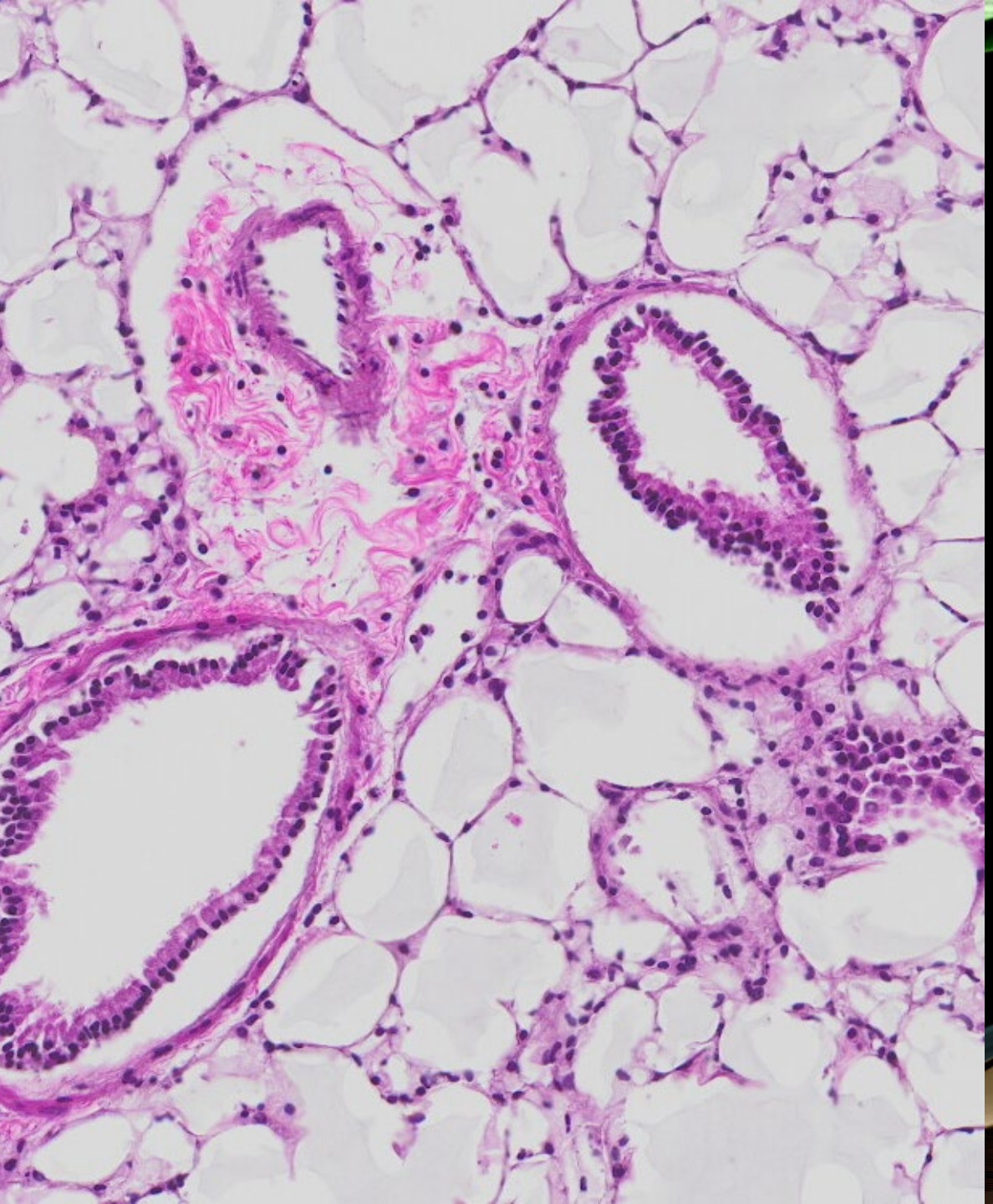

THE MISSING LINK: *EX VIVO* E-CIGARETTE TOXICITY MODELING

Jacklyn "Skye" Kelty, PhD

Postdoctoral Fellow

Environmental and Occupational Health Sciences Institute

Rutgers University

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Research Team
Julia Herbert, DVM, PhD

Alyssa Bellomo
Jeffrey Ho

Andrew Gow, PhD
Debra Laskin, PhD
Jeffrey Laskin, PhD
John McGann, PhD
Reynold Panettieri, PhD

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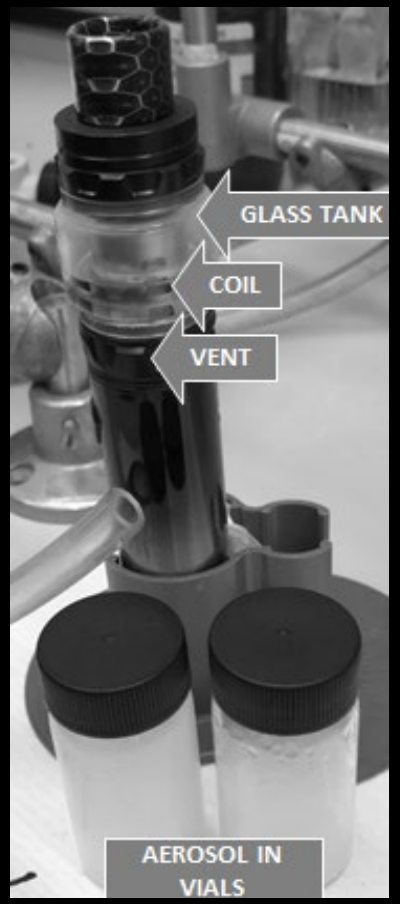
OUR PATH FOR TODAY



Pulmonary toxicity testing spectrum

in vitro ↔ *ex vivo* precision-cut lung slices ↔ *in vivo*

Best practices in e-cig toxicity testing

gaps in biological components of existing models

Q&A

Menthol contribution to acute e-cig toxicity

Q&A

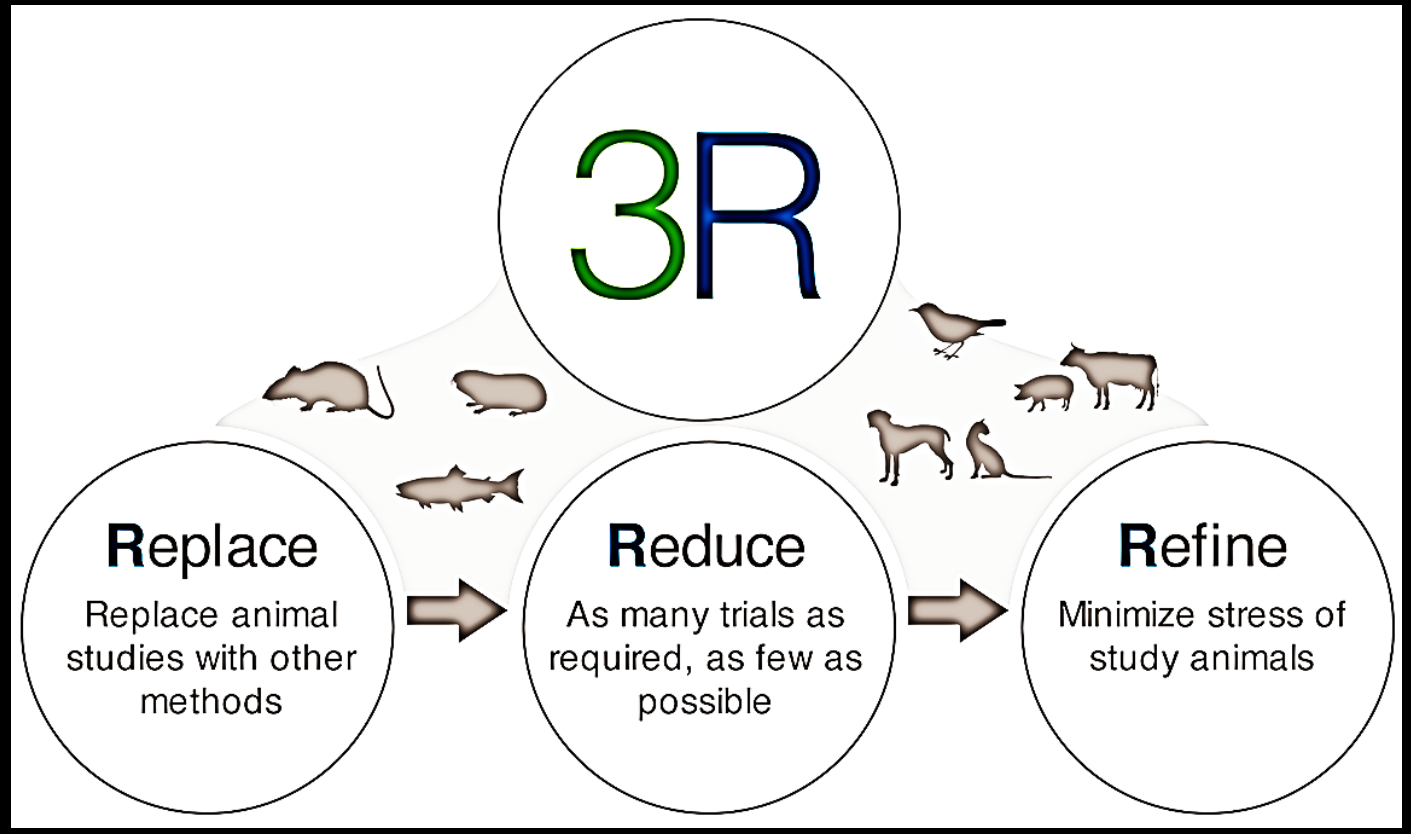
Identifying susceptible subpopulations for e-cig toxicity

Q&A

3R PUSH FOR PULMONARY TOXICITY TESTING

Laboratory animals are not people.

People are diverse so simplify with caution!



https://ehe.jhu.edu/graduate/masters-programs/master-of-science-in-toxicology-for-human-risk-assessment/research-and-practice-in-risk-assessment.html

SHORTFALLS FOR LUNG MODEL VALIDATION

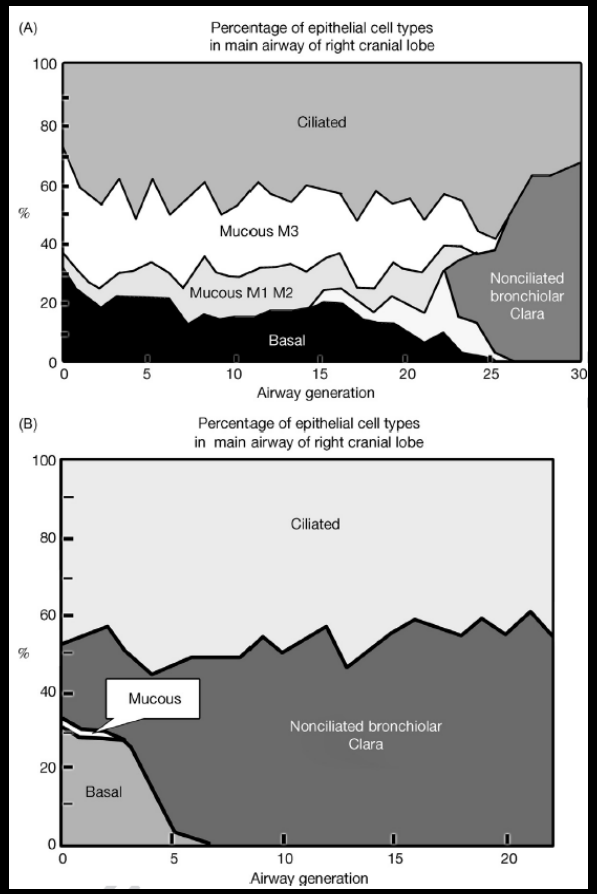
No *in vitro* models are approved by US regulatory agencies for pulmonary toxicity testing (McMullen 2018, NTP 2020, NIEHS 2020)

- *Function?* Very few pulmonary models are validated relative to source tissue baselines or *in vivo* exposure responses
- *Complexity?* Respiratory system physiological conditions and structure are complicated to replicate in the lab
- *Dose extrapolation?* Inhaled toxicant distribution in the lung is tricky

Opportunity to improve translation? A good investment?

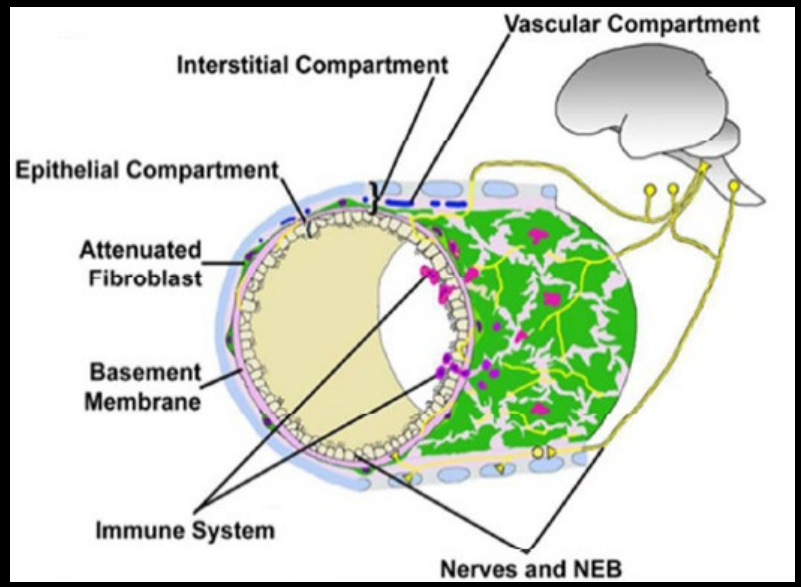
COMPLEXITY OF THE LUNG

40 cell types with varied abundance by airway generation and species



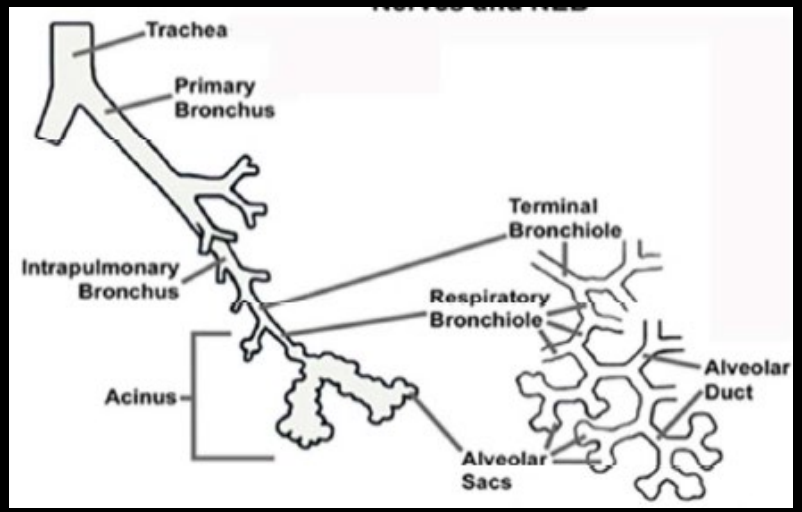
Van Winkle 2017. DOI: 10.1016/B978-0-12-801238-3.65841-5

Complicated tissue structure and function



Van Winkle 2017. DOI: 10.1002/cptx.18

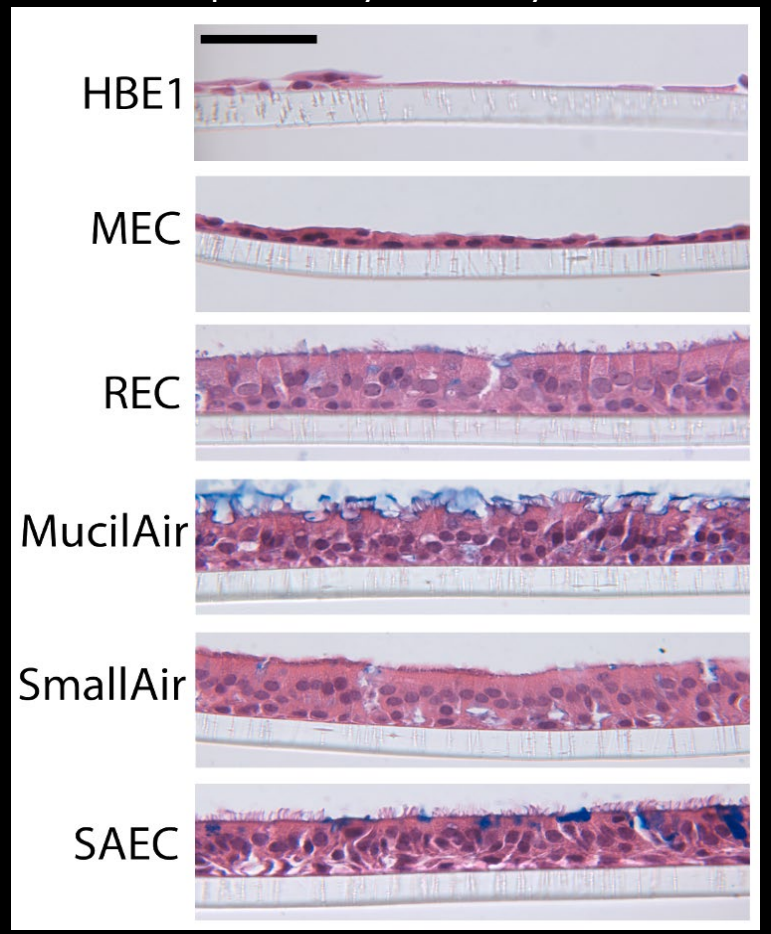
Complicated organ structure at interface to environment and full cardiac output



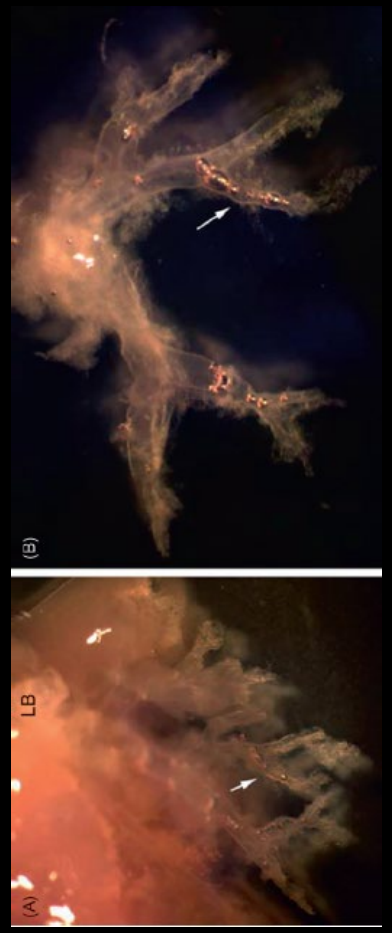
Van Winkle 2017. DOI: 10.1002/cptx.18

IN VITRO => IN VIVO AXIS

Can you guess which is the cell line? Others are primary airway cells at ALI.



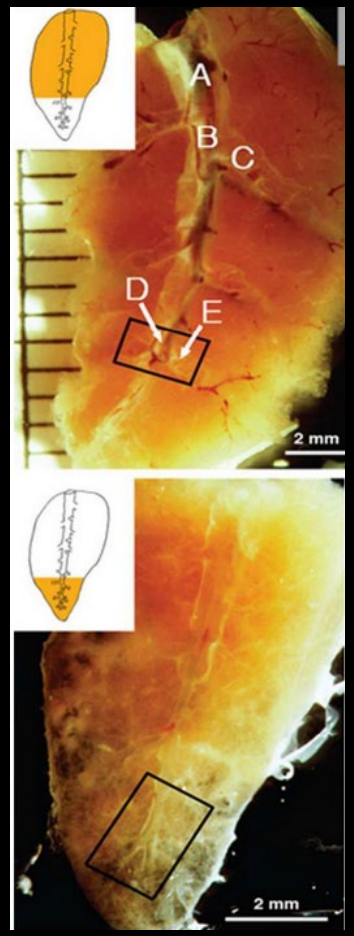
Microdissection



Van Winkle 2017. DOI: 10.1002/cptx.18

Precision-Cut Lung Slices

Whole Lung



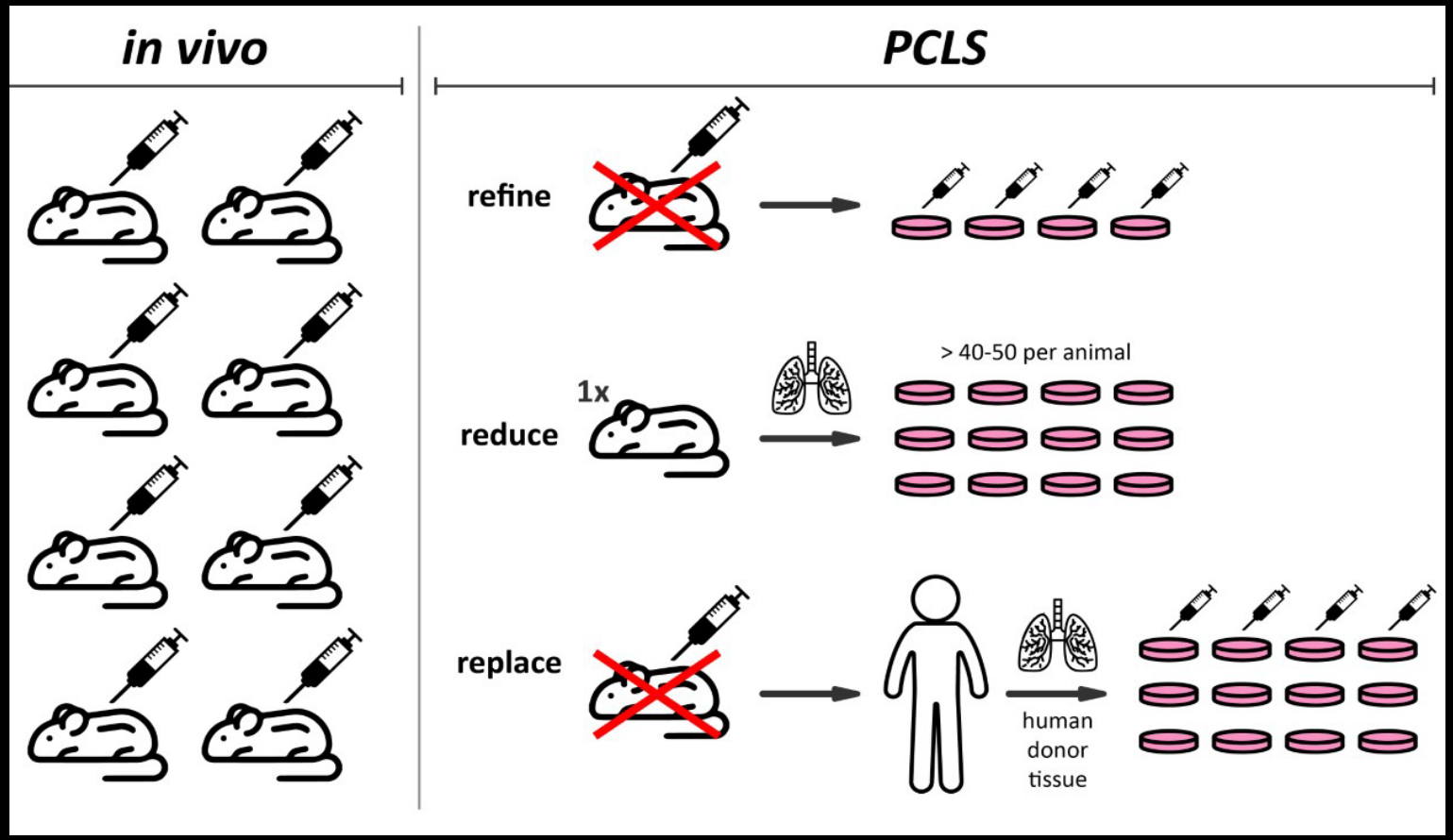
Van Winkle 2017. DOI: 10.1002/cptx.18

QUICK NOTE ON SPECIES DIFFERENCES

Species differences in lung structure and function are site specific.

	TRACHEA	PROXIMAL BRONCHI (branches 2-6)	DISTAL BRONCHI (branches 7+)
Epithelial Structure*	ciliated pseudo-stratified columnar	ciliated pseudo-stratified columnar	simple columnar or cuboidal
Submucosal Glands*	PRESENT	Mouse- ABSENT Rhesus- PRESENT Human- PRESENT	ABSENT
Club Cell Density*	Mouse (49%) Rhesus (0%) Human (0%)	Mouse (61%) Rhesus (0%)	Mouse (>50%) Rhesus (0%, RB >90%) Human (11-41%, RB 22%)
Goblet Cell Density*	Mouse (<1%) Rhesus (17%) Human (9%)	Mouse (0%) Rhesus (15%)	Mouse (0%) Rhesus (14%, RB +) Human (2%, RB 0%)
Ciliated Cell Density*	Mouse (39%) Rhesus (33%) Human (49%)	Mouse (36%) Rhesus (47%)	Mouse (<50%) Rhesus (49%, RB<10%) Human (+, RB +)
Basal Cell Density*	Mouse (10%) Rhesus (42%) Human (33%)	Mouse (1%) Rhesus (32%)	Mouse (0%) Rhesus (29%, RB +) Human (+, RB +)

PCLS AS A 3R SCREENING MODEL



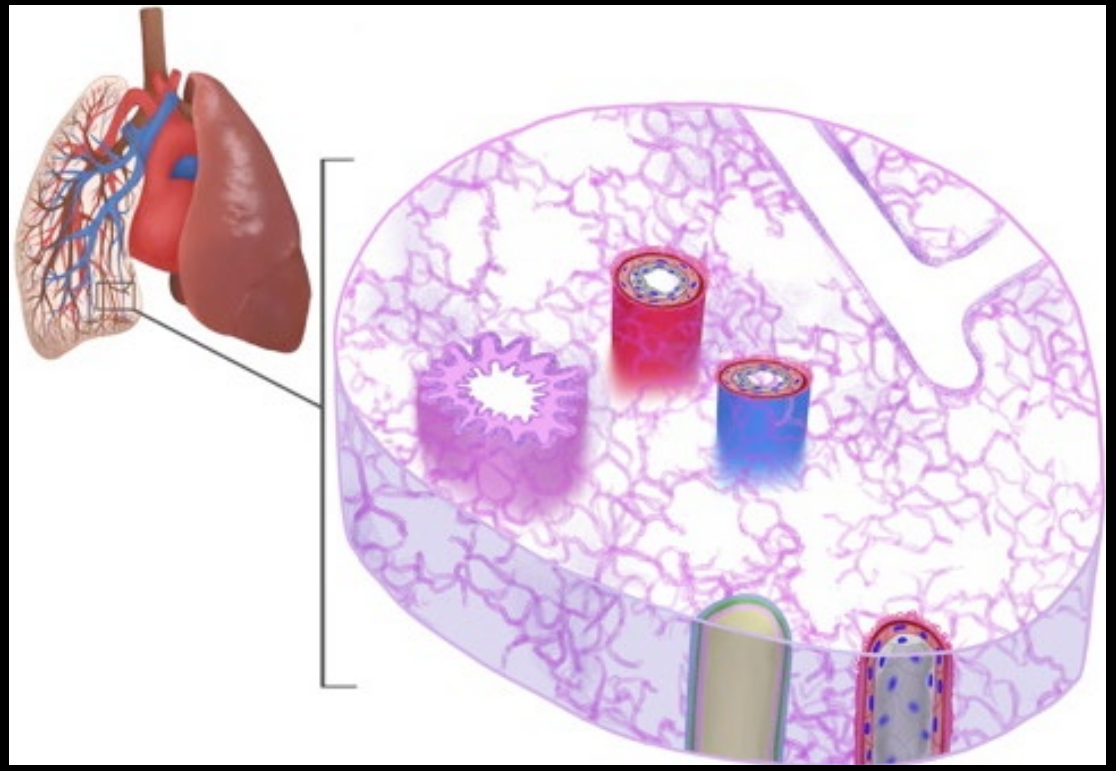
Refines by potentially avoiding direct animal exposure.

Reduces by generating of many technical replicates.

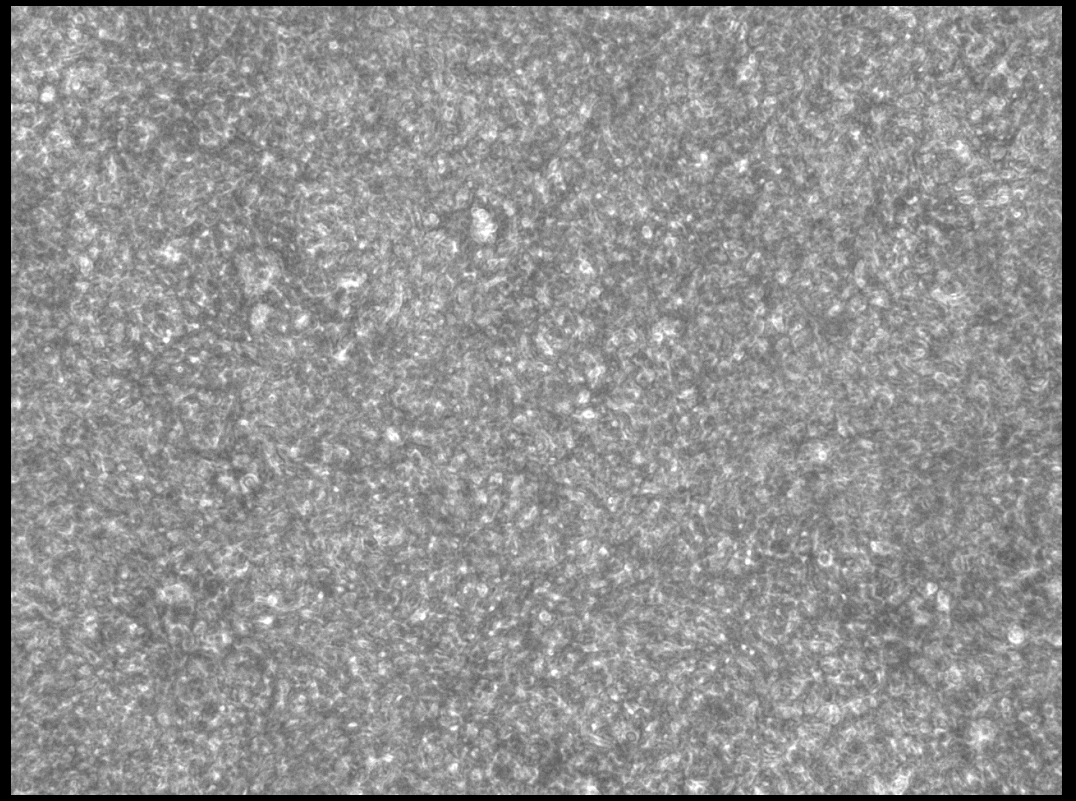
Relaces by using human source tissue.

PCLS FUNCTIONAL AND CELLULAR ENDPOINTS

PCLS contain all pulmonary resident cells in the structural orientation found in lung lobes.

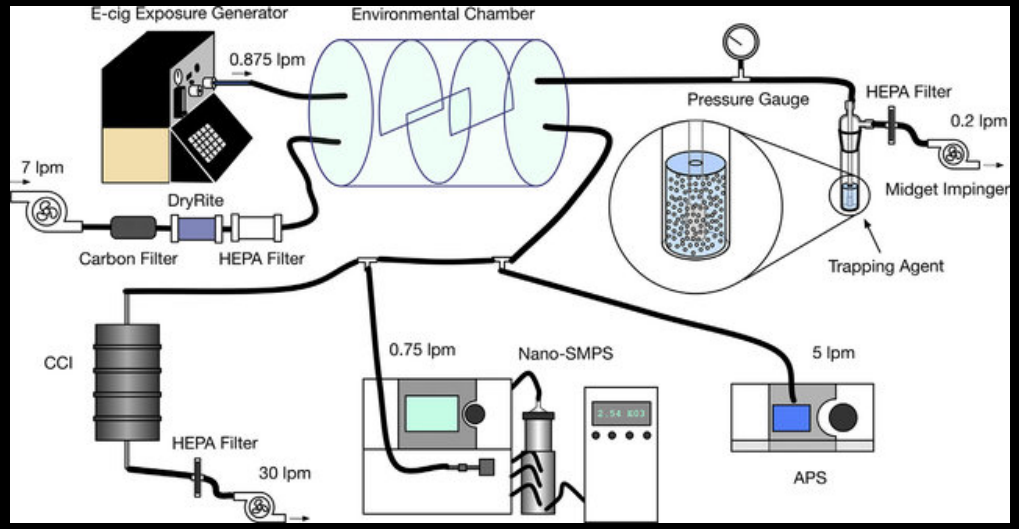


Bronchial cross sections in PCLS can contract. Bronchial cilia are beating in PCLS.



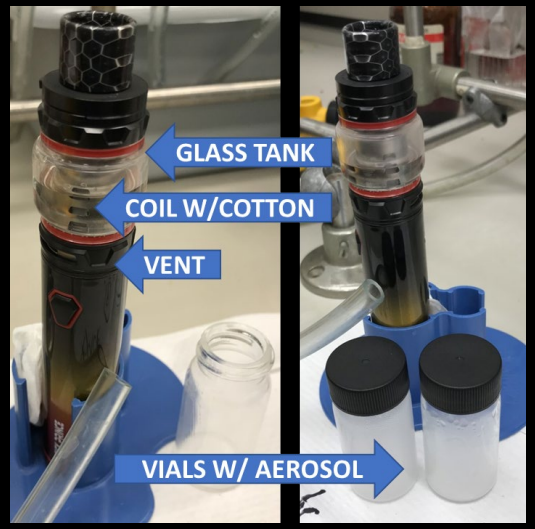
E-CIG TOXICITY TESTING STRONG CHEM + ENGINEERING

Chemists and engineers created standardized and human-relevant exposure systems…



Zhao 2017. DOI: 10.1016/j.jhazmat.2017.10.057

Adamson 2016. DOI: 10.1186/s13065-016-0221-9



E-CIG TOXICITY TESTING WEAK BIOLOGY

…then most labs used submerged, immortalized cell lines that are far from a functioning lung tissue…

Without clear understanding of dose relevance…

Without exploring time-course or dose-response patterns…

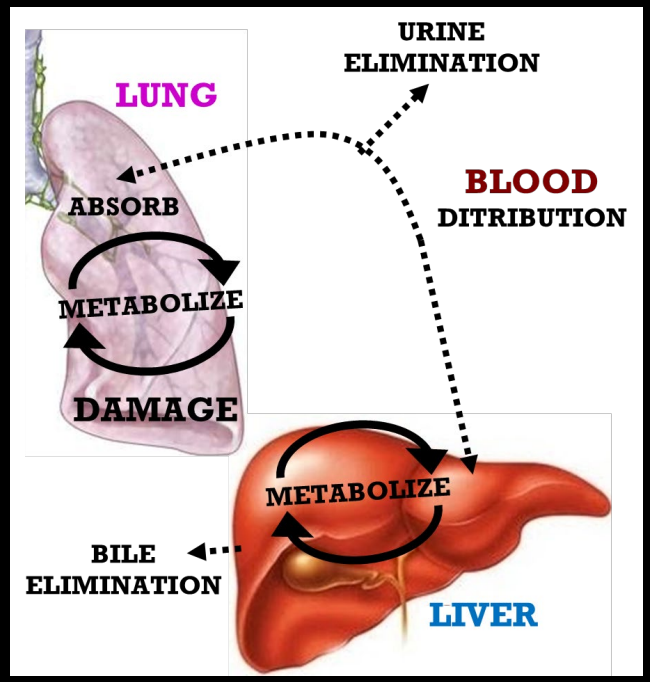
Without discovering the toxic components of the mixture…

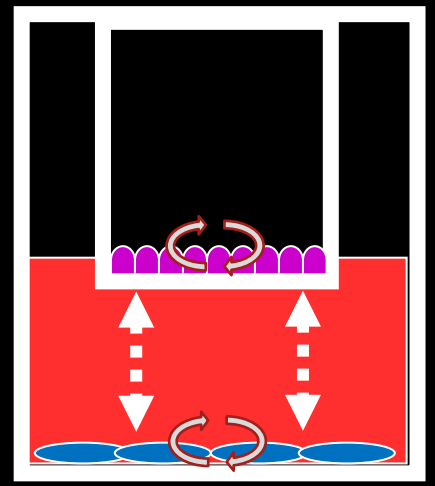
Without identifying potentially susceptible populations…

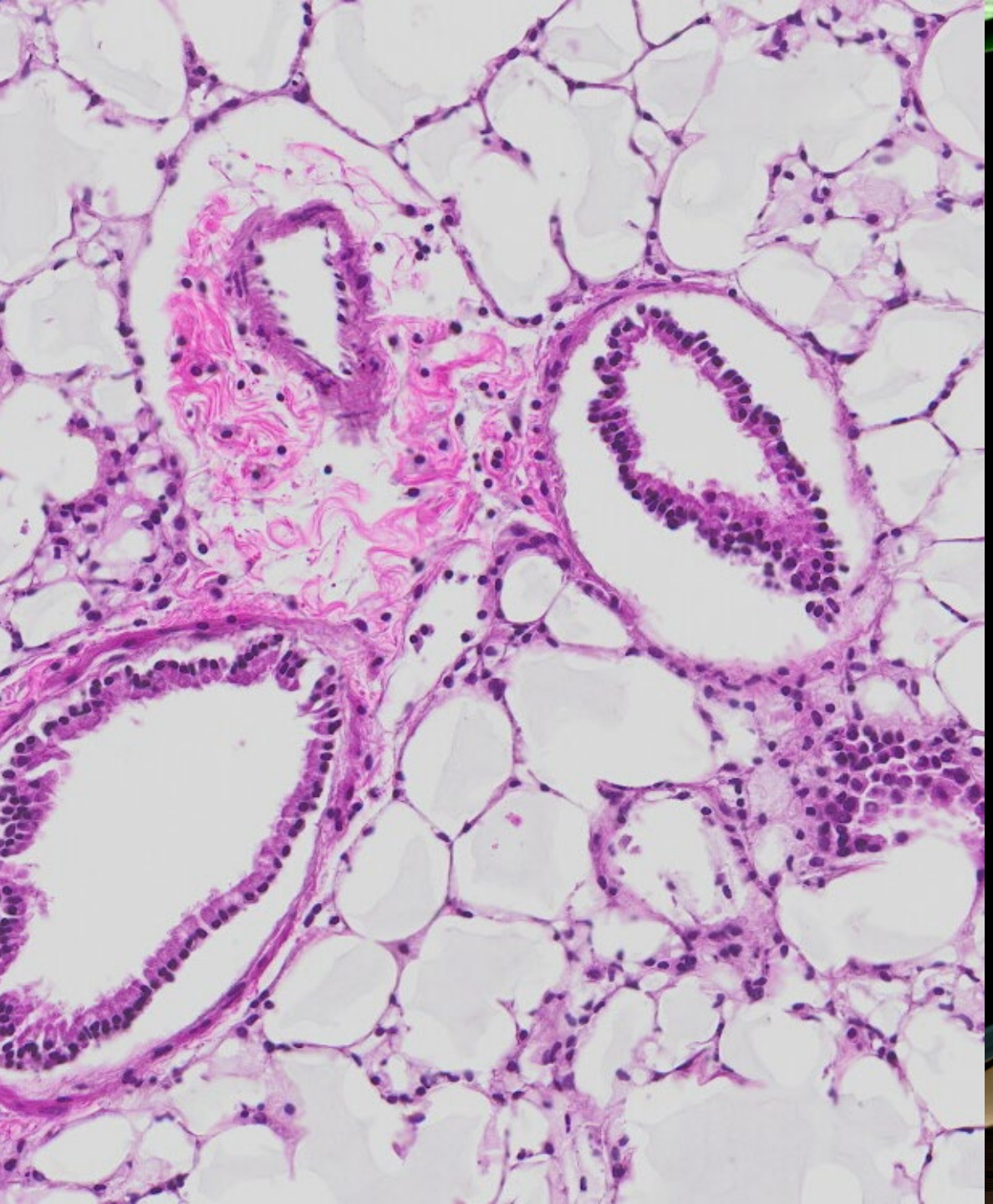
E-CIG TOXICITY TESTING DEFINED LOCAL DOSE

Local dose of nicotine is undefined:

- Heterogeneous consumption preferences
- Varied distribution throughout airway tree
- Local xenobiotic metabolism clearance
- Clearance and recirculation through pulmonary circulation
- Air-liquid interface vs. submerged exposure
- Normalization is debated– total particulate matter? Nicotine levels?



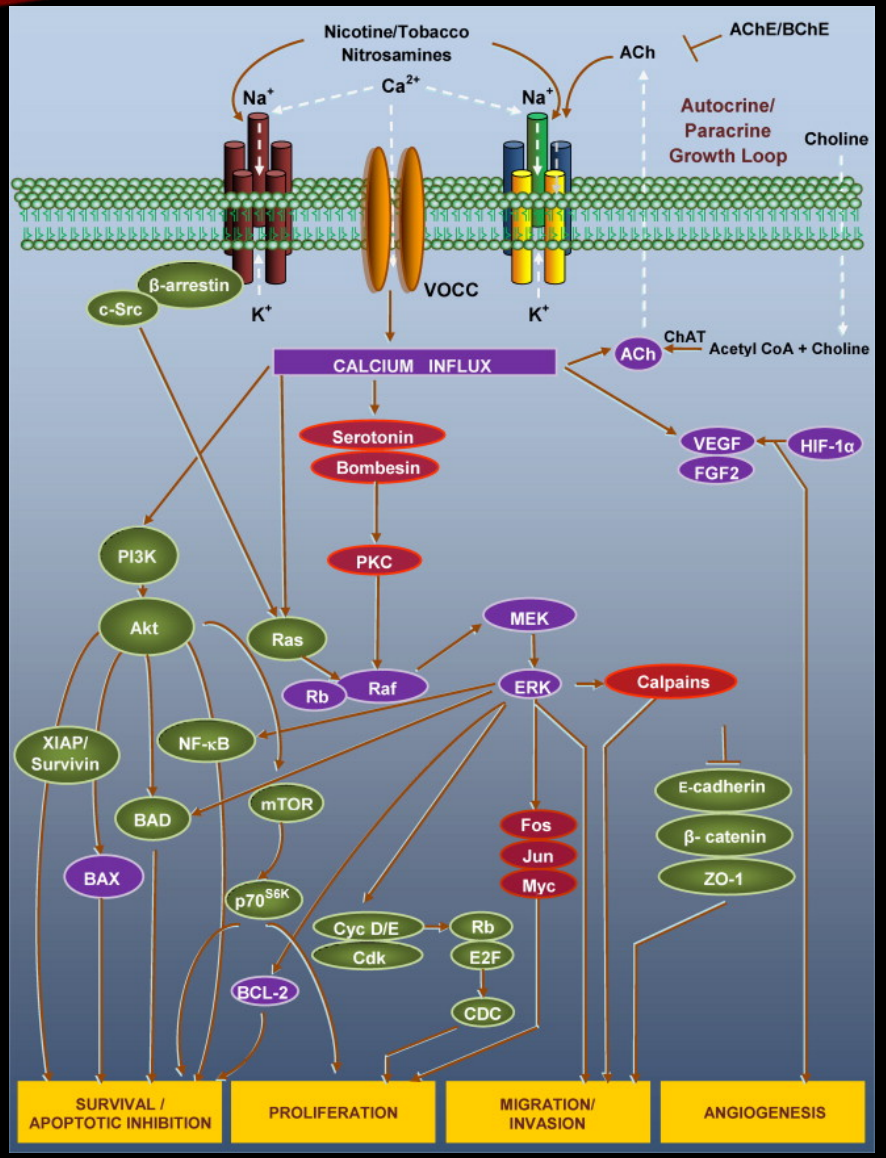




Q & A

IN VITRO PULMONARY AND E-CIG TOXICITY MODELS

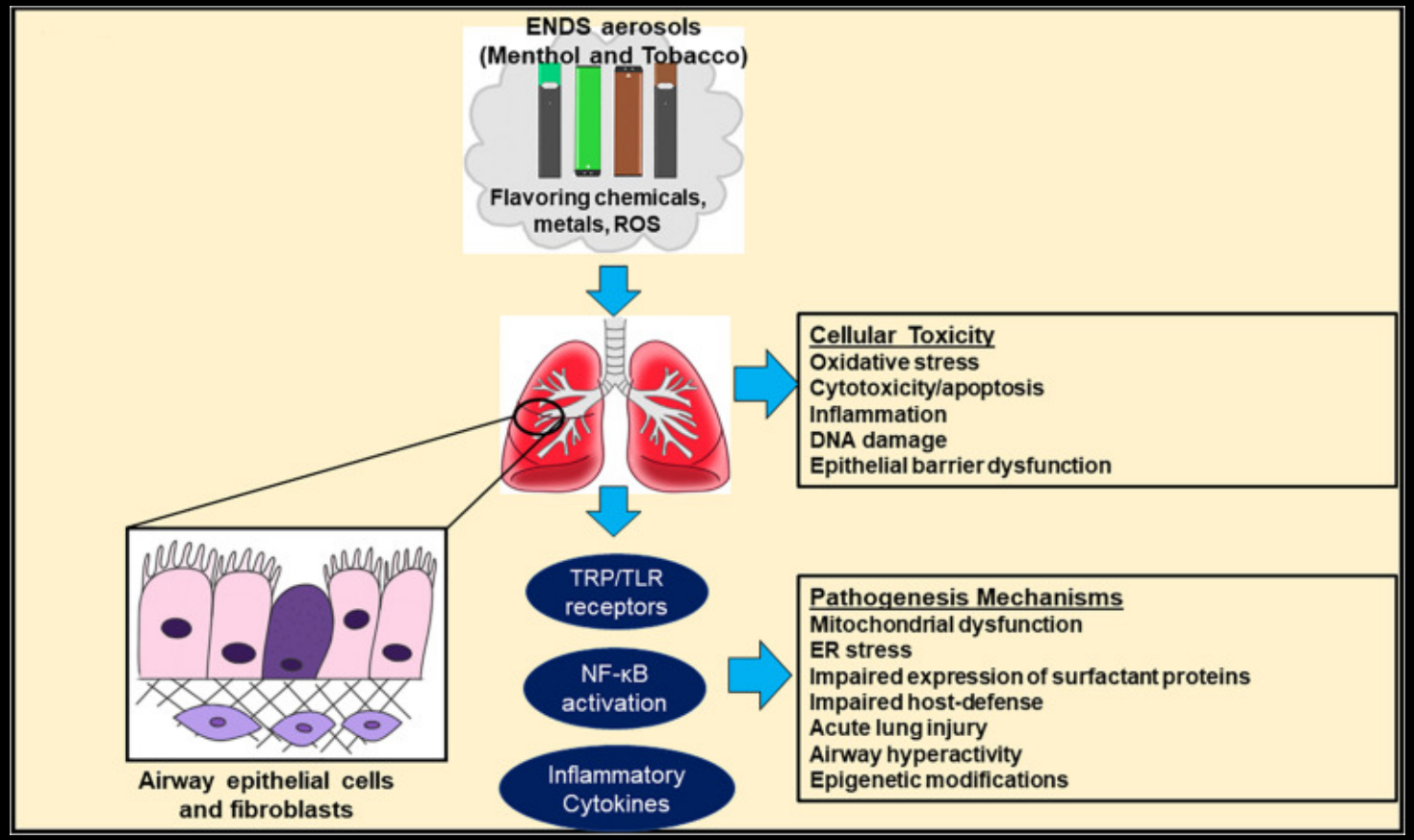
KNOWN NICOTINE E-CIG TOXICITY



Improgo 2011. doi: 10.1016/j.bcp.2011.05.020

- nAchR ion channel
- Ca2+ intracellular signaling
- G-coupled protein signaling
- Non-receptor effects
- Clearance through xenobiotic metabolism

KNOWN MENTHOL E-CIG TOXICITY



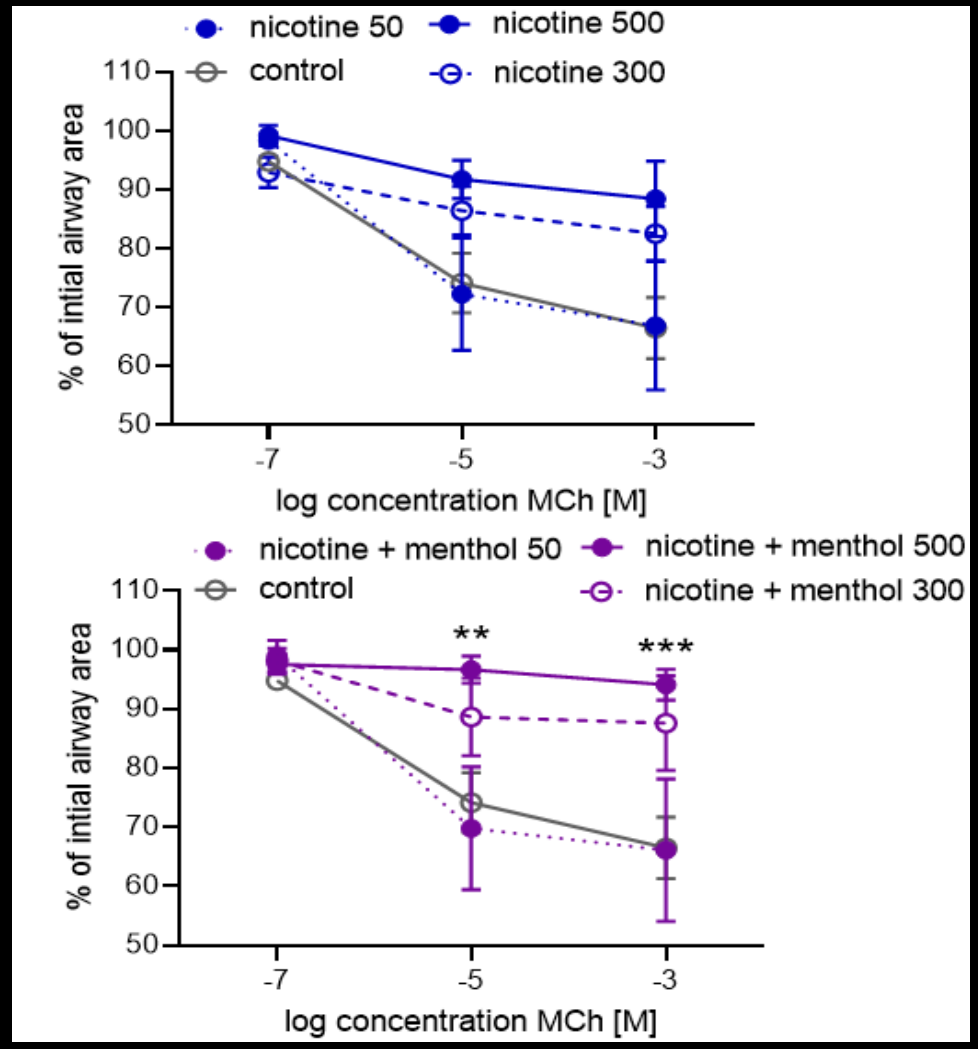
Kaur 2020. doi: 10.3389/fphys.2020.613948

- TRPM8 ion channel
- Ca2+ intracellular signaling
- G-coupled protein signaling
- Non-receptor effects
- Clearance through xenobiotic metabolism

NOTES FOR ALL EXPERIMENTS

- Experiments presented today are 4 h exposures to e-cig condensate unless otherwise stated.
- Each group represents at least 4 mice with at least 3 unique batches of condensate.
- Dose is normalized to the glycerin concentration of each condensate. Range of no response at 50 mM dose to total loss of function at 500 mM.
- Exposure groups are vehicle, nicotine, nicotine + menthol or menthol
- Group comparisons were 2-way ANOVA with Dunnett's Multiple Comparison post-hoc test compared to a media control group without condensate.
- Hill slope dose response curves were generated using least squares regression.

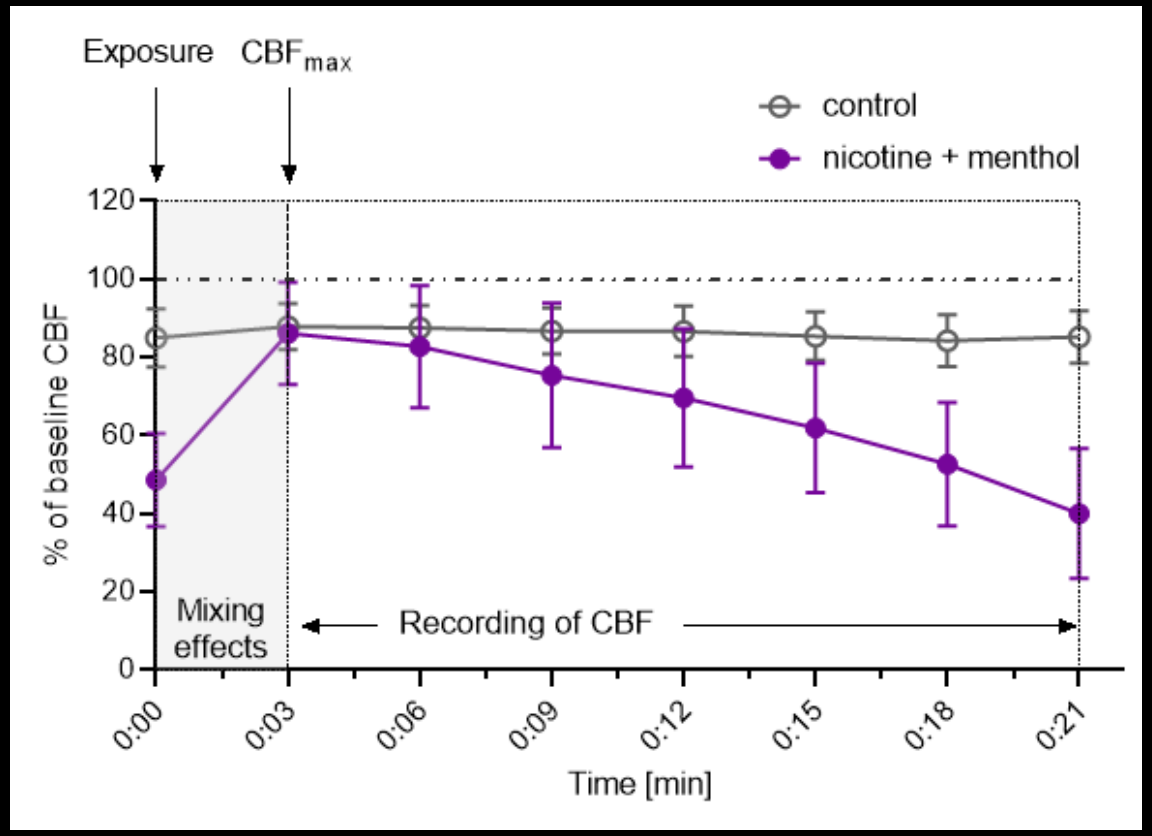
E-CIG CONDENSATE REDUCES AIRWAY RESPONSIVENESS



Nicotine e-cig condensates impaired airway responsiveness to methacholine (MCh).

Menthol containing e-cig condensates ablated airway responsiveness.

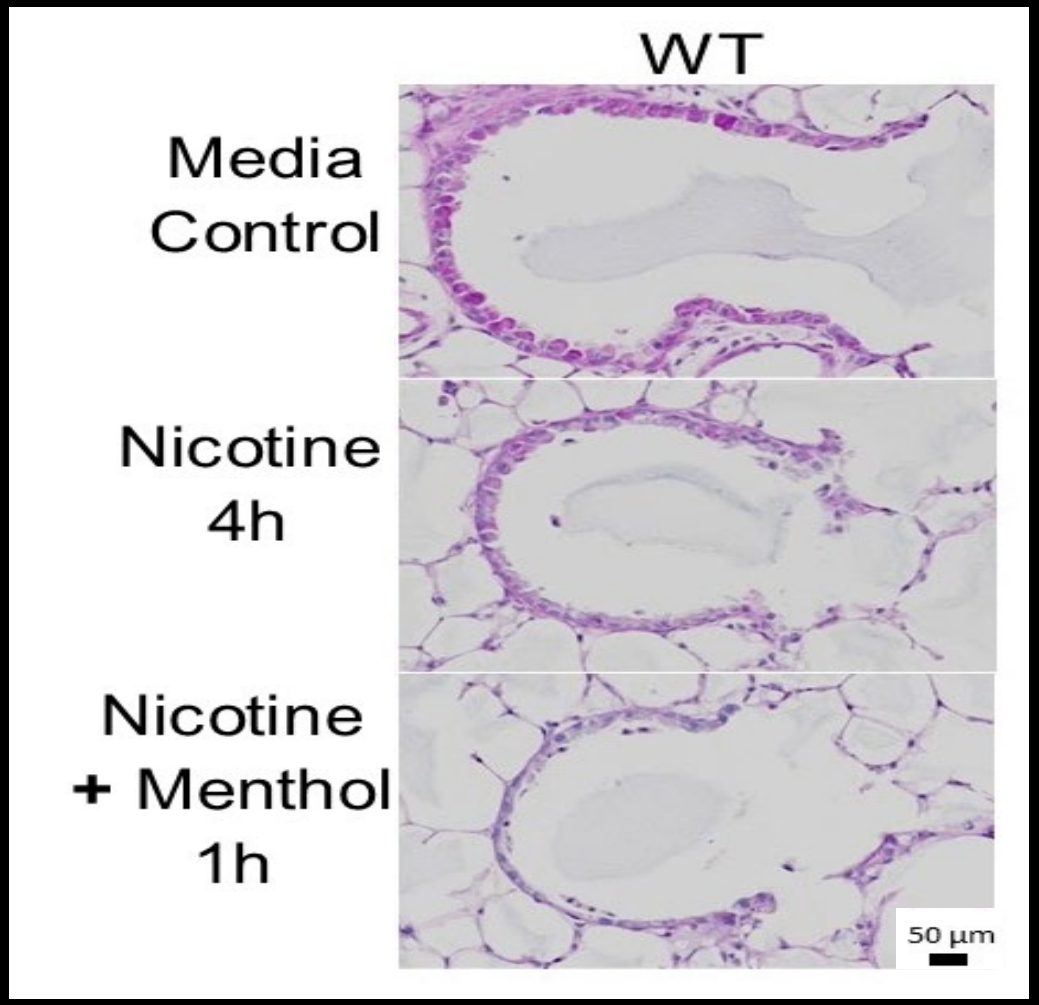
MENTHOL E-CIG CONDENSATE REDUCES CILIA BEAT FREQUENCY



Nicotine e-cig condensates did not substantially alter whole field mean CBF.

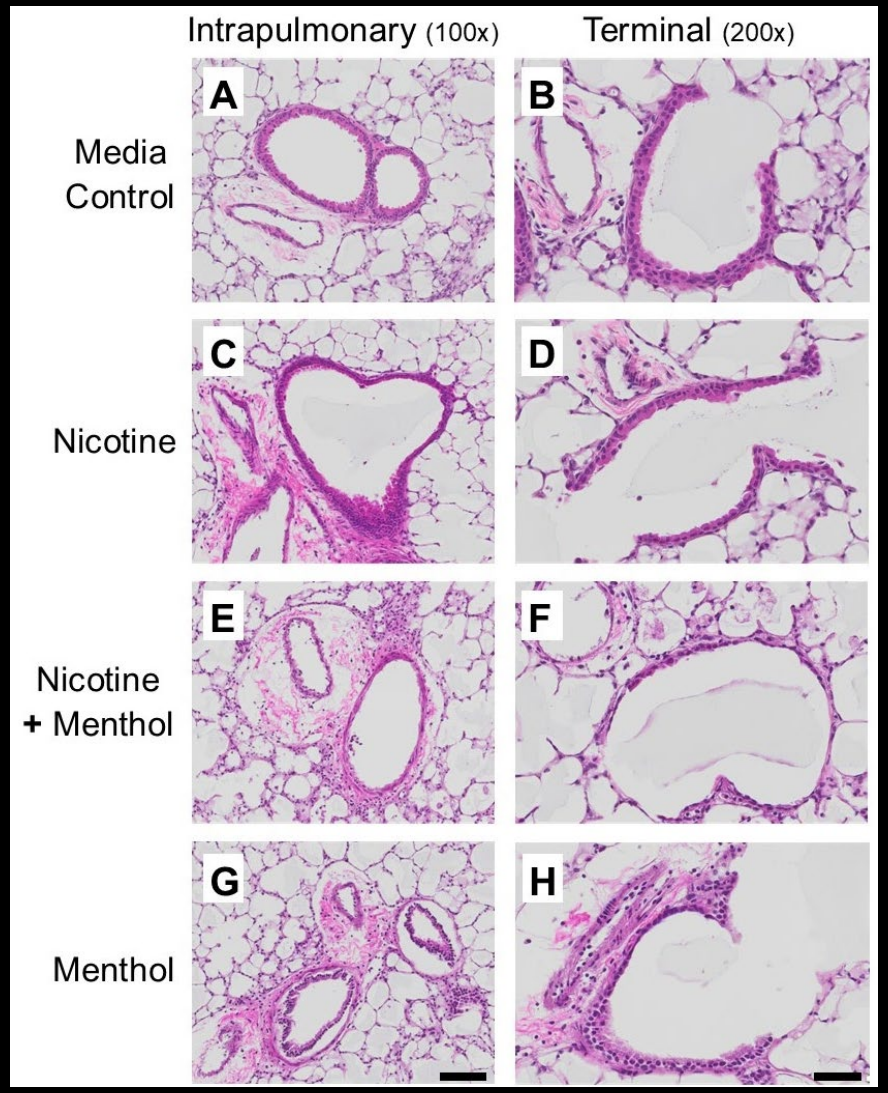
Menthol containing e-cig condensates caused dramatic loss of CBF within minutes of exposure.

MENTHOL E-CIG CONDENSATE REDUCES MUCUS RESERVES



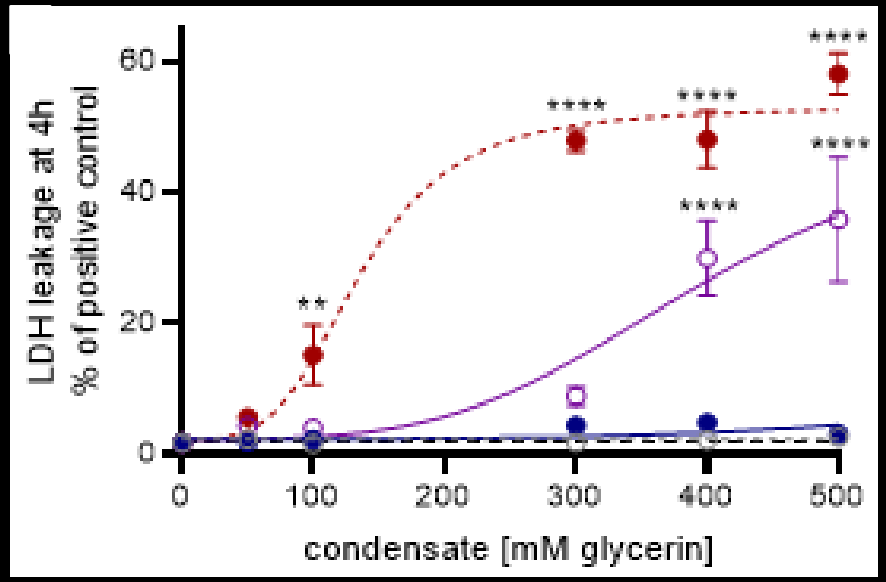
Nicotine and menthol containing e-cig condensates led to loss of mucus (bright pink PAS stain).

E-CIG CONDENSATE REDUCES EPITHELIAL VIABILITY

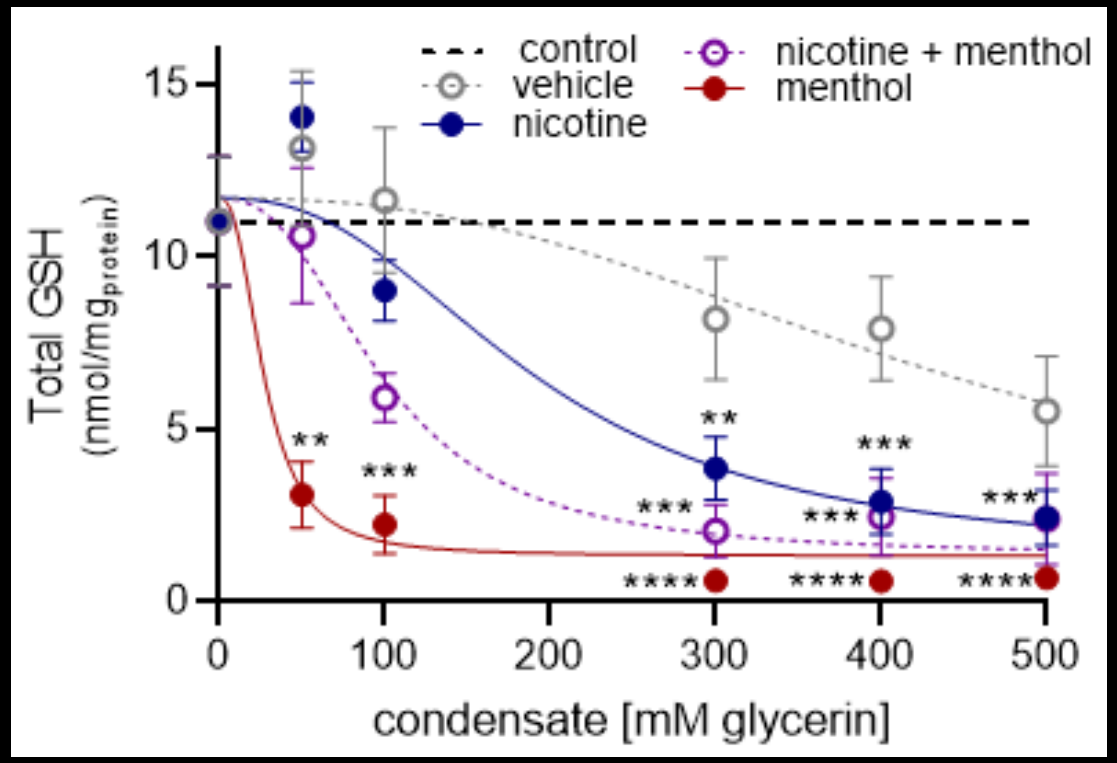


No evidence of nicotine cytotoxicity.

Menthol-containing condensates caused severe epithelial toxicity at high dose.



E-CIG CONDENSATE REDUCES ANTIOXIDANT STORES



Nicotine led to dose-responsive depletion of intracellular glutathione (GSH).

Menthol-containing condensates were more potent relative to nicotine.

NICOTINE AND MENTHOL MECHANISMS OF INTERACTION

Desensitization?

- Ca2+ intracellular signaling
- G-coupled protein signaling

Signaling cascade crosstalk?

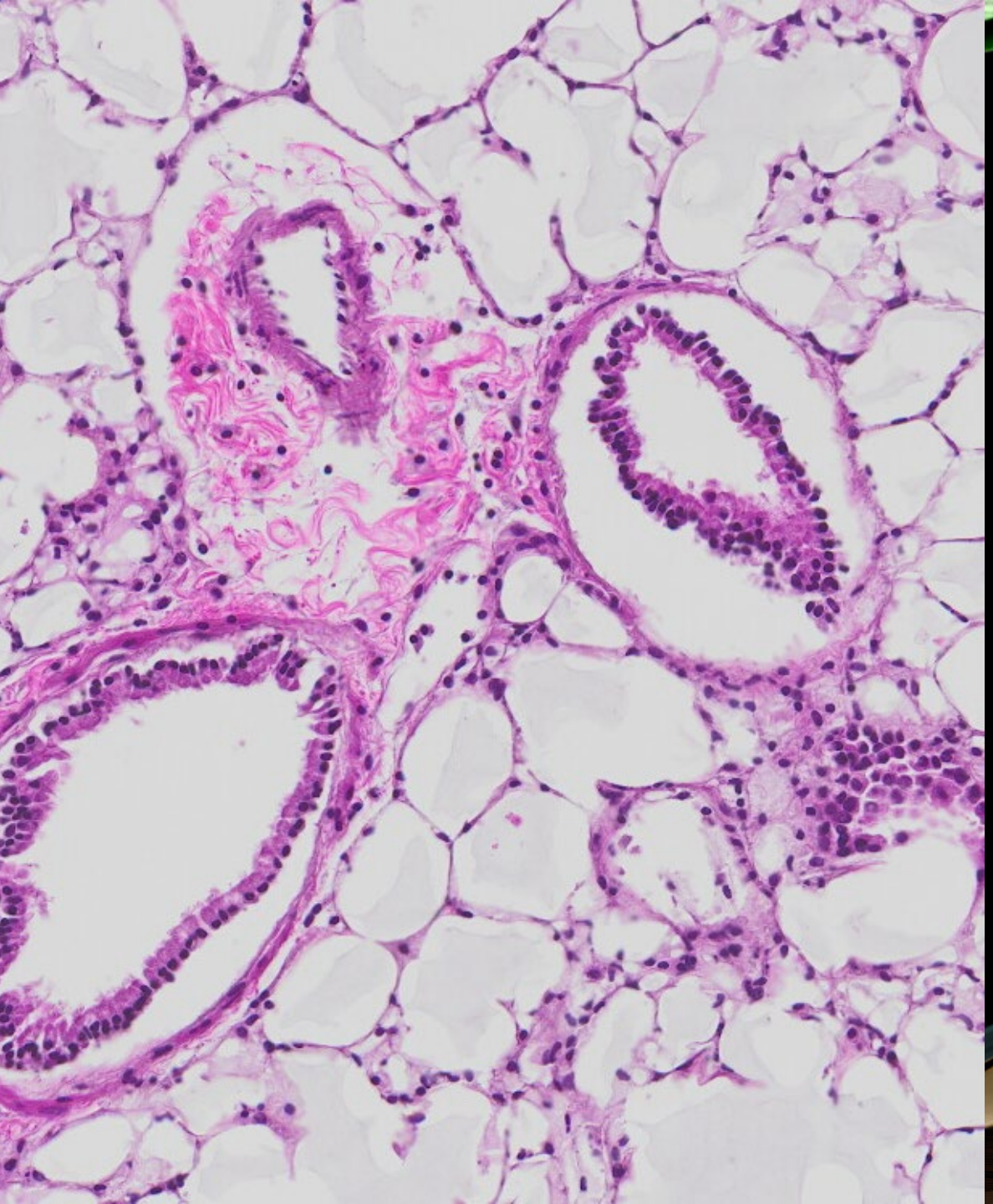
- Shared negative feedback loops
- Counteracting pathways

Antioxidant compensation?

- Glutathione synthesis enzymes
- Reduction in degradation of glutathione

Xenobiotic metabolism competition?

- Shared oxidation and glucuronidation clearance
- Nicotine is known to influence xenobiotic metabolism enzyme activity



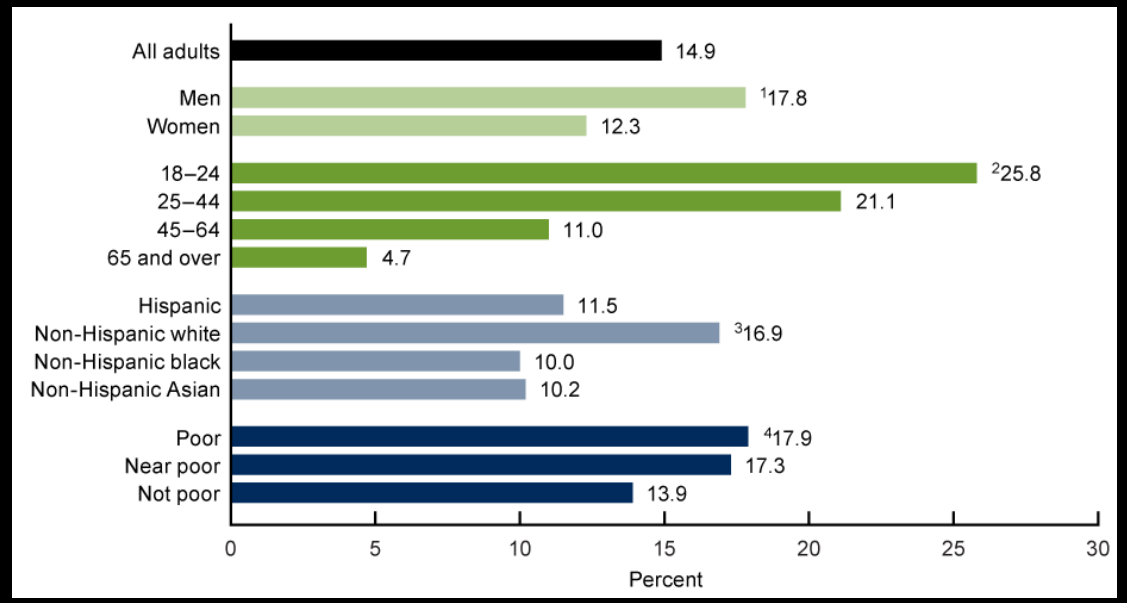
Q & A

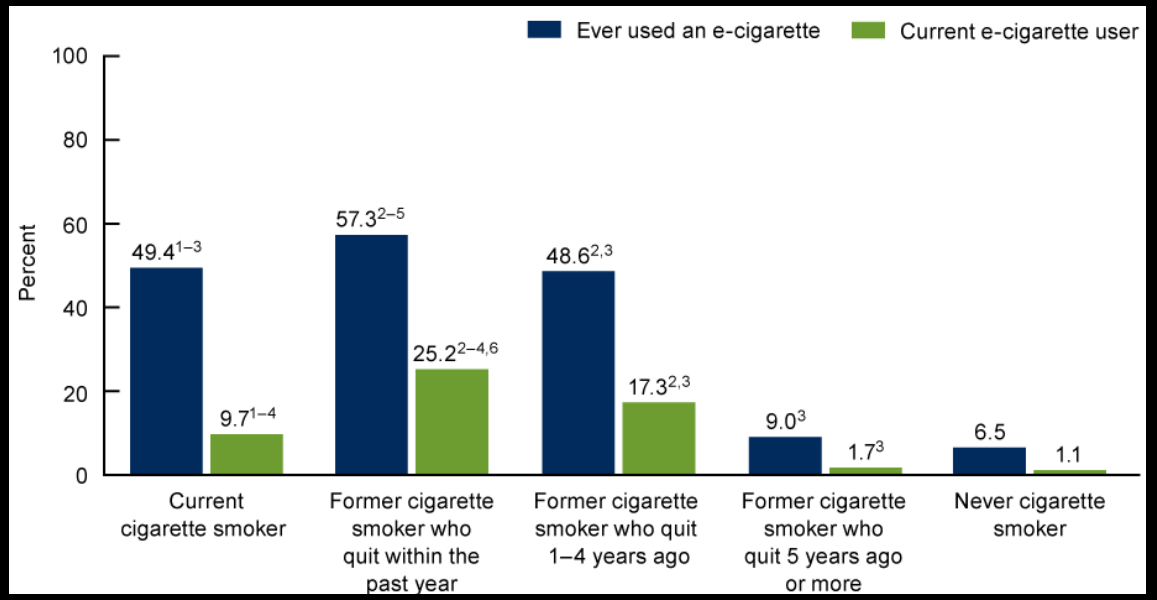
NICOTINE AND MENTHOL E-CIG TOXICITY

E-CIG CONSUMER POPULATION

Folks with intersectional identities use e-cigs.

Should *in vitro* models cover potential variations to susceptibility…
or do we continue to use uncertainty factors?





Percentage of US adults that have ever tried e-cigarettes in 2018.

https://www.cdc.gov/nchs/products/databriefs/db365.htm

TISSUE VARIATION IN VITRO

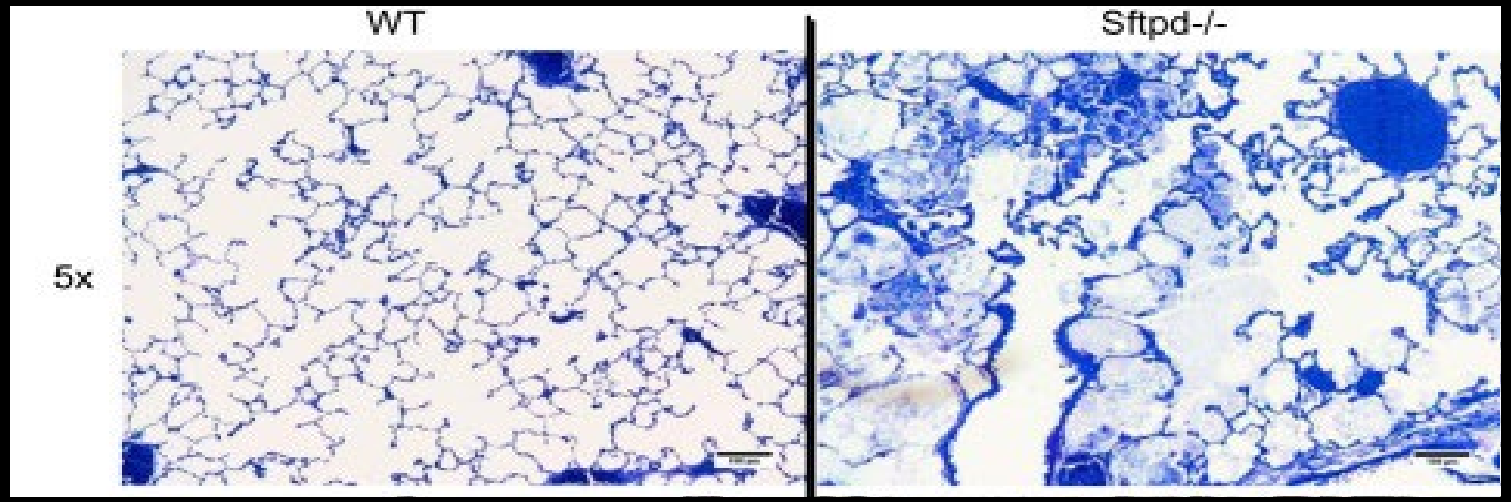
Human lung diversity is often lost in cell culture conditions.

SPD-/- MICE AS AN EXAMPLE OF POTENTIALLY SUSCEPTIBLE LUNGS

Healthy lung tissue

vs.

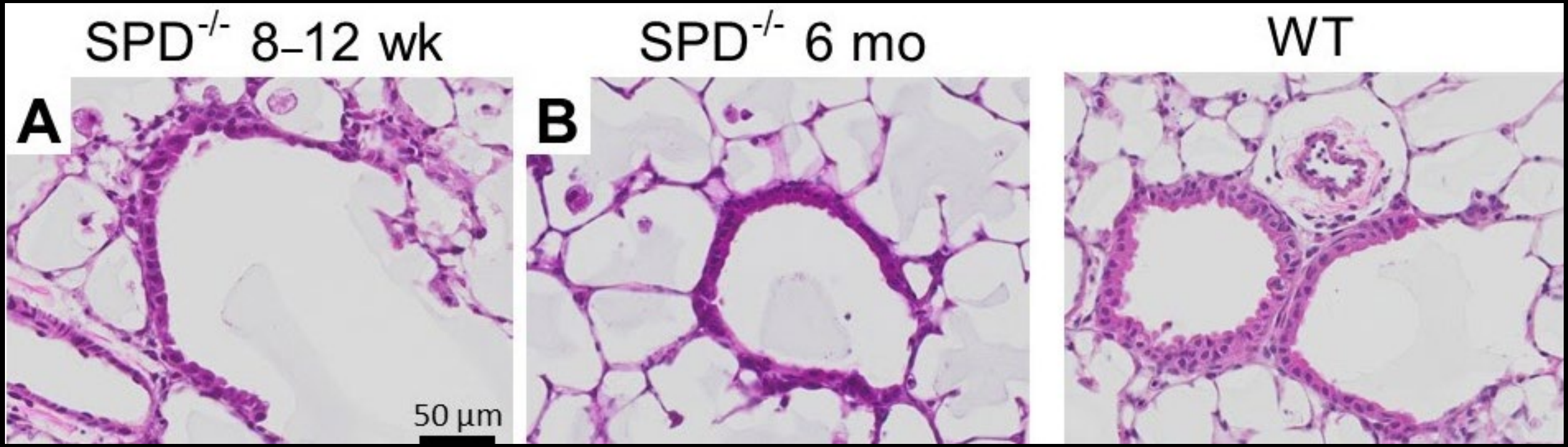
chronic lung inflammation in SPD-/- mice



Knudsen 2015. DOI: 10.1152/ajplung.00017.2015

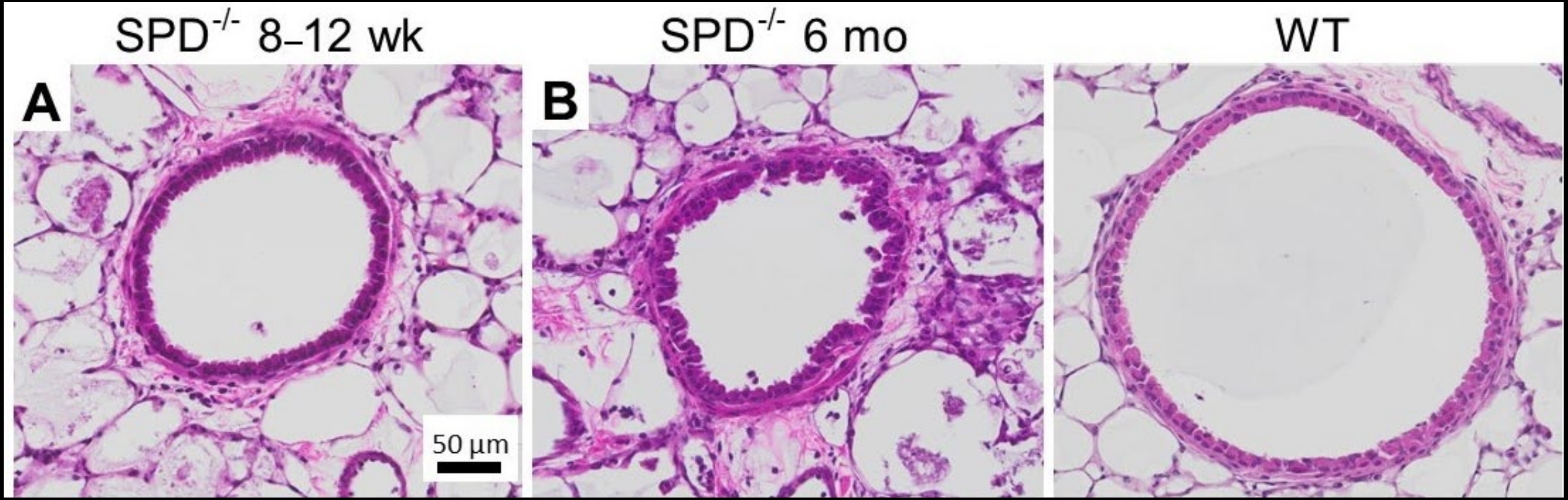
SPD-/- PCLS AS AN EXAMPLE OF POTENTIALLY SUSCEPTIBLE LUNGS

Chronic lung inflammation in SPD-/- mice is apparent in H&E stained PCLS



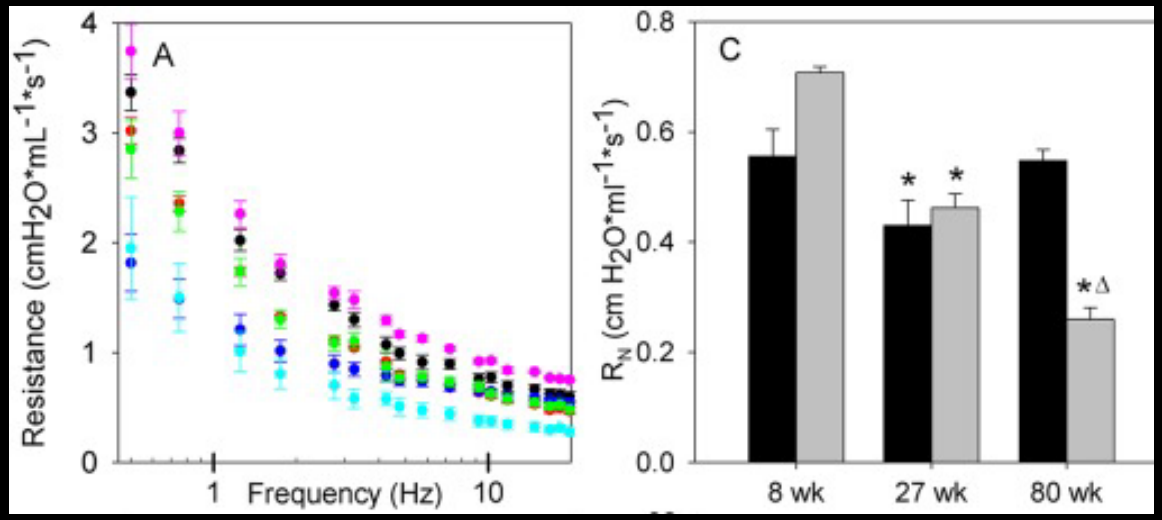
SPD-/- PCLS AS AN EXAMPLE OF POTENTIALLY SUSCEPTIBLE LUNGS

Chronic lung inflammation in SPD-/- mice is apparent in H&E stained PCLS



BASELINE DIFFERENCES IN SPD-/- PCLS: RESPIRATORY MECHANICS

STEP 1:
Fresh tissue vs
in vitro samples

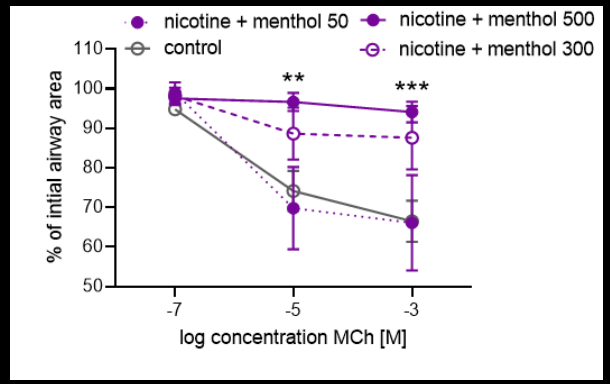


WT = black, red, blue
SPD-/- = pink green, cyan, gray

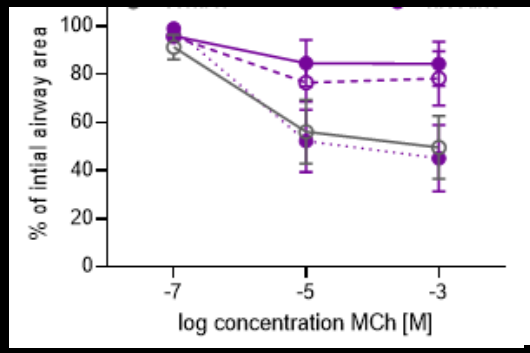
Massa 2017. doi: 10.1371/journal.pcbi.1005570

STEP 2:
Baseline differences
between in vitro groups

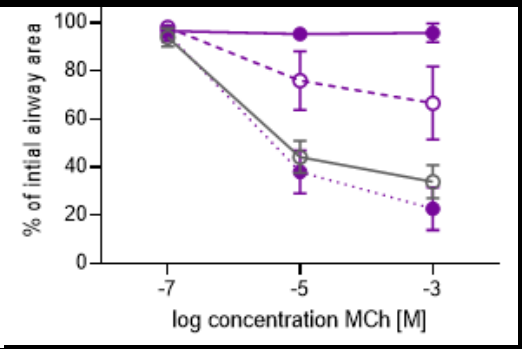
WT



SPD-/- 8-12 wk



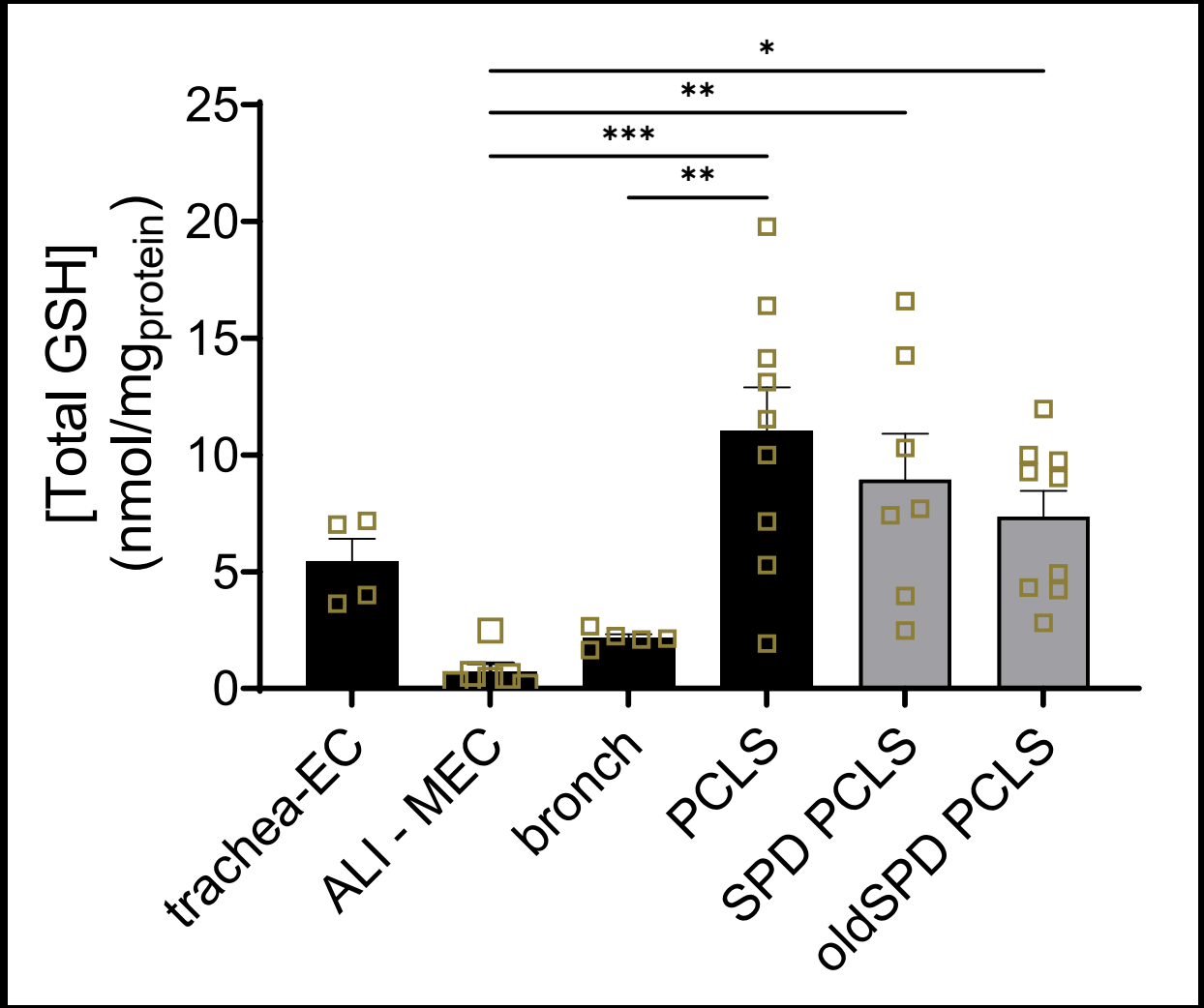
SPD-/- 6 mo



BASELINE DIFFERENCES IN SPD-/- PCLS: ANTIOXIDANTS

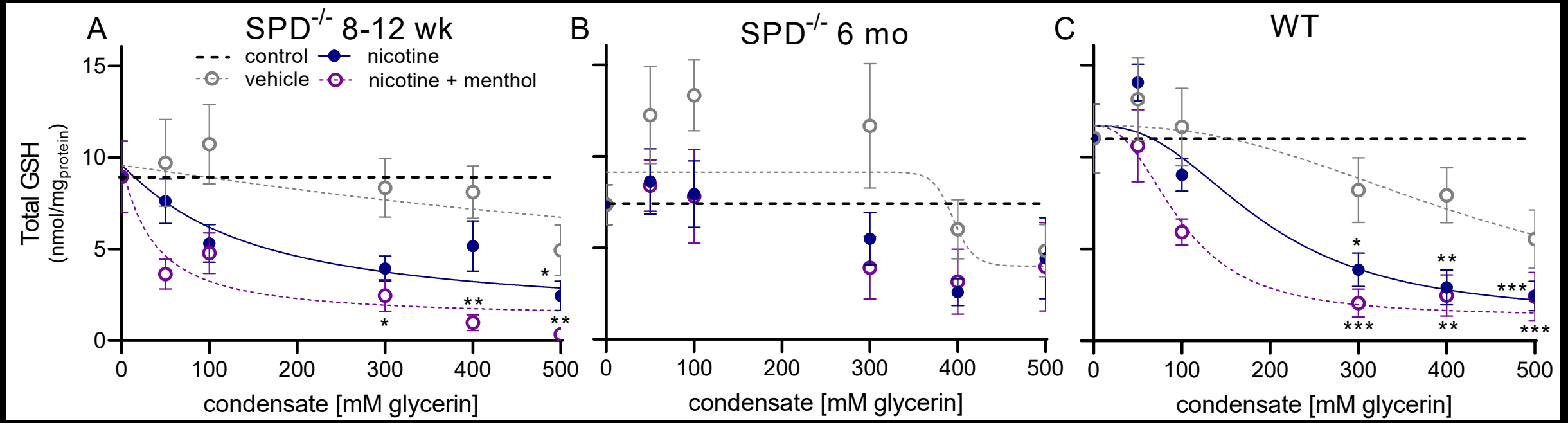
STEP 1:
Fresh tissue vs
in vitro samples

STEP 2:
Baseline differences
between in vitro groups



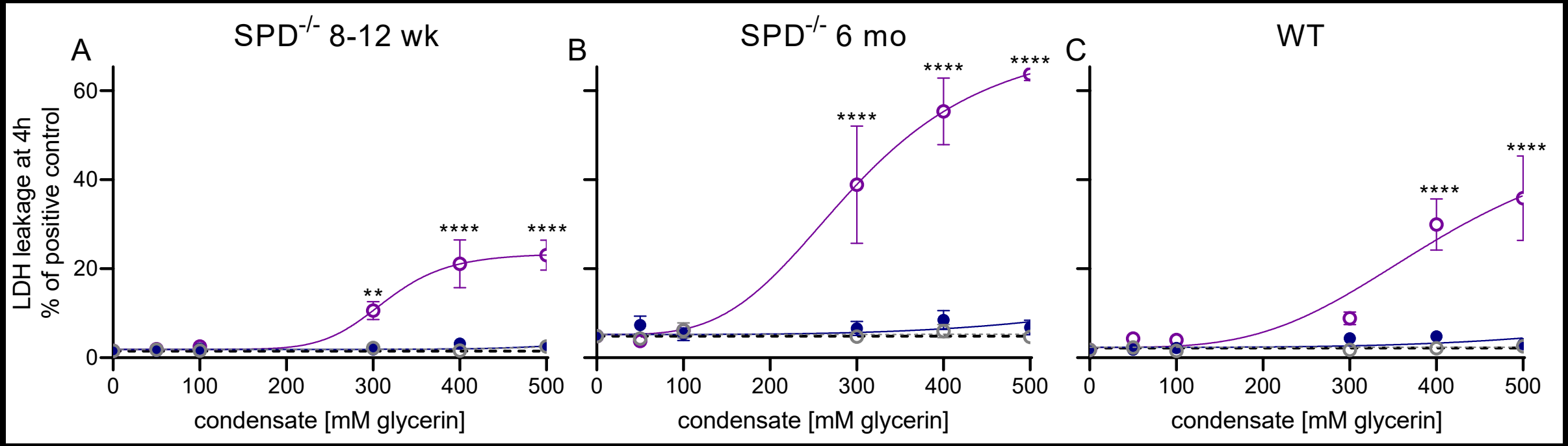
GLUTATHIONE DEPLETION BY E-CIG FOR SPD-/- PCLS

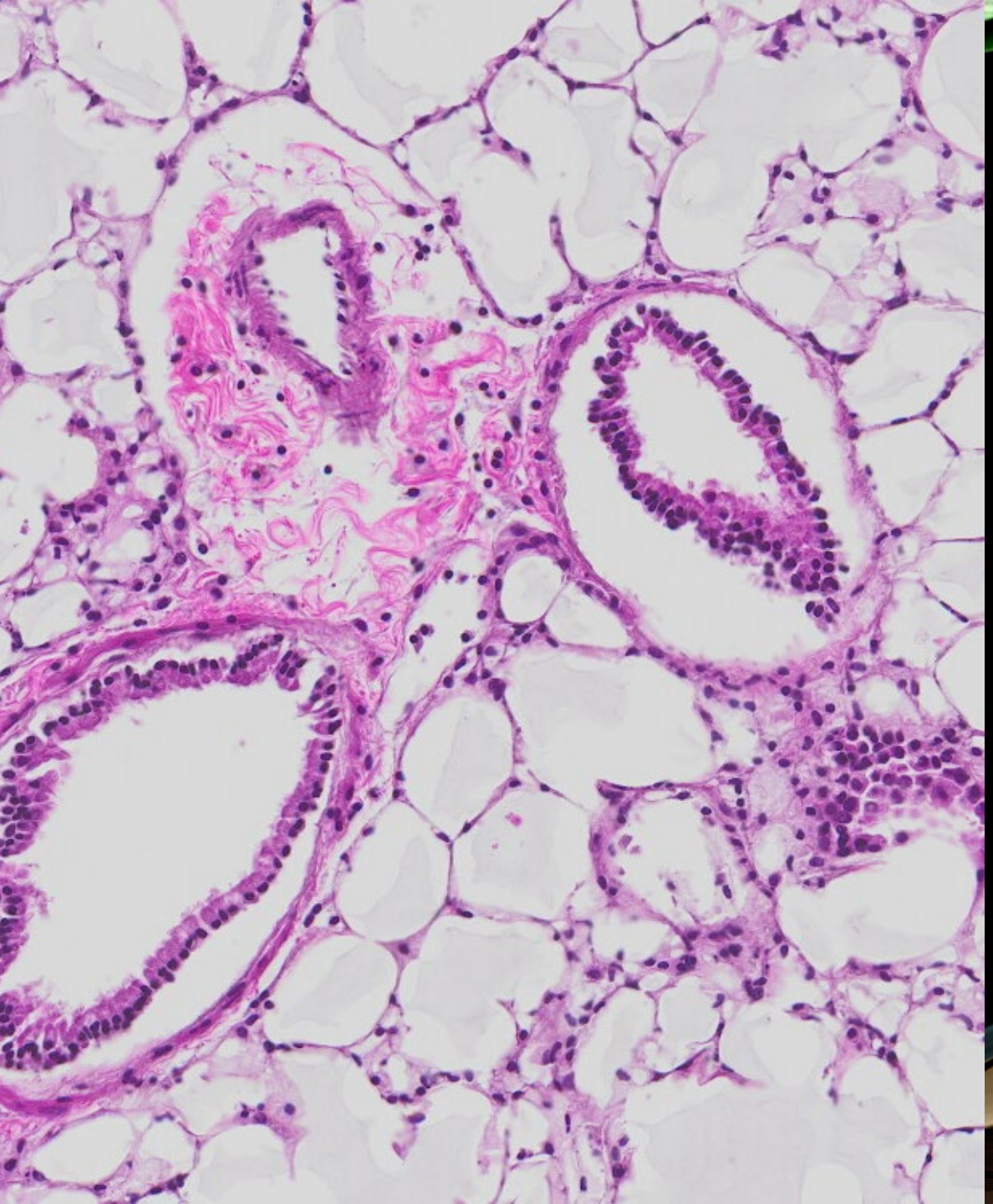
Antioxidant responses to e-cig toxicants were most severe in PCLS from WT mice.



CYTOTOXICITY BY E-CIG FOR SPD-/- PCLS

Cytotoxicity responses to e-cig toxicants most severe in PCLS from old SPD-/- mice.





Q & A

SUSCEPTIBILITY TO E-CIG TOXICITY