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Advanced *in vitro* models and approaches for toxicity testing of electronic cigarette aerosol exposure

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Systems toxicology assessment of electronic cigarettes

Various types of electronic cigarettes

There are 8,000 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.

Challenges in toxicity assessment of electronic cigarettes

Lack of standards for the selection of chemicals to be monitored

- The list of harmful and potentially harmful constituents—established for combustible cigarettes is, with some exception, not applicable to electronic nicotine delivery systems (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" or "Food grade" as currently used for food ingredients is informative but may 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

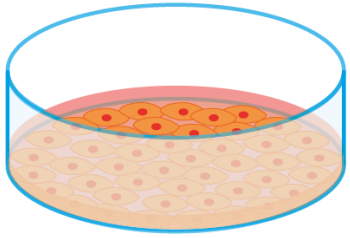
- FARSALINOS, K. E. & LE HOUEZEC, J. 2015. Regulation in the face of uncertainty: the evidence on electronic nicotine delivery systems (e-cigarettes). Risk Manag Healthc Policy, 8, 157-67.FLORA, J. W., MERUVA, N., HUANG, C. B., WILKINSON, C. T., BALLENTINE, R., SMITH, D. C., WERLEY, M. S. & MCKINNEY, 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.

Relevant test systems in *in vitro* toxicology

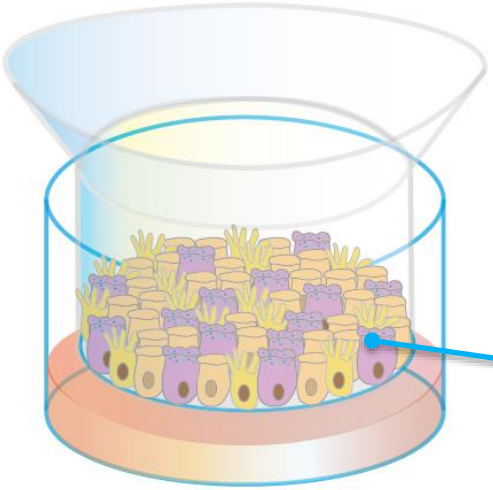
An EU directive "on the protection of animals used for scientific purposes" (EU Directive 2010/63/EU) strongly promotes the use of alternative animal test methods.

In the context of the 3Rs—to Replace, Reduce, and Refine the use of animal in research—relevant test systems offer a strategy to significantly minimize the use of animals in research.

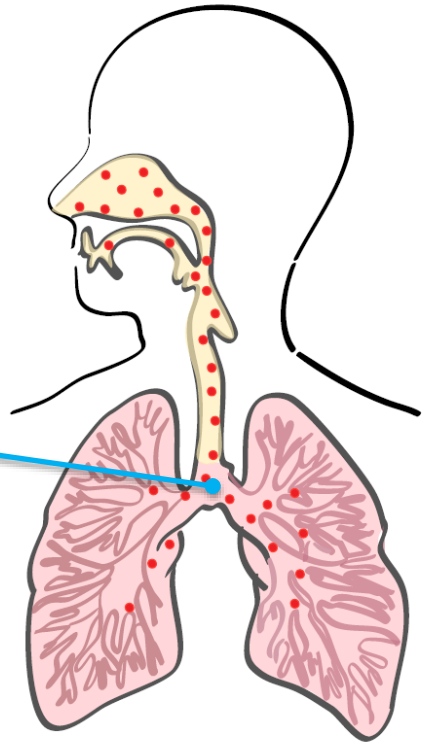
Relevant biological test systems which best represent human tissue and allow for aerosol exposure are available.



Two-dimensional submerged human bronchial epithelial cultures

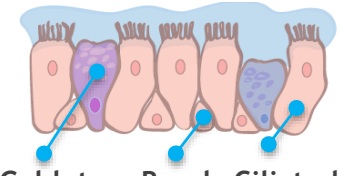


Three-dimensional organotypic human bronchial epithelial cultures



Organotypic cultures mimic the morphology and functionality of their *in vivo* tissue counterparts

Pseudostratified columnar epithelium



Goblet **Basal** **Ciliated cells**

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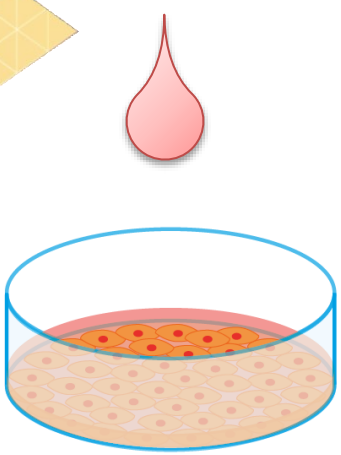
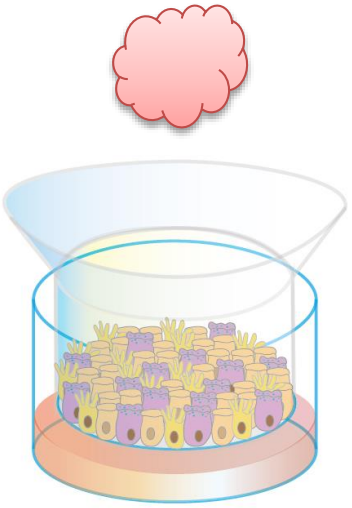
Differences in exposure

Liquid

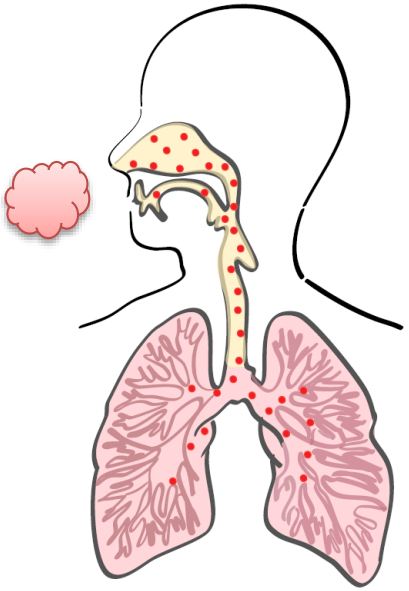


Two-dimensional submerged cell cultures



Three-dimensional organotypic cell cultures

Aerosol

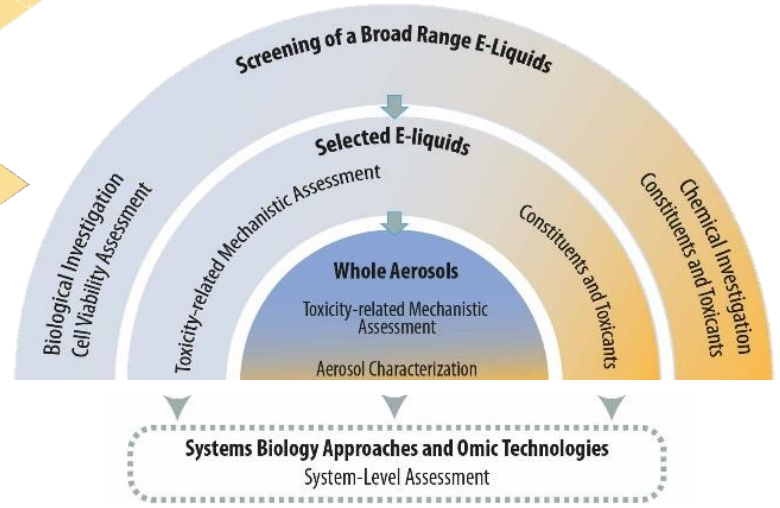


Respiratory airways

- Higher throughput
- Lack of physiological relevance due to submerged condition
- Osmotic stress at high concentration
- The liquid composition may vary when aerosolized

- Increased physiological relevance of the exposure
- Effect of the vaping device can be accounted
- Different efficiency among exposure systems
- Lack of differential site deposition

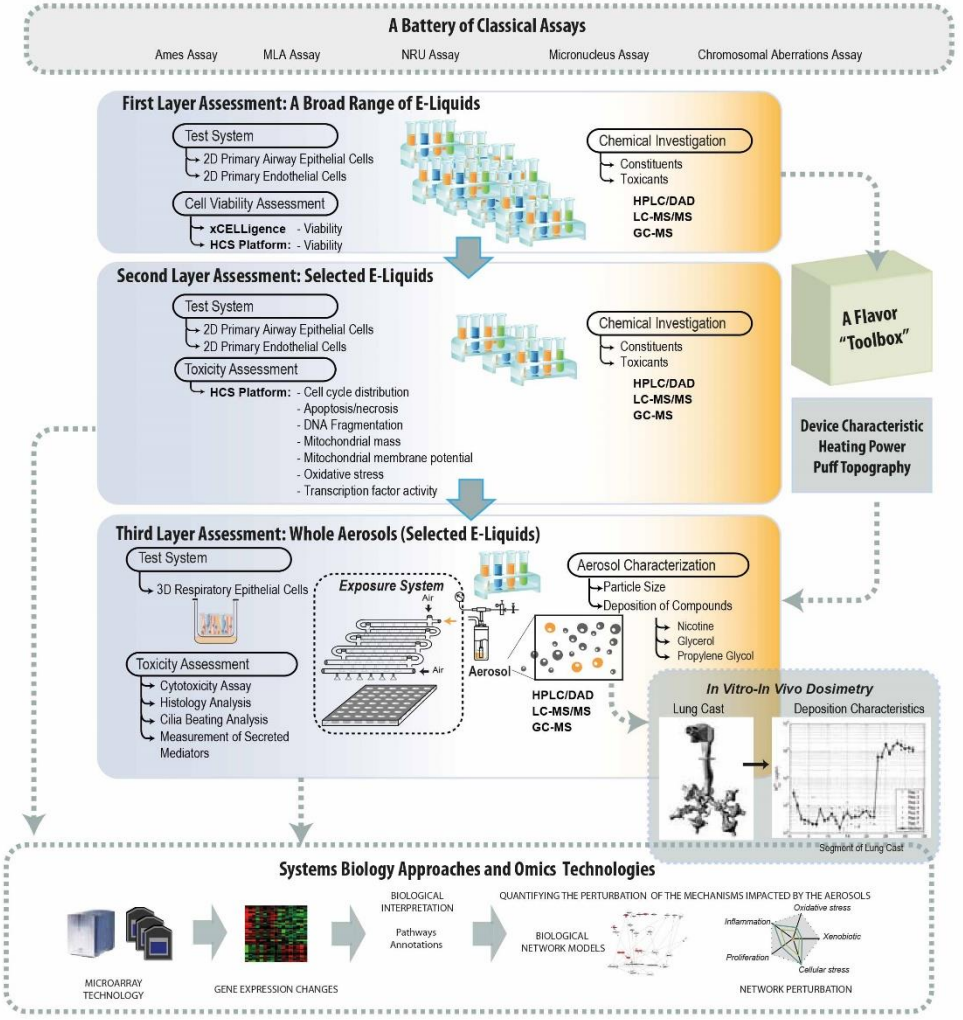
A use case for *in vitro* system toxicology assessment of e-liquids framework



Iskandar et al. 2016

IQOS® MESH™

- P4M3 electronic device using *MESH* technology
- Liquid-based, contains nicotine
- No combustion, low temperature



- ISKANDAR, A. R., GONZALEZ-SUAREZ, I., MAJEED, S., MARESCOTTI, D., SEWER, A., XIANG, Y., LEROY, P., GUEDJ, E., MATHIS, C., SCHALLER, J. P., VANSCHEEUWIJCK, P., FRENTZEL, S., MARTIN, F., IVANOV, N. V., PEITSCH, M. C. & HOENG, J. 2016. A framework for in vitro systems toxicology assessment of e-liquids. Toxicol Mech Methods, 26, 389-413.

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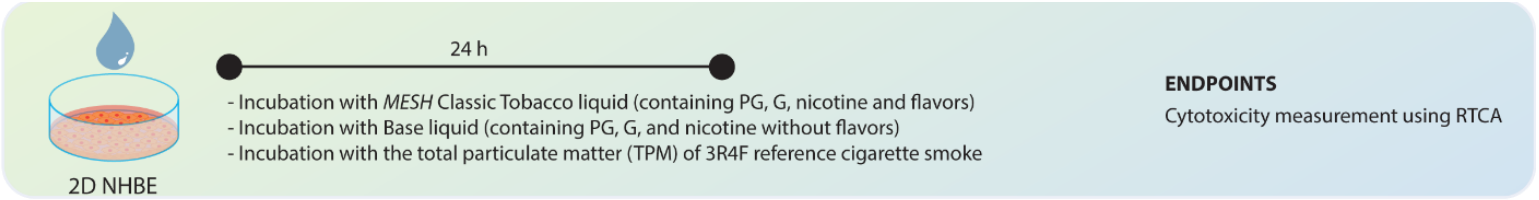
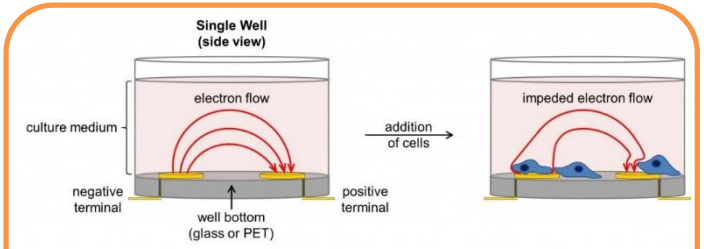
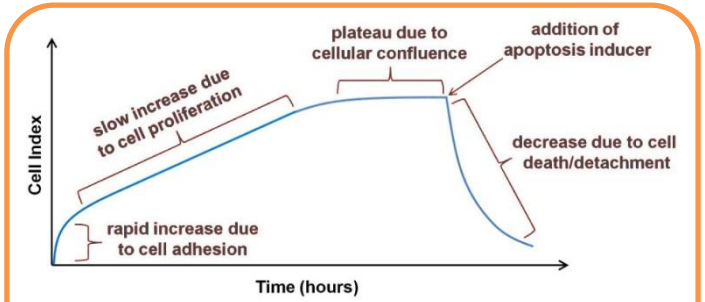
First layer assessment





Real-time impedance – Gold microelectrode biosensors are linked to form an array allowing simultaneous monitoring of the whole cell population.



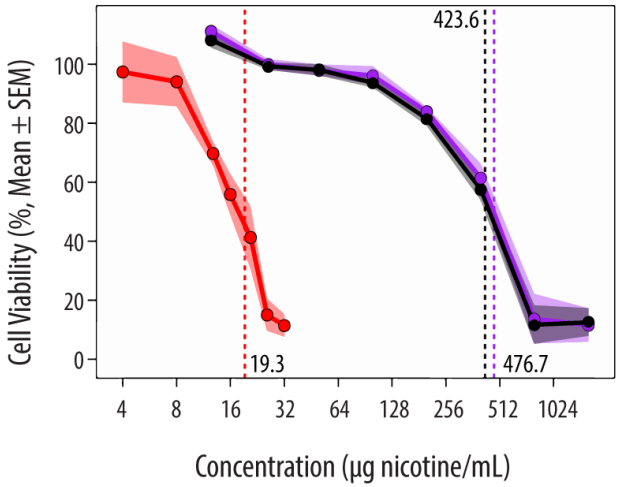
Cell index – Unitless parameter is used to report the impedance of electron flow caused by adherent cells.

Images: https://www.aceabio.com/products/rtca-mp/

Results

- Both e-liquids showed comparable cytotoxic effects → addition of flavors did not profoundly alter the toxicity profile of the Base liquid

- With reference to their nicotine content, 3R4F TPM showed approximately 20-fold lower cytotoxic EC_{50} compared to that of the e-liquids (19 vs. >400 µg nicotine/mL).



New data; not submitted
"Prepared for submission, June 2019"

3R4F TPM
MESH Classic Tobacco liquid
Base liquid

Manuscript in preparation

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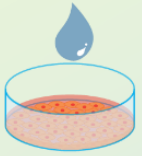
Second layer assessment



2D NHBE

4 h

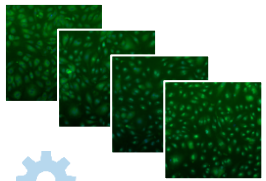
24 h

- 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

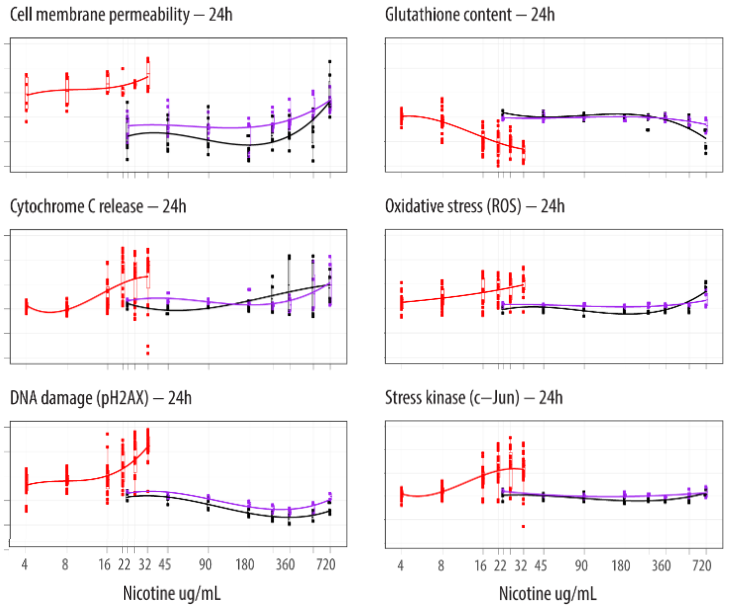
ENDPOINTS

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

Automated Image Acquisition



and Analysis



3R4F TPM MESH Classic Tobacco liquid Base liquid

New data; not submitted
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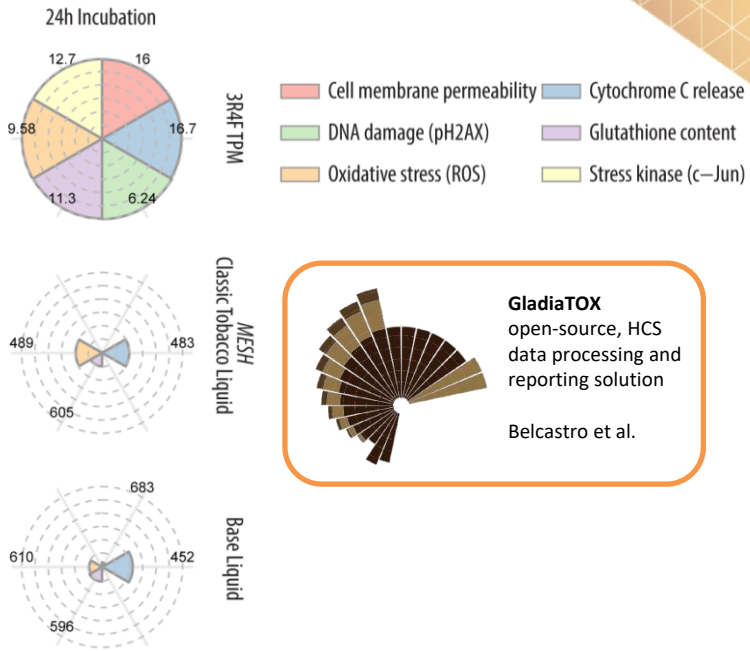

GladiaTOX
open-source, HCS data processing and reporting solution

Belcastro et al.

- Both e-liquids (Base and *MESH* Classic Tobacco) showed comparable effects → addition of flavors did not profoundly alter the effect on the phenotypic profile of the Base liquid.
- Only few of the phenotypic effects are obtained at a much higher level of nicotine exposure.

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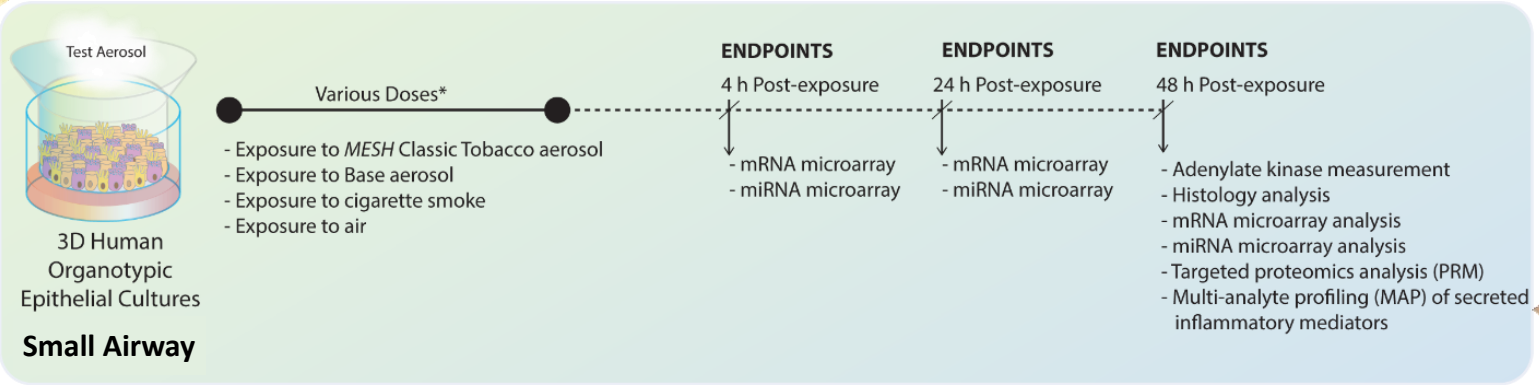
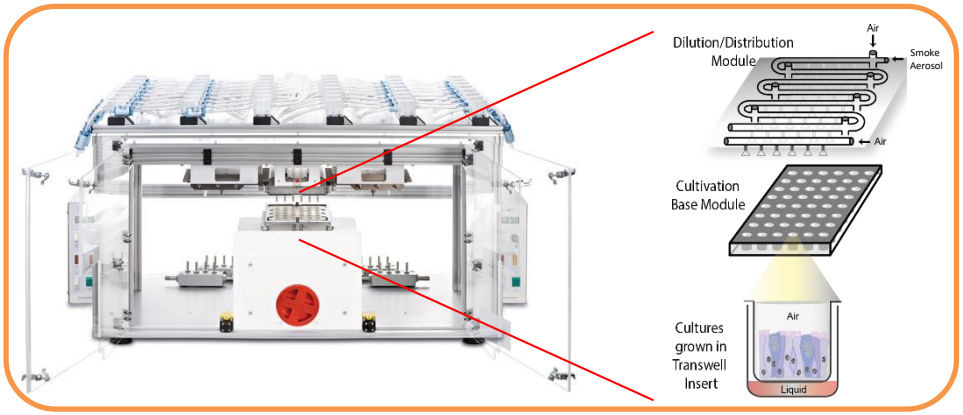
Third layer assessment (1/3)

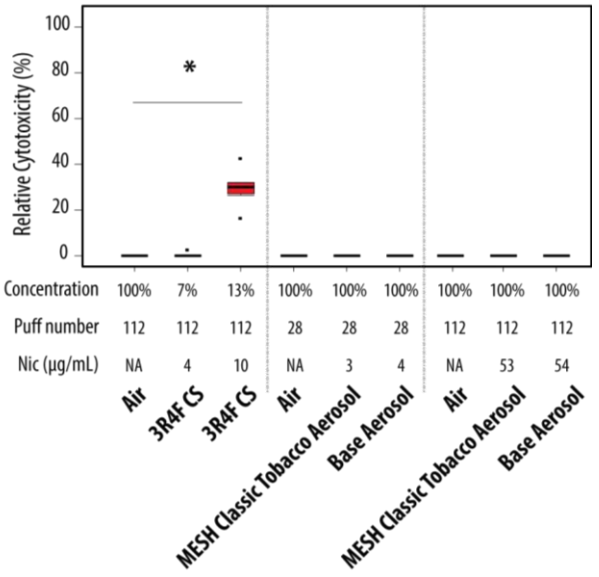




New data; not submitted

"Prepared for submission, June 2019"

Cytotoxicity (adenylate kinase)



Air Exposure
Undiluted (100%) for 112puffs

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

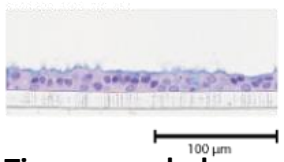
3R4F CS
Diluted (13%) for 112 puffs
10 µg nicotine/mL

MESH Classic Tobacco Aerosol
Undiluted (100%) for 28 puffs
3 µg nicotine/mL

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

Base Aerosol
Undiluted (100%) for 28 puffs
4 µg nicotine/mL

Base Aerosol
Undiluted (100%) for 112 puffs
54 µg nicotine/mL



Tissue morphology
(hematoxylin and eosin-stained section)

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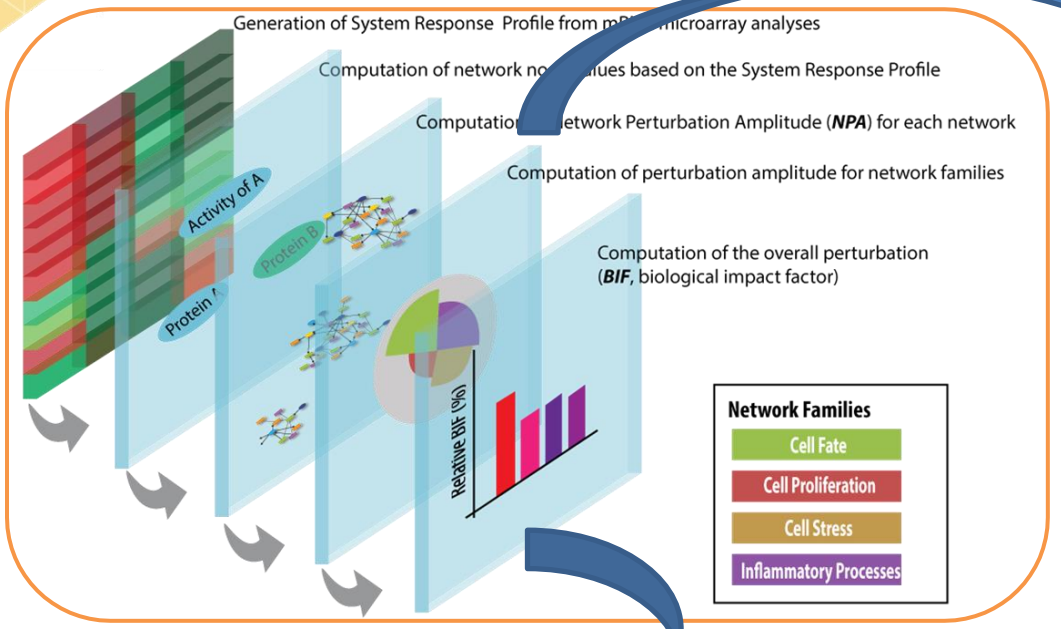
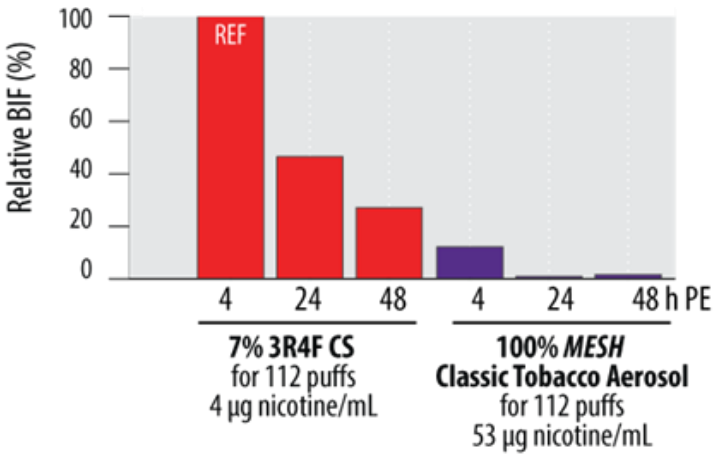
Third layer assessment (2/3)

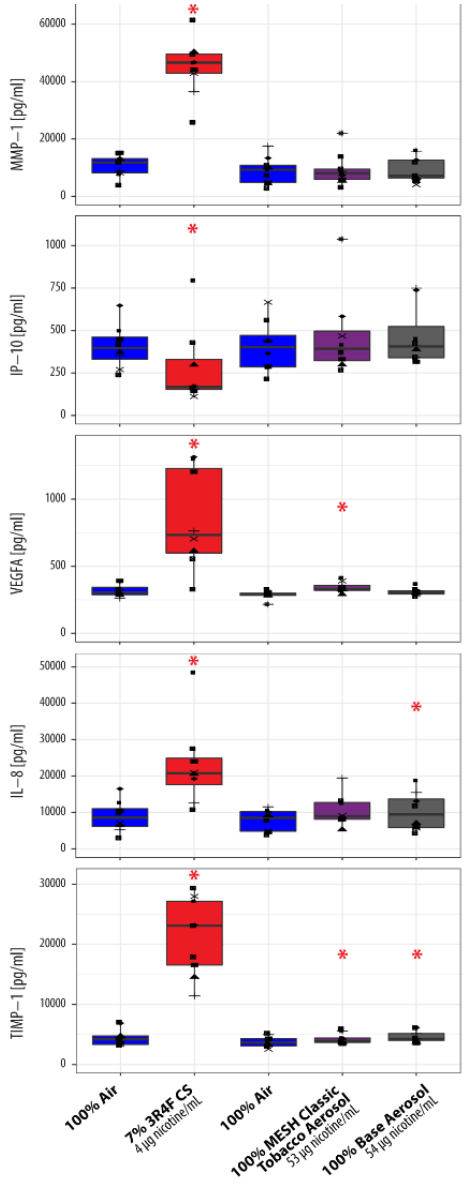


Biological Impact Factor



New data; not submitted
"Prepared for submission, June 2019"

Multi-analyte profiling



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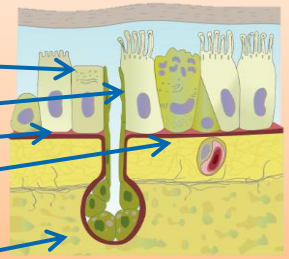
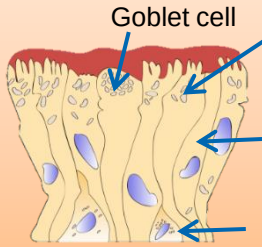
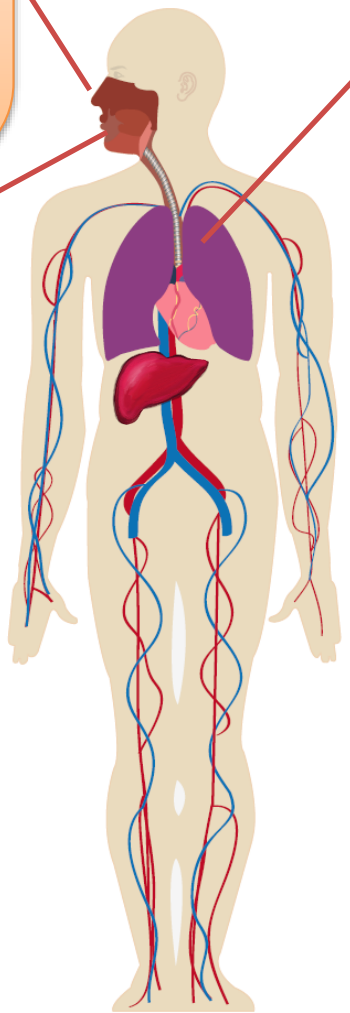
Nasal epithelium

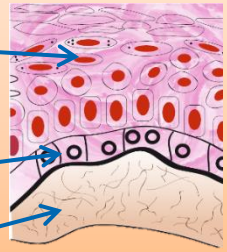


Bronchial epithelium





Oral epithelium

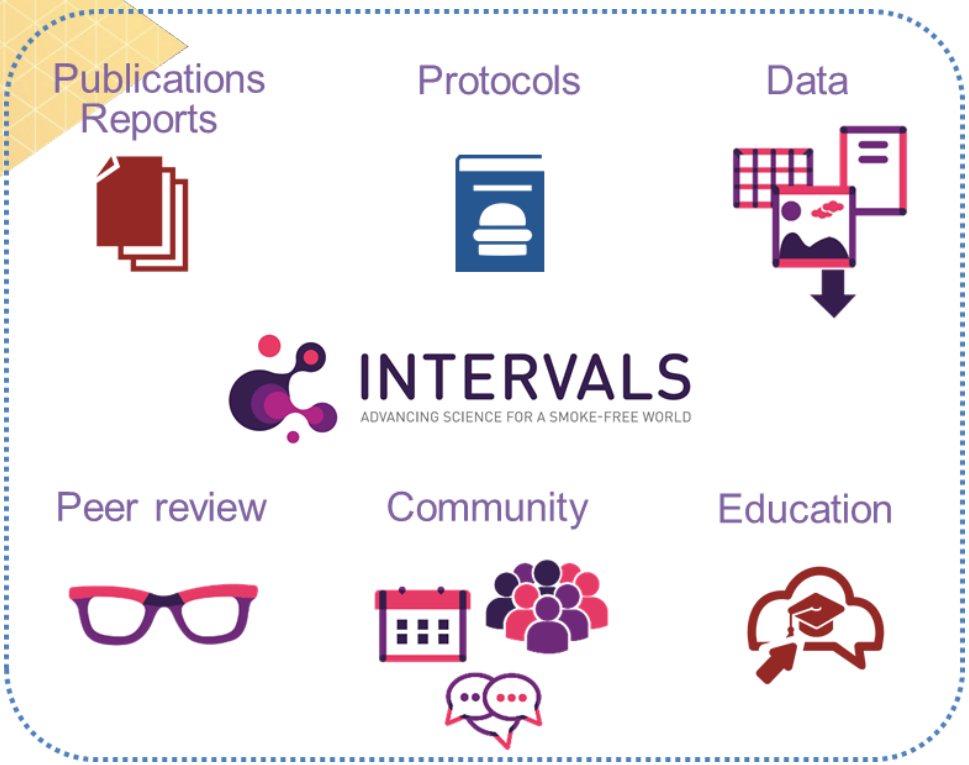


- Investigation can be expanded to additional relevant organs for a holistic approach.
- Multiple organs can be connected to create a more physiological response → organ-on-a-chip

Conclusion

- While ENDS have the potential to lower the exposure to harmful chemicals compared to smoking, their long term effects are not yet fully explored.
- Testing the potential toxic effects of exposure to e-liquid aerosols requires the use of relevant test systems and exposure models.
- Systems biology approaches uncover changes at the cellular and molecular levels, which are meant to complement standard toxicity assays.
- Collaborative efforts between the scientific community, industry, and regulatory stakeholders are facilitating the adoption of 21st Century Toxicology approaches.

INTERVALS - enabling science to support designing a smoke-free future



INTERVALS is a public **repository** for 21st-century pre-clinical and clinical (systems) **inhalation toxicology assessment** data and results that supports open data principles

Website
http://intervals.science/

Boué S et al., Supporting evidence-based analysis for modified risk tobacco products through a toxicology data-sharing infrastructure 2017, 6:12

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Acknowledgments

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