicity assays (e.g., Ames assay and mouse lymphoma assay). It is designed to screen a large number of e-liquids for potential toxicity prior to performing whole-aerosol assessment on human organotypic tissue cultures. The Flavor Toolbox workflow comprises the following three steps: (i) STEP 1, which quantifies the toxicity of the exposure by using a real-time impedance-based measurement, expressed as Tox-Score; (ii) STEP 2, which measures and investigates the phenotypic impact of the exposure by HCS image analysis; and (iii) STEP 3, which combines transcriptomic data and computable biological networks in a systems toxicology approach.



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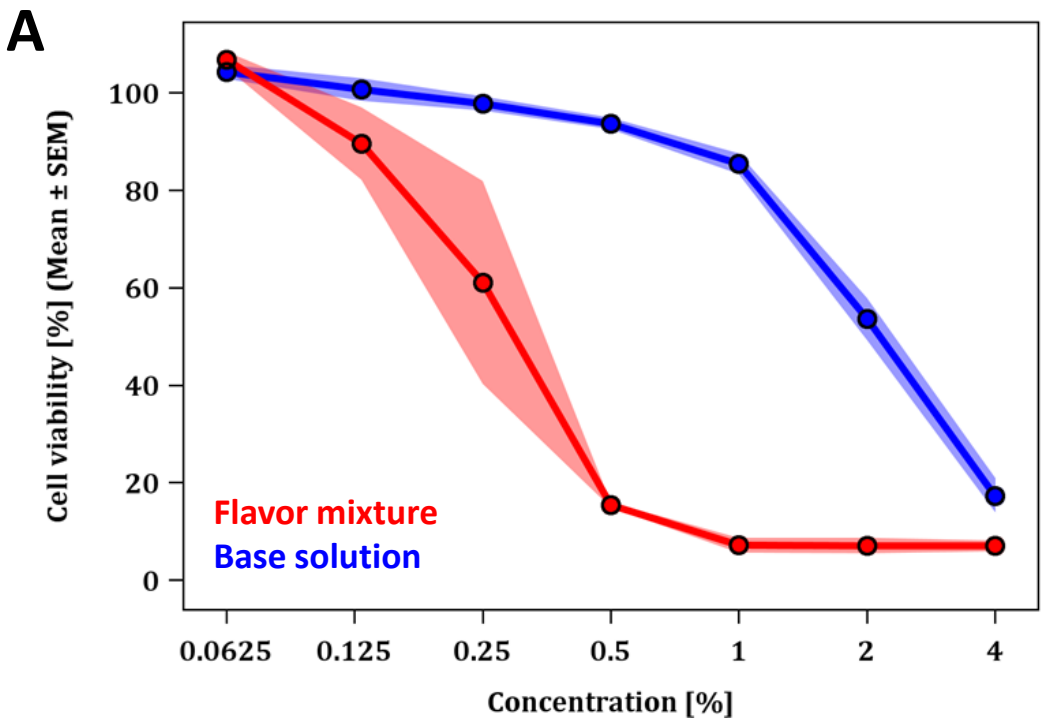
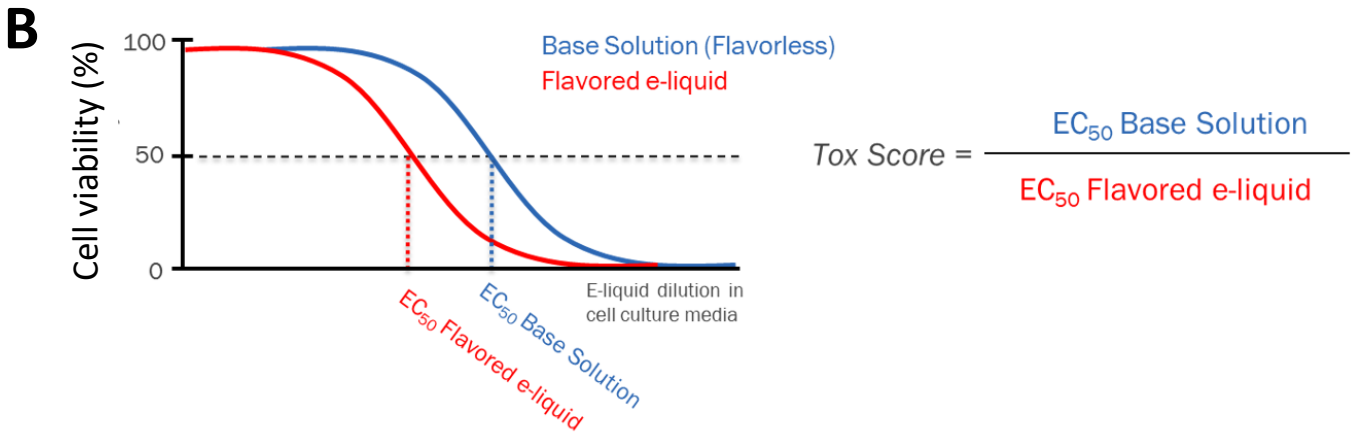
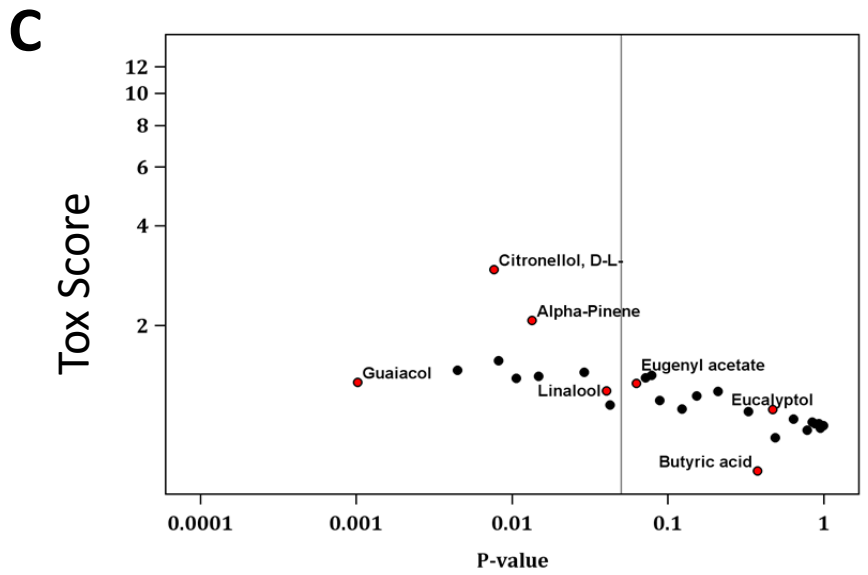
Tox-Score Computation for E-Liquid Cytotoxicity Profile







(A) RTCA-based cell viability dose–response curves for the28-flavor mixture (white dots) and the corresponding base solution (black dots).
(B) Computation of Tox-Score based on cell viability data.
(C) Tox-Scores (y axis) are shown as a function of their corresponding *p* values computed for each flavoring substance present in the 28-flavor mixture. The vertical line indicates a *p* value of 0.05. Each dot corresponds to one flavor solution, with those selected for subsequent HCS-based investigation shown by white dots.

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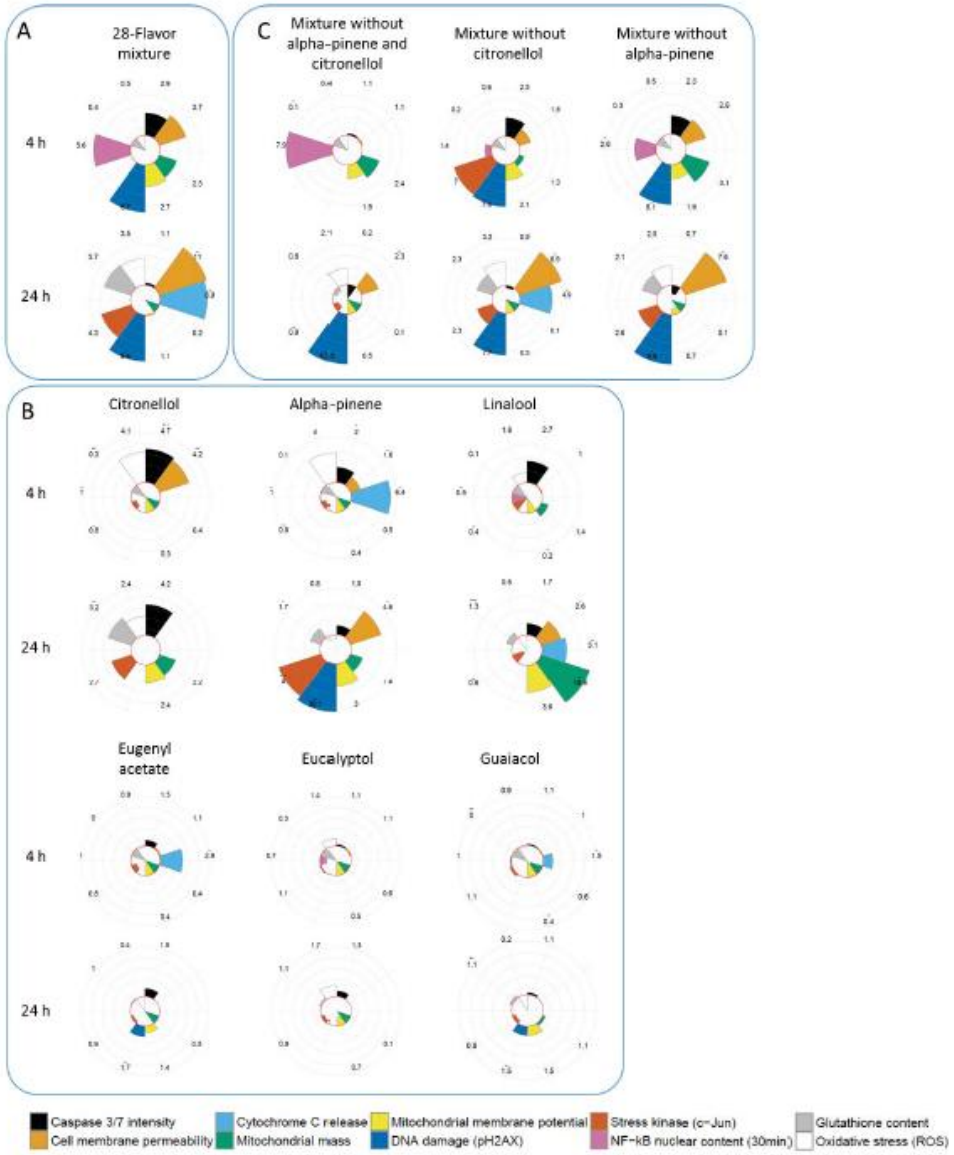
High-Content Screening



Circular bar plots of HCS-endpoint MEC ratios for (A) the 28-flavor mixture, (B) various individual flavoring substances with different Tox-Scores, and (C) flavor mixture without citronellol and/or alpha-pinene. Each MEC ratio (reported next to each segment of the circular chart for each HCS endpoint) was computed by dividing the mean base solution MEC (from n = 3 replicates) by the mean flavor mix MEC. A unilateral *t*-test was computed with null hypothesis: The base solution MEC mean is higher than the flavor mix MEC mean. The *t*-test *p* values are reported as follows: ***<0.001, **<0.01, *<0.05. The "-" sign on top of an MEC ratio denotes an imputed MEC value. Red circles correspond to an MEC of 1. Abbreviations: pH2AX, phosphorylated H2A histone family member X; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ROS, reactive oxygen species.

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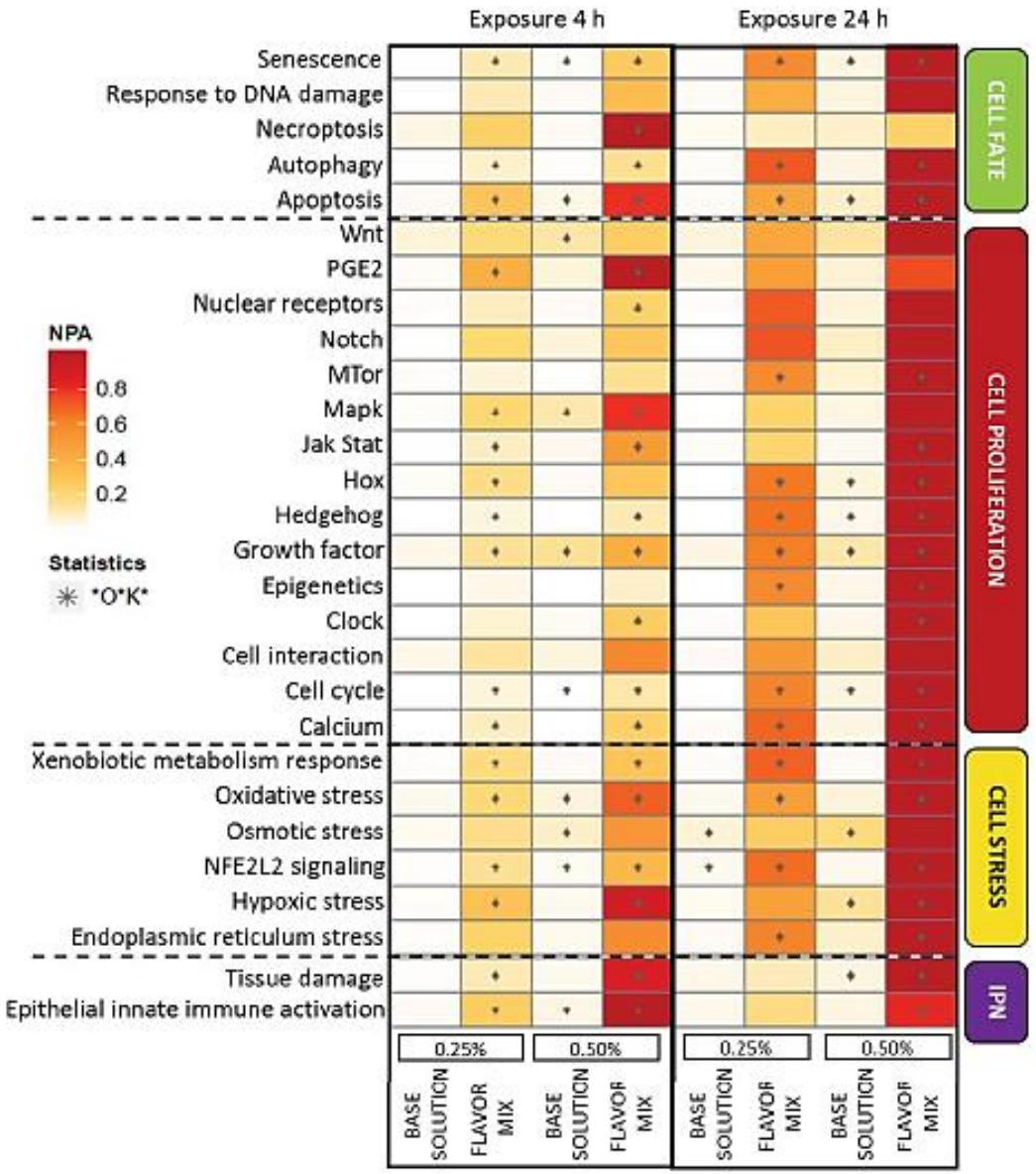
Biological Network Perturbation Amplitudes



Heatmap of the biological networks perturbed by the base solution alone or by the flavor mixture at two dilutions (0.25% and 0.50% v/v) at the 4- and 24-h time points. A network is considered perturbed if, in addition to the significance of the NPA score with respect to the experimental variation, the two companion statistics (O and K) that report the specificity of the NPA score with respect to the biology described in the network are also significant (as indicated by an asterisk). The darker the color the stronger the perturbation. Abbreviation: IPN: Inflammatory Processes Network.

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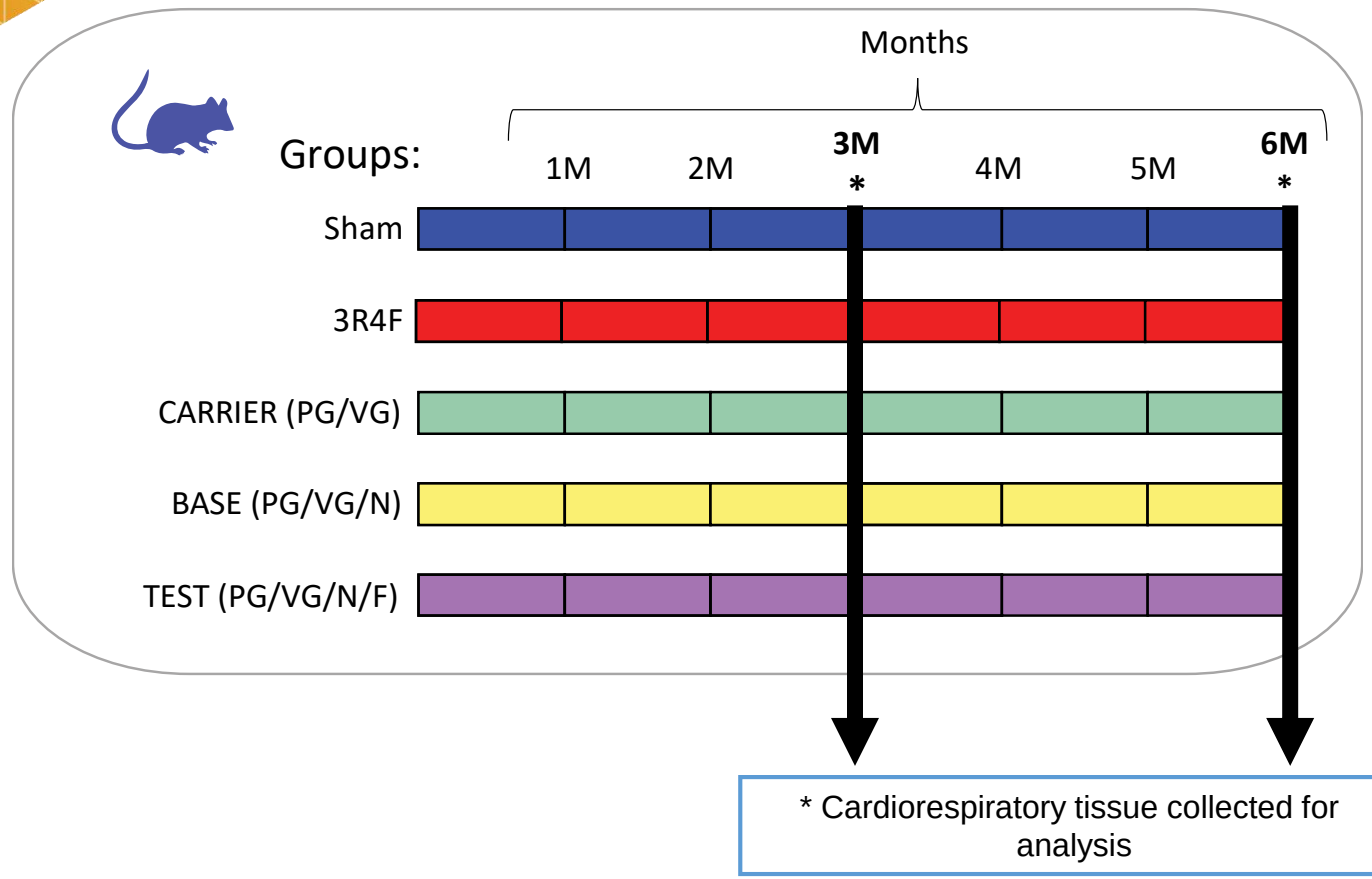
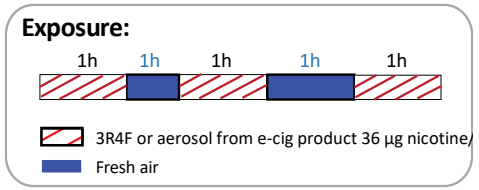
Animal Models of Disease

A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

EXPERIMENTAL DESIGN



- Female ApoE$^{-/-}$ mice (12–14 weeks at initial dosing) were exposed to air (sham), 3R4F cigarette smoke (CS), or e-vapor aerosols generated from CARRIER (PG/VG/water), BASE (CARRIER plus 4% nicotine), and TEST (BASE plus flavors) by using CAG (capillary aerosol generator) system.
- ApoE$^{-/-}$ mice were exposed via a whole-body inhalation system for up to 3 h/day, 5 days/week for 6 months.
- Fresh air breaks between 1-h exposure



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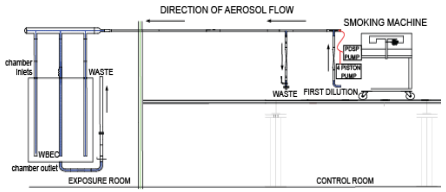
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

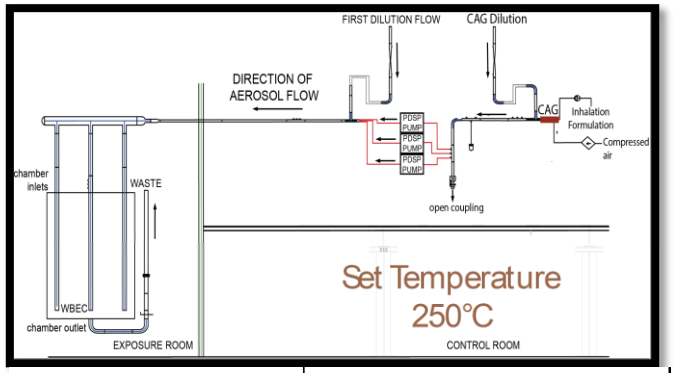
SMOKE MACHINE FOR GENERATING CS FROM 3R4F



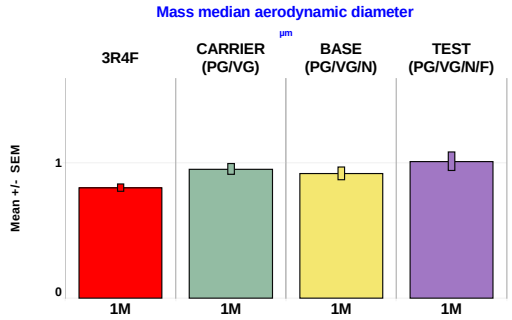
The 3R4F cigarettes were smoked in accordance with the **Health Canada intense smoking protocol (Health_Canada, 1999)**.

CAG (capillary aerosol generator) SYSTEM TO GENERATE E-VAPOR AEROSOLS

The CAG system was successfully set up to generate and consistently deliver respirable e-vapor aerosols to the whole-body mouse exposure system.



			CARRIER (PG/VG)	BASE (PG/VG/N)	TEST (PG/VG/N/F)
CAG temperature	6M	°C	250.03 (+/-) 1.7588	249.19 (+/-) 2.4870	249.74 (+/-) 1.8527



The CAG was used to generate e-vapor from various e-liquids: "CARRIER" containing PG/VG alone, "BASE" containing PG/VG and 4% nicotine, and "TEST" containing PG/VG, 4% nicotine, and flavors.

- ❑-Target **TPM 600 µg/L**, for the 3R4F group.
- ❑-PG/VG/N and PG/VG/N/F at matching nicotine concentrations to 3R4F **35 µg/L.**

		Sham	3R4F	CARRIER (PG/VG)	BASE (PG/VG/N)	TEST (PG/VG/N/F)
Nicotine	µg/L	<LOD	35.15 (+/-) 4.8	<LOD	35.53 (+/-) 4.9	35.73 (+/-) 5.6
Total particulate matter	µg/L	-5.93 (+/-) 7.2	562.43 (+/-) 64.8	1 093.11 (+/-) 150.9	1 103.23 (+/-) 161.4	1 083.40 (+/-) 176.7

Gupta, R., et al., Investigation of a Novel Condensation Aerosol Generator: Solute and Solvent Effects. Aerosol Science and Technology, 2003. 37(8): p. 672-681.
Werley, M.S., et al., Non-clinical safety and pharmacokinetic evaluations of propylene glycol aerosol in Sprague-Dawley rats and Beagle dogs. Toxicology, 2011. 287(1-3): p. 76-9

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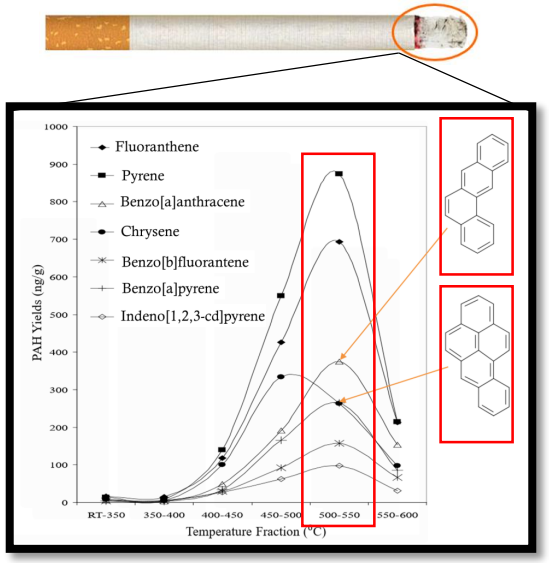
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

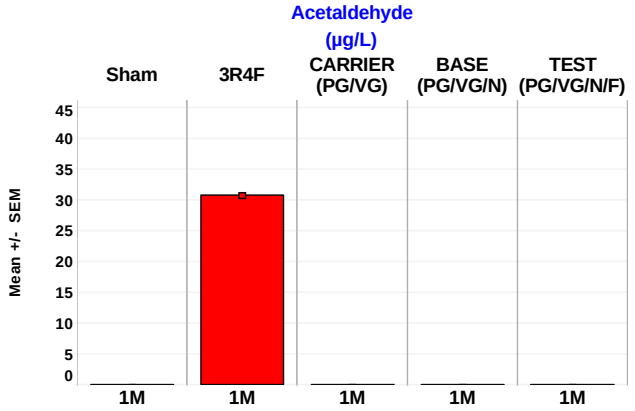
COMBUSTION IS A KEY

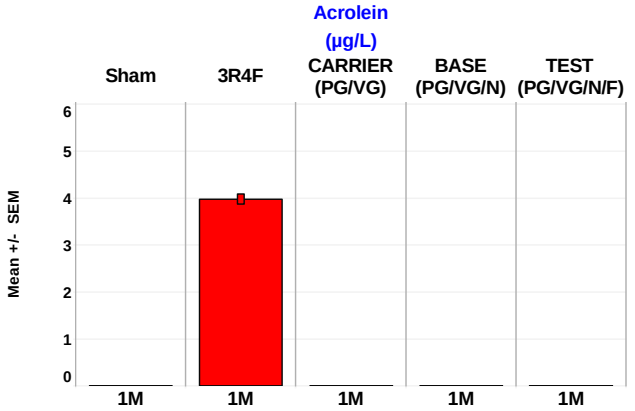
Scientific studies have shown that, as the temperature of tobacco increases, the levels of harmful potentially harmful chemicals formed increase

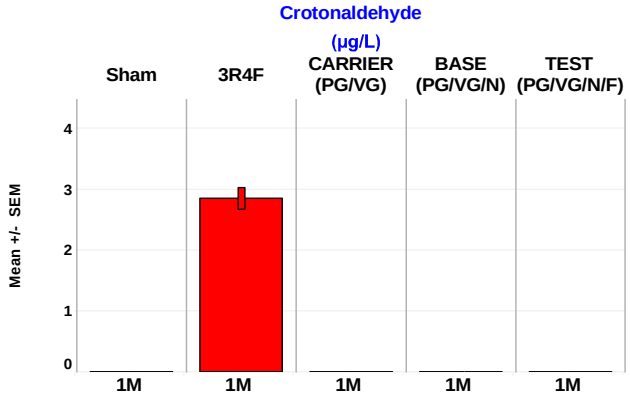


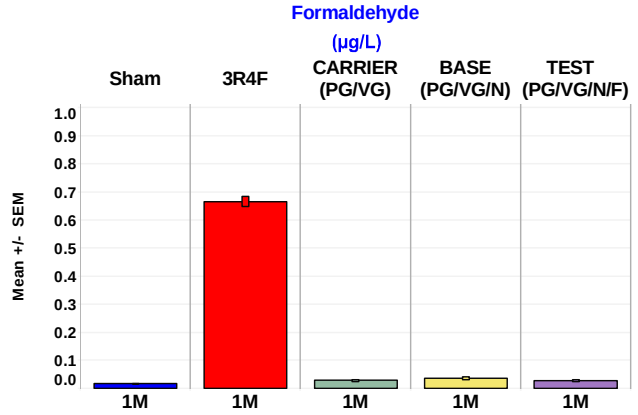
Source: McGrath, T.E., Wooten, J.B., Chan W.G. and Hajaligol, M.R., 2007, Formation of polycyclic Aromatic Hydrocarbons from Tobacco: the "Link" between Low Temperature Residual Solid and PAH Formation, Food and Chemical Toxicology, 45,6,1039-1050

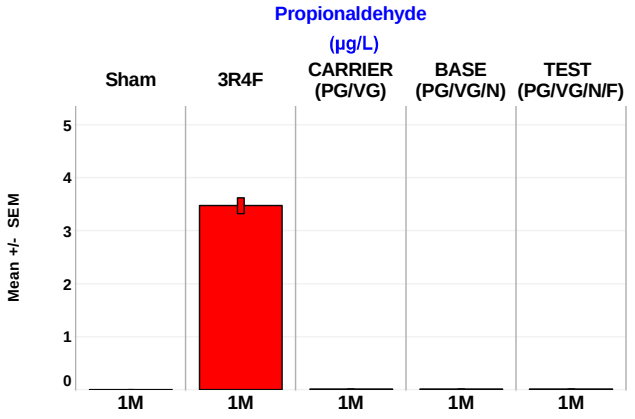
AEROSOL CONSTITUENTS IN TEST ATHMOSTHERE











Compared with cigarette smoke exposure, e-vapor aerosol (CARRIER, BASE, and TEST) exposure presents lower levels of harmful smoke constituents in the atmosphere.

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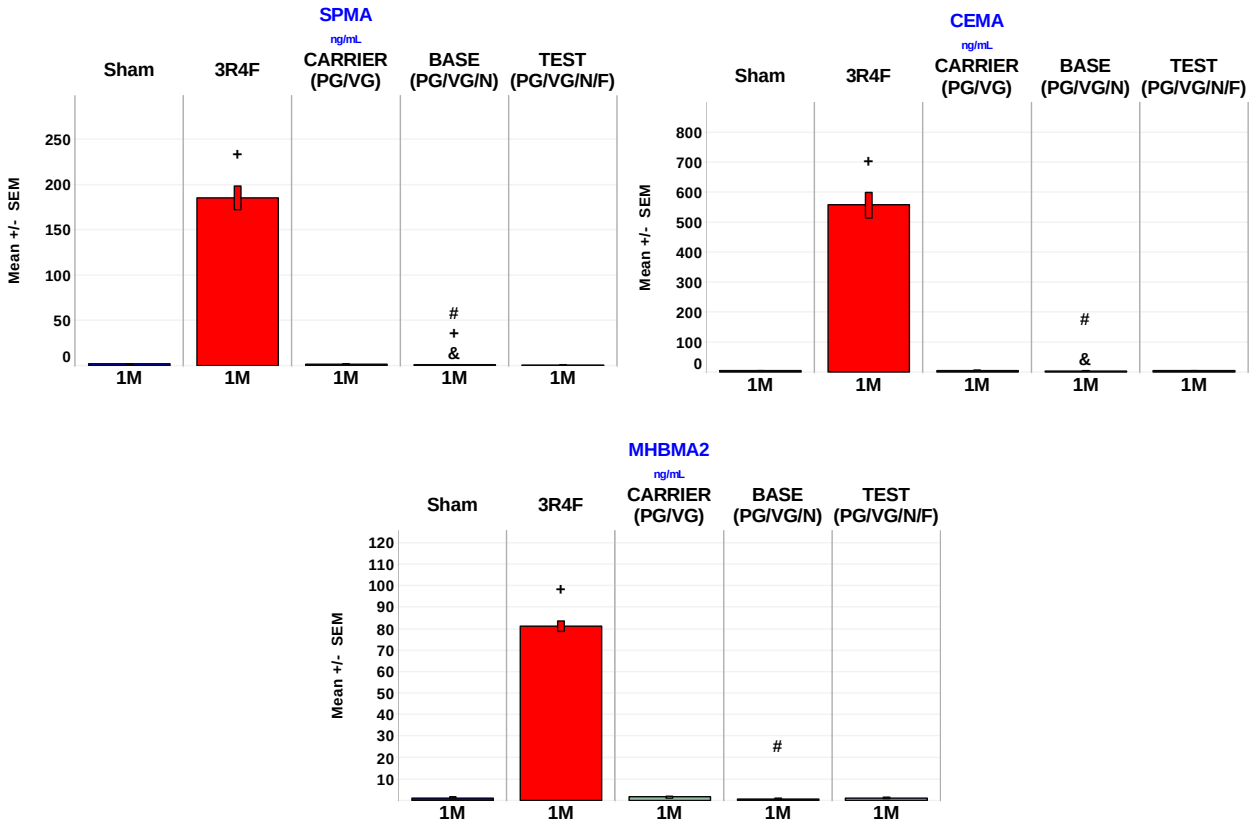
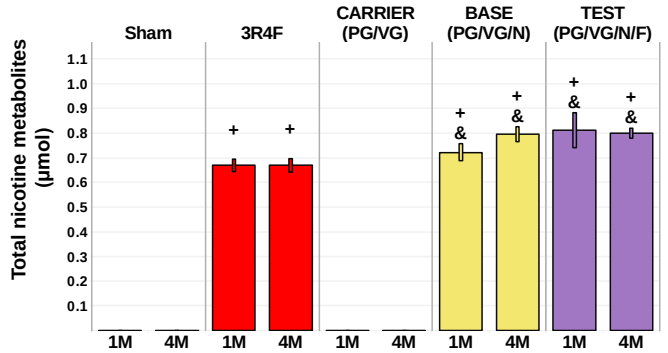
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

BIOMARKER OF CS

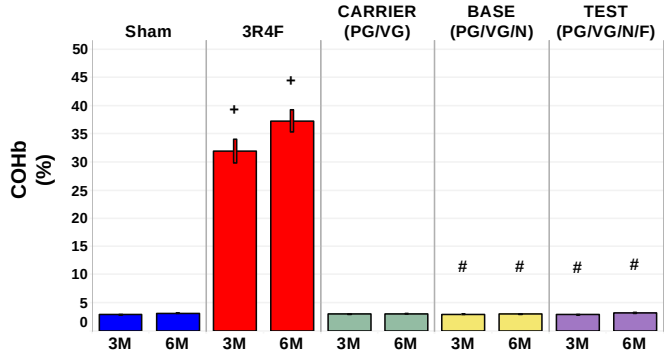
In urine:



In plasma:



In blood:



\+ p<0.05 significant versus Sham
\# p<0.05 significant versus 3R4F
& p<0.05 significant versus PG/VG

Compared with cigarette smoke exposure, e-vapor aerosol (CARRIER, BASE, and TEST) exposure presents lower levels of potentially harmful biomarkers in urine and blood.

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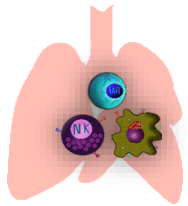
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

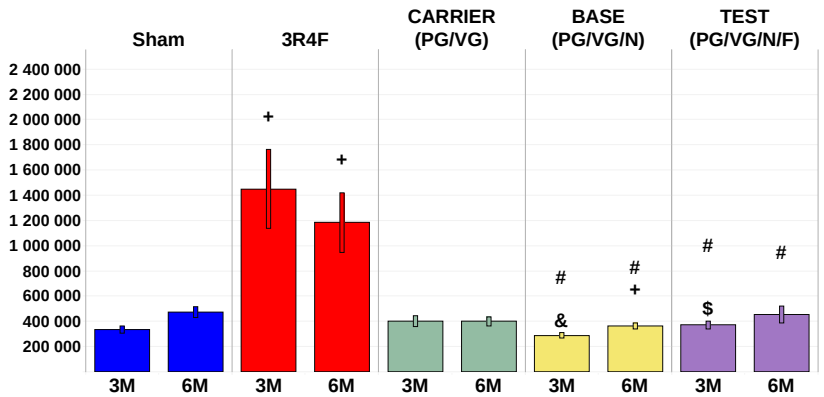
Assessment of E-Vapor Aerosols in a 6-Month ApoE$^{-/-}$ Mouse Study -- Lung Effects

LUNG INFLAMMATION



Cell-free BALF supernatants were analyzed using a multiplexed bead array

Total cells



\+ p<0.05 significant versus Sham
\# p<0.05 significant versus 3R4F
& p<0.05 significant versus PG/VG

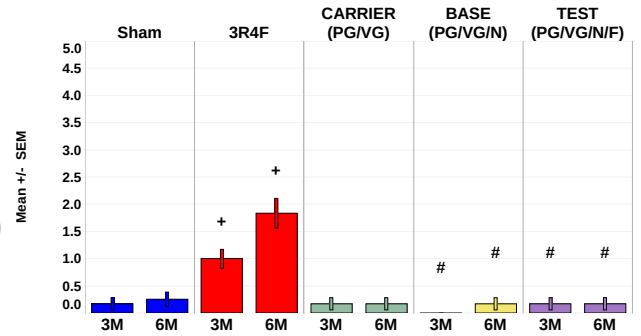
Endpoint_Name	3R4F 3M	3R4F 6M	CARRIER (PG/VG) 3M	CARRIER (PG/VG) 6M	BASE (PG/VG/N) 3M	BASE (PG/VG/N) 6M	TESTMIX (PG/VG/N/F) 3M	TESTMIX (PG/VG/N/F) 6M
G-CSF	3.59	2.72	0.74	0.50	0.82	0.52	0.78	1.09
GM-CSF	2.14	1.57	0.98	0.78	1.99	0.83	2.18	1.06
IFN-g	0.84	1.26	0.79	2.89	0.95	1.67	0.54	1.91
IL-1a	0.54	0.53	1.22	1.47	0.93	1.32	0.89	1.24
IL-1b	1.03	0.94	0.75	0.77	0.84	0.88	1.11	0.95
IL-2	0.49	0.68	1.05	1.29	0.92	1.11	0.79	0.98
IL-4	1.22	0.96	0.60	1.52	1.07	1.54	0.89	1.30
IL-5	1.68	0.87	1.30	2.01	0.93	0.98	1.24	0.83
IL-6	3.88	4.84	2.32	2.77	0.50	1.72	0.82	1.46
IL-7	0.52	0.74	0.97	0.69	0.92	1.07	1.05	0.56
IL-9	0.93	0.84	0.98	1.87	1.09	1.14	0.82	1.14
IL-10	0.32	0.29	1.05	1.04	0.70	0.94	0.63	0.72
IL-12	1.24	3.36	0.62	1.93	0.57	1.33	0.90	0.95
IL-12b	0.60	0.58	0.90	1.29	0.96	1.16	0.69	0.93
IL-13	0.63	0.85	0.70	2.06	0.96	1.25	0.77	1.28
IL-15	0.93	1.03	0.87	1.20	1.55	0.88	0.72	0.68
IL-17	1.79	2.70	0.58	1.55	0.49	0.65	0.56	2.11
IP-10	3.48	3.66	0.82	1.47	0.88	1.12	0.81	1.23
KC	4.99	8.20	0.66	1.09	0.63	1.02	0.61	1.73
MCP-1	6.15	4.77	1.05	0.62	1.04	0.88	1.30	0.60
MIP-1a	2.28	2.70	0.86	0.94	1.02	1.04	0.98	0.71
MIP-1b	10.52	12.82	1.02	0.90	1.39	1.57	0.80	1.48
MIP-2	1.06	0.85	0.91	1.25	0.95	0.88	1.04	1.09
MMP total	1.70	2.19	1.10	0.96	0.98	1.04	0.98	1.00
PECAM-1	1.24	1.07	1.09	0.88	0.83	1.01	0.74	1.04
pro-MMP-9	61.87	17.54	1.93	0.50	0.98	0.29	1.07	0.51
RANTES	0.55	0.79	0.65	1.69	0.77	0.91	0.83	0.96
sE-Selectin	1.04	0.88	0.84	2.66	0.92	0.91	0.89	1.01
sICAM1	2.23	2.13	1.08	0.98	0.97	0.93	1.05	0.89
sP-Selectin	0.94	1.04	0.89	1.26	0.92	0.98	1.18	0.86
Thrombomodulin	1.90	2.34	0.91	1.09	0.86	0.95	0.95	1.06
TNF-a	2.10	2.77	0.71	1.45	0.82	1.17	1.12	0.97
Total PAI-1	2.54	2.60	0.97	0.95	0.92	1.00	1.15	1.14

Compared with cigarette smoke exposure, exposure to e-vapor aerosols resulted in a lower number of inflammatory cells in lung BALF.

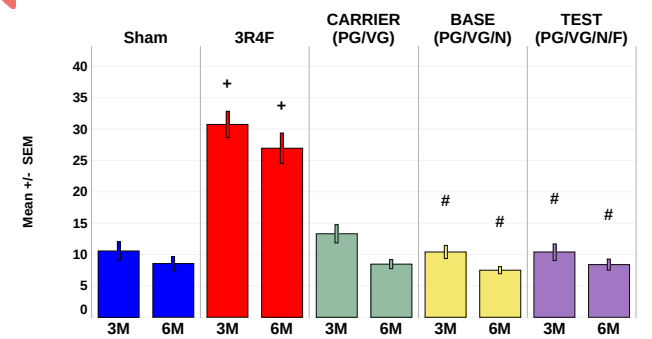
Compared with cigarette smoke exposure, exposure to
e-vapor aerosols resulted in lower levels of inflammatory mediators.

LUNG STRUCTURAL DAMAGE

Emphysema
Score



Destructive index
%



Compared with cigarette smoke exposure, exposure to e-vapor aerosols resulted in lower emphysematous changes in the lungs, as demonstrated by histopathological semi-quantitative scoring.

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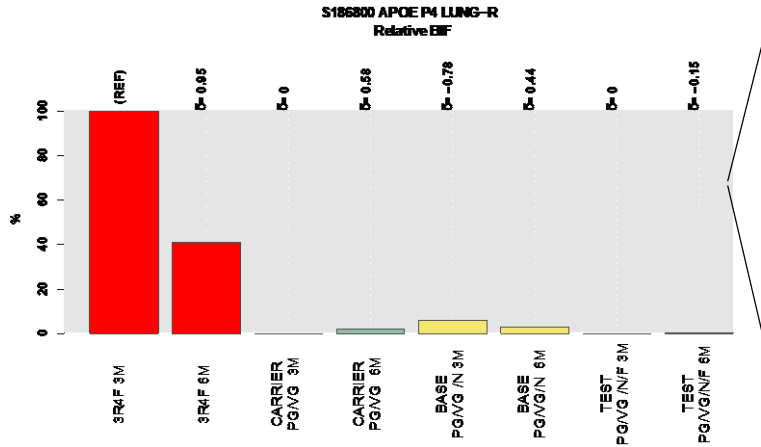
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

Assessment of E-Vapor Aerosols in 6-Month ApoE$^{-/-}$ Mouse Study -- Lung Effects: Transcriptomics

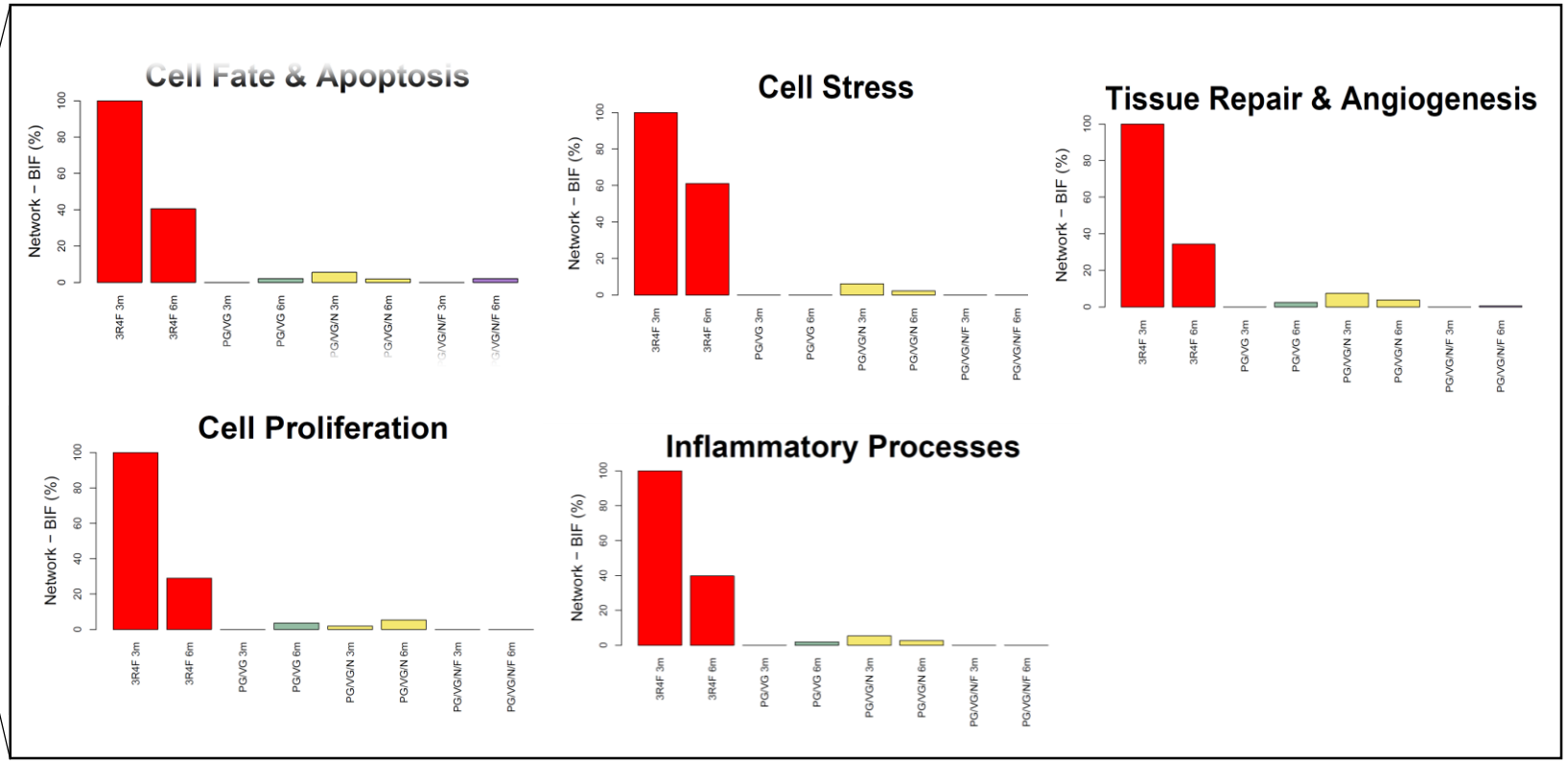
LUNG MOLECULAR CHANGES

Differentially expressed genes in the lungs

	Group of Mice		3R4F		PG/VG		PG/VG/N		PG/VG/N/F	
Target	Tissue	TimePoint	3	6	3	6	3	6	4	6
	LUNG-R	Up	848	351	0	0	0	0	0	0
		Down	477	93	0	0	0	0	0	0



Compared with cigarette smoke exposure, exposure to e-vapor aerosol resulted in lower molecular changes in lung tissue.



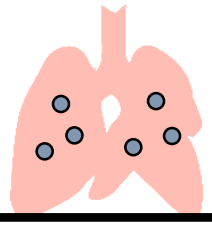
Compared with cigarette smoke exposure, exposure to e-vapor aerosols induced significantly less molecular changes related to stress responses, cell proliferation, and inflammation in lung tissue.

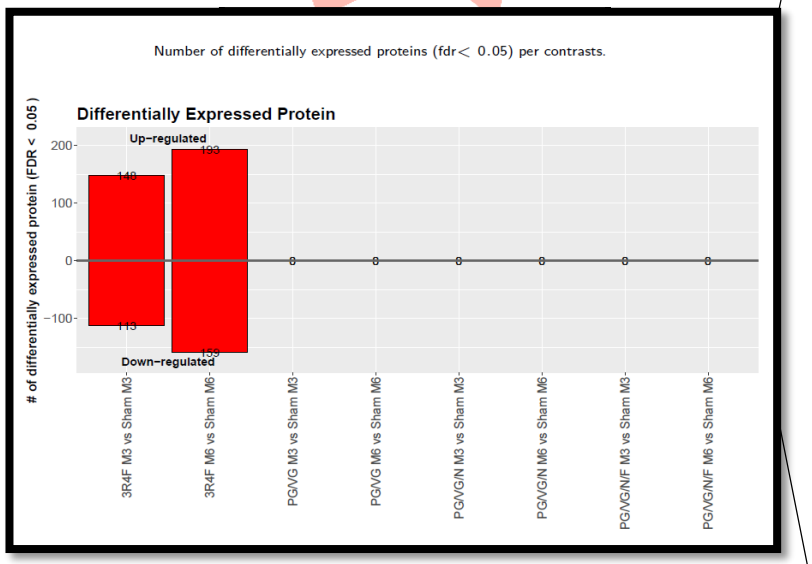
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

Assessment of E-Vapor Aerosols in 6-Month ApoE$^{-/-}$ Mouse Study – Lung Effects- Proteomics

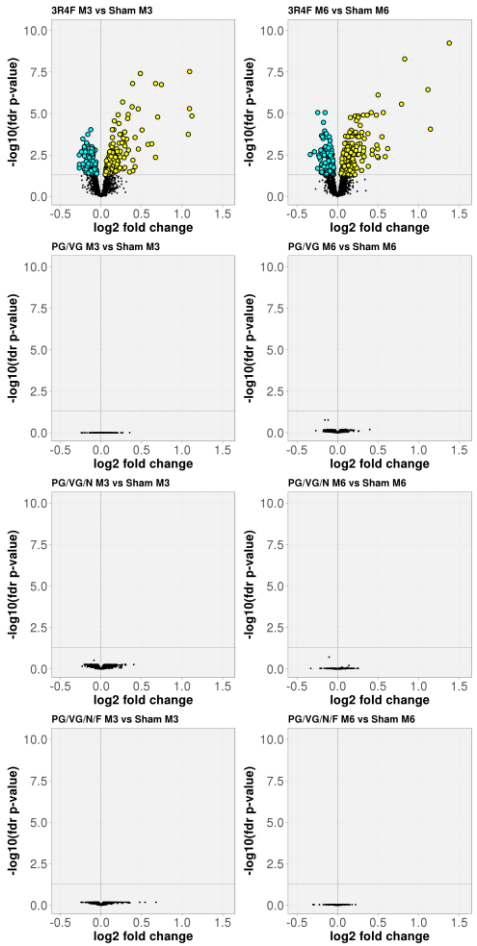
LUNG MOLECULAR CHANGES

Proteomics in the lungs

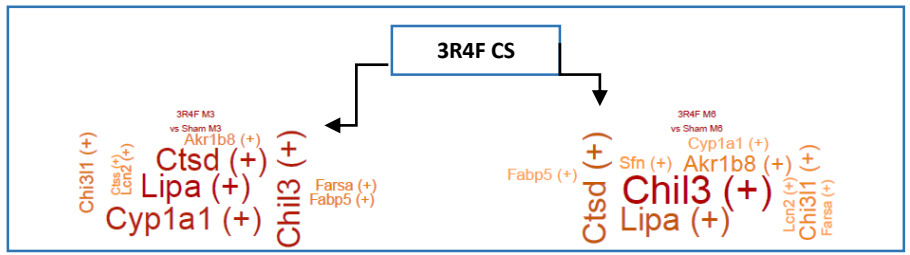




Compared with cigarette smoke exposure, exposure to e-vapor aerosols resulted in fewer differentially expressed proteins in lung tissue.



Protein response to 3R4F CS included upregulation **of xenobiotic enzymes** (e.g., Cyp1a1 and Akr1b8), **immune-related proteins** (e.g., Ctsd, Lcn2, and Chi3l1), and **lipid metabolism proteins** (Lipa and Fabp5).



PG/VG M3 vs Sham M3: No DEG

PG/VG M6 vs Sham M6: No DEG

PG/VG/N M3 vs Sham M3: No DEG

PG/VG/N M6 vs Sham M6: No DEG

PG/VG/N/F M3 vs Sham M3: No DEG

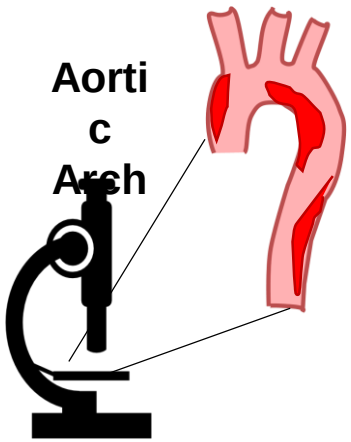
PG/VG/N/F M6 vs Sham M6: No DEG

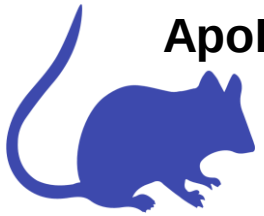
Top 10 Differentially Abundant Proteins

A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

Cardiovascular Diseases

- Atherosclerosis is an inflammatory disease characterized by the accumulation of lipoproteins and leucocytes as plaque in the arterial layer. Uncontrolled, it can lead to coronary heart disease (CHD) and underlying clinical events such as heart attack and angina.
- Development of CHD is accelerated by a variety of risk factors, including male sex, smoking, dyslipidemia, elevated blood pressure, physical inactivity, obesity, and diabetes.
- Patients with COPD have an increased risk cardiovascular morbidity and mortality.





The ApoE$^{-/-}$ mouse model permits the concomitant evaluation of:

Emphysema (COPD)
- Lung function
- Pulmonary inflammation
- Pathology

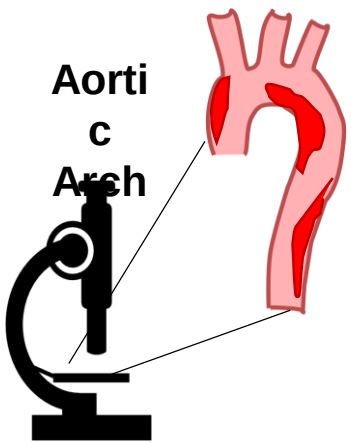
Cardiovascular disease
- Clinical chemistry
- Plaque development

Lo Sasso, G., Schlage, W.K., Boue, S., Veljkovic, E., Peitsch, M.C. and Hoeng, J. (2016): The Apoe(-/-) mouse model: a suitable model to study cardiovascular and respiratory diseases in the context of cigarette smoke exposure and harm reduction. Journal of translational medicine, 14, 146.

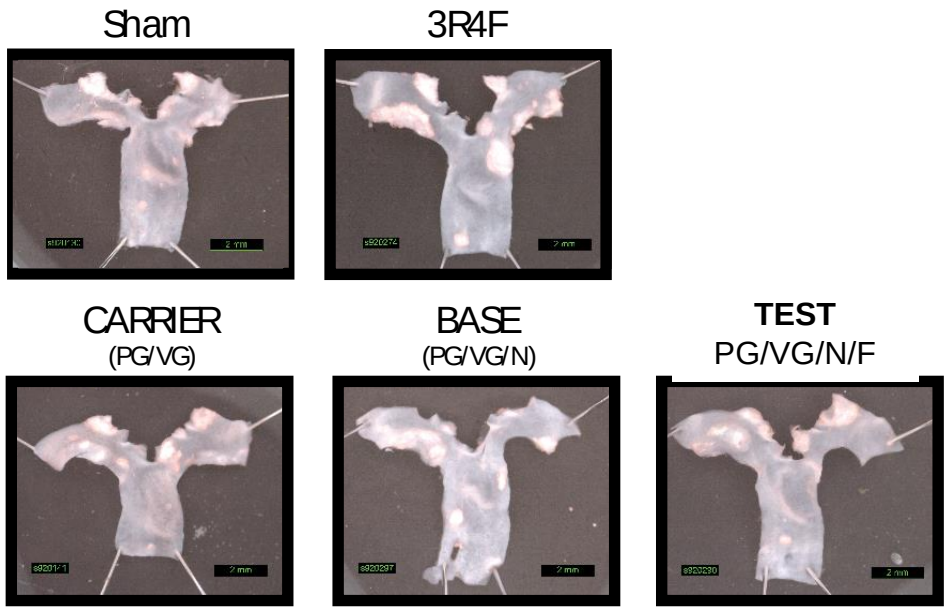
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

Effects of 3R4F CS and e-vapor aerosols on atherosclerotic plaque formation

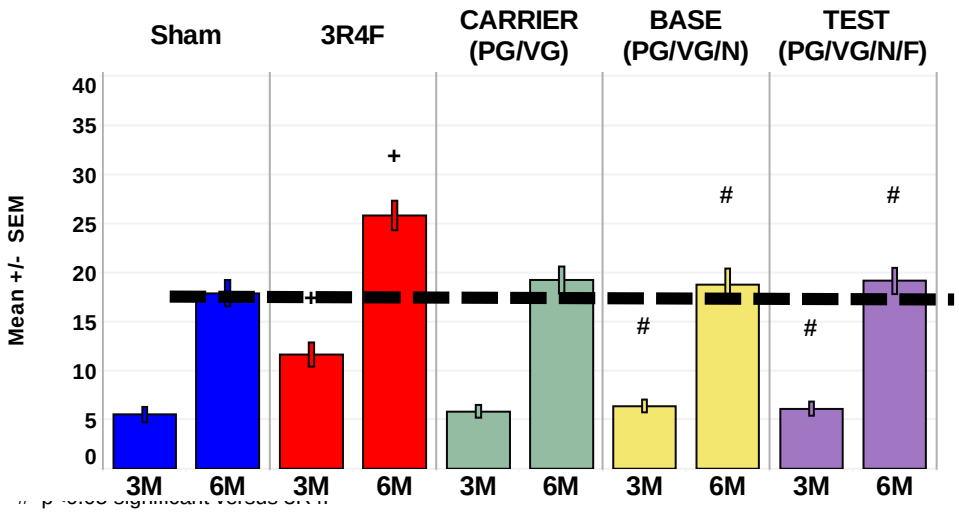
STRUCTURAL DAMAGE



6 months



Percentage of plaque (%)



Compared with cigarette smoke exposure, exposure to e-vapor aerosols (CARRIER, BASE, and TEST) induced lower atherosclerotic plaque formation.

There was no difference in plaque area between animals exposed to CARRIER, BASE, or TEST aerosol for 6 months and those treated with fresh air.

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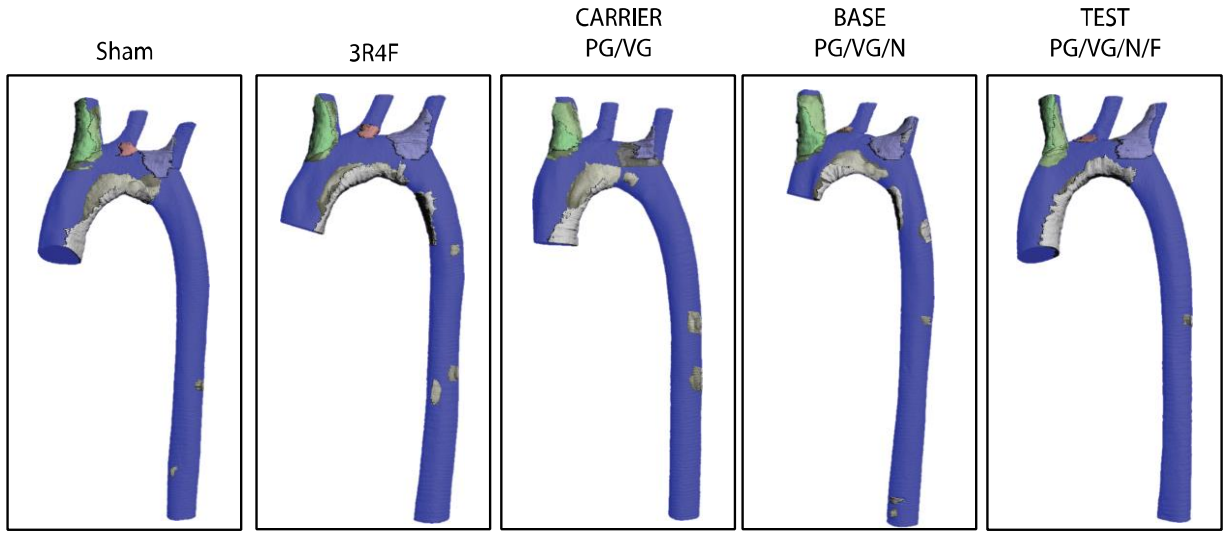
A Six-Month Inhalation Study in ApoE$^{-/-}$ Mice to Investigate Cardiovascular and Respiratory Exposure Effects of E-Vapor Aerosols Compared with Cigarette Smoke

Effects of 3R4F CS and e-vapor aerosols on atherosclerotic plaque formation

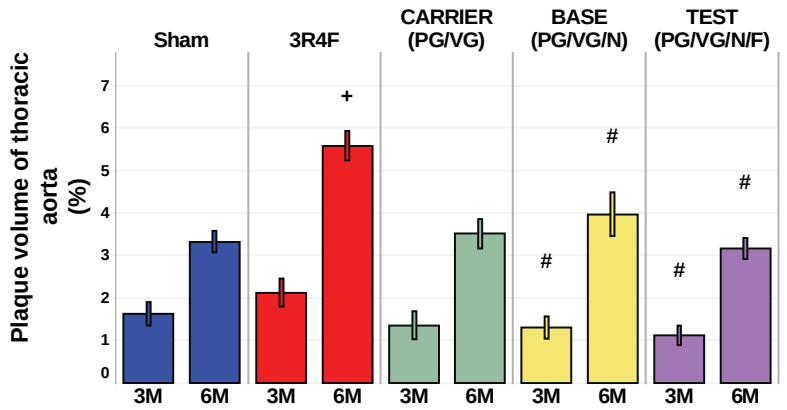
STRUCTURAL DAMAGE

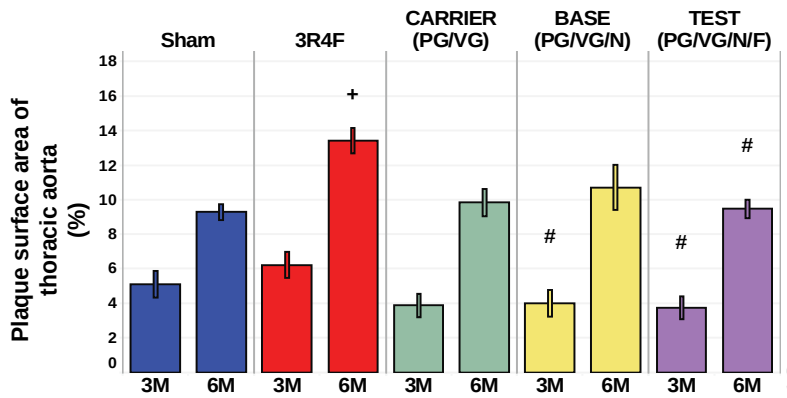
Representative pictures of atherosclerotic lesions and thoracic aorta acquired by CT



Compared with cigarette smoke exposure, exposure to e-vapor aerosols (CARRIER, BASE, and TEST) induced lower atherosclerotic plaque formation.

There was no difference in plaque area between animals exposed to CARRIER, BASE, or
TEST aerosol for 6 months and those exposed to fresh air.





\+ p < 0.05 significant versus sham
\# p < 0.05 significant versus 3R4F

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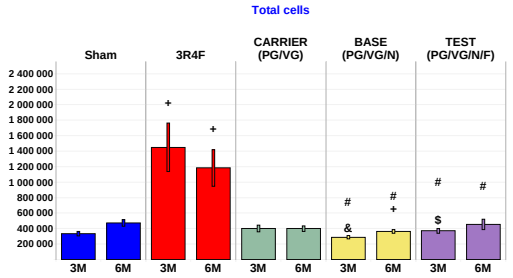
Summary

Compared with cigarette smoke, exposure to e-vapor aerosols resulted in:

LUNG INFLAMMATION

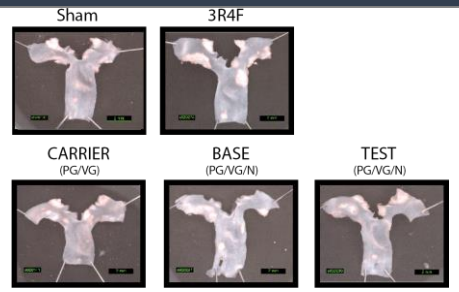
Total Lung Cells



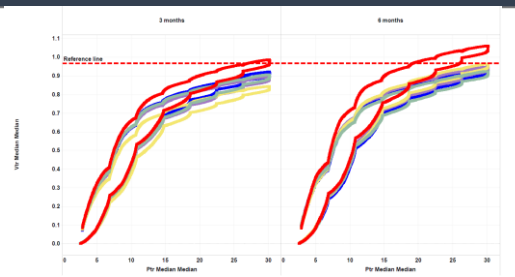
Mediators in BALF

Endpoint_Name	3R4F 3M	3R4F 6M	BASE (PG/VG/N) 3M	BASE (PG/VG/N) 6M	CARRIER (PG/VG) 3M	CARRIER (PG/VG) 6M	TESTMIX (PG/VG/N/F) 3M	TESTMIX (PG/VG/N/F) 6M
G-CSF	3.59	2.72	0.82	0.52	0.74	0.50	0.78	1.09
GM-CSF	2.14	1.57	1.99	0.83	0.98	0.78	2.18	1.06
IFN-g	0.84	1.26	0.95	1.67	0.79	2.89	0.54	1.91
IL-1a	0.54	0.53	0.93	1.32	1.22	1.47	0.89	1.24
IL-1b	1.03	0.94	0.84	0.88	0.75	0.77	1.11	0.95
IL-2	0.49	0.68	0.92	1.11	1.05	1.29	0.79	0.98
IL-4	1.22	0.96	1.07	1.54	0.60	1.52	0.89	1.30
IL-5	1.68	0.87	0.93	0.98	1.30	2.01	1.24	0.83
IL-6	3.88	4.84	0.50	1.72	2.32	2.77	0.82	1.46
IL-7	0.52	0.74	0.92	1.07	0.97	0.69	1.05	0.56
IL-9	0.93	0.84	1.09	1.14	0.98	1.87	0.82	1.14
IL-10	0.32	0.29	0.70	0.94	1.05	1.04	0.63	0.72
IL-12	1.24	3.36	0.57	1.33	0.62	1.93	0.90	0.95
IL-12b	0.60	0.58	0.96	1.16	0.90	1.29	0.69	0.93
IL-13	0.63	0.85	0.96	1.25	0.70	2.06	0.77	1.28
IL-15	0.93	1.03	1.55	0.88	0.87	1.20	0.72	0.68
IL-17	1.79	2.70	0.49	0.65	0.58	1.55	0.56	2.11
IP-10	3.48	3.66	0.88	1.12	0.82	1.47	0.81	1.23
KC	4.99	8.20	0.63	1.02	0.66	1.09	0.61	1.73
MCP-1	5.15	4.77	1.04	0.88	1.05	0.62	1.30	0.60
MIP-1a	2.28	2.70	1.02	1.04	0.86	0.94	0.98	0.71
MIP-1b	10.52	12.82	1.39	1.57	1.02	0.90	0.80	1.48
MIP-2	1.06	0.85	0.95	0.88	0.91	1.25	1.04	1.09
MMP total	1.70	2.19	0.98	1.04	1.10	0.96	0.98	1.00
PECAM-1	1.24	1.07	0.83	1.01	1.09	0.88	0.74	1.04
pro-MMP-9	61.67	17.58	0.98	0.29	1.93	0.50	1.07	0.51
RANTES	0.55	0.79	0.77	0.91	0.65	1.69	0.83	0.96
sE-Selectin	1.04	0.88	0.92	0.91	0.84	2.66	0.89	1.01
sICAM1	2.23	2.13	0.97	0.93	1.08	0.98	1.05	0.89
sP-Selectin	0.94	1.04	0.92	0.98	0.89	1.26	1.18	0.86
Thrombomodulin	1.90	2.34	0.86	0.95	0.91	1.09	0.95	1.06
TNF-a	2.10	2.77	0.82	1.17	0.71	1.45	1.12	0.97
Total PAI-1	2.54	2.68	0.92	1.00	0.97	0.95	1.15	1.14

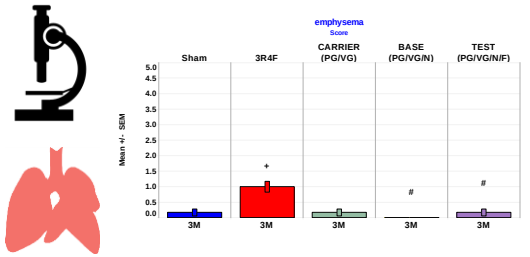
CARDIOVASCULAR



LUNG FUNCTION



LUNG HISTOPATHOLOGY



Biomarkers/Endpoints		CARRIER (PG/VG)	BASE (PG/VG/N)	TEST (PG/VG/N/F)
HPHC	*Acrolein*	↓	↓	↓
	Acetaldehyde	↓	↓	↓
	Formaldehyde	↓	↓	↓
	Propionaldehyde	↓	↓	↓
	Crotonaldehyde	↓	↓	↓
	4-(methylnitrosamino)1-(3-pyridyl)-1-butanone	↓	↓	↓
	N-Nitrosonornicotine	↓	↓	↓
Cardio vascular disease	*The rate of atherosclerotic plaque growth*	↓	↓	↓
	Transcriptomics analysis of the aorta - molecular dysregulation	↓	↓	↓
	Red blood cells - Hematocrite level	↓	↓	↓
	Platelets level	↓	↓	↓
	Pulse wave velocity (carotid artery)	↓	↓	↓
	Transcriptomics analysis of the heart ventricle - molecular dysregulation	↓	↓	↓
	Systolic-Diastolic dysfunctiom -Myocardial performance index	↓ ↓	↓ ↓	↓ ↓
Respiratory disease	*Lung inflammation-inflammatory cells in BALF*	↓	↓	↓
	Lung inflammation-inflammatory mediators	↓	↓	↓
	Lung function measured using FlexiVent system	↓	↓	↓
	Lung emphysematous changes	↓	↓	↓
	Transcriptomics analysis of the lung -molecular dysregulation of xenobiotic metabolism, inflammation, hypoxia apoptosis, cell proliferation.	↓	↓	↓
	Transcriptomics analysis of the RNE -molecular dysregulation of xenobiotic metabolism, inflammation, hypoxia apoptosis, cell proliferation.	↓	↓	↓

In comparison with 3R4F cigarette smoke exposure, e-vapor aerosol exposure results in:

- ✓ Lower levels of inflammatory cells and mediators
- ✓ Lower atherosclerotic plaque formation
- ✓ Lower emphysematous changes in the lungs

This study suggests that e-vapor aerosols induce significantly lower biological responses associated with smoking-related cardiovascular and pulmonary diseases.

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Thank you for your attention

Acknowledgements

The Singapore Team