In vivo Genotoxicity Testing of Aerosolized ENDS E-Liquids

Presenter: Jingjie Zhang

Co-authors: James Randazzo, Kamala Pant, Viktoriya Lagoda, Willie McKinney, K Monica Lee

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Altria Client Services

REGULATORY SCIENCES

Supporting business strategies with evidence & innovation

Content

- Background

  - E-vapor Product Regulatory Testing (US)

  - Altria Client Services LLC (ALCS) Evaluation Approach

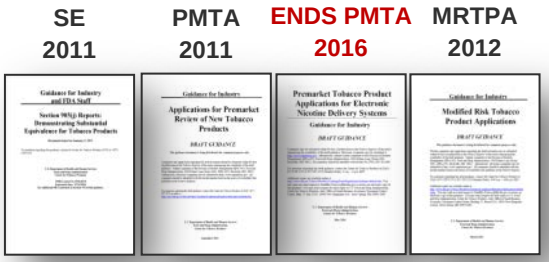
- E-vapor Formulation (e-Liquid) Testing

  - *In vitro* cytotoxicity and genotoxicity

  - *In vivo* follow-up genotoxicity

- Summary

E-Vapor Product Evaluation



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ENDS | **E-Liquid** | **Product (Aerosols)**

Individual Health Risk

Population Health Risk

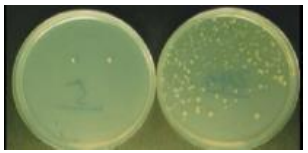
ALCS e-Vapor Evaluation Approach

Constituent Reduction | **Risk Reduction Individual** | **Harm Reduction Population**

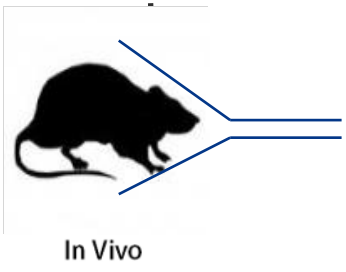
- Product Design and Control
- Chemical and Physical Characterization
- Toxicology and Risk Assessment
- Studies in Adult Human Subjects
- Perception and Behavior Assessment
- Risks and Benefits to Health of the Population

The Product | **Individual Health Risk** | **Population Impact**

Hazard Characterization



In Vitro



In Vivo

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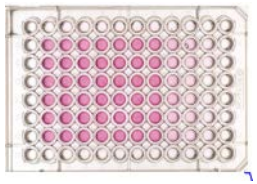
ALCS Nonclinical: Tox Evaluation

Toxicological evaluation of e-liquids & aerosols

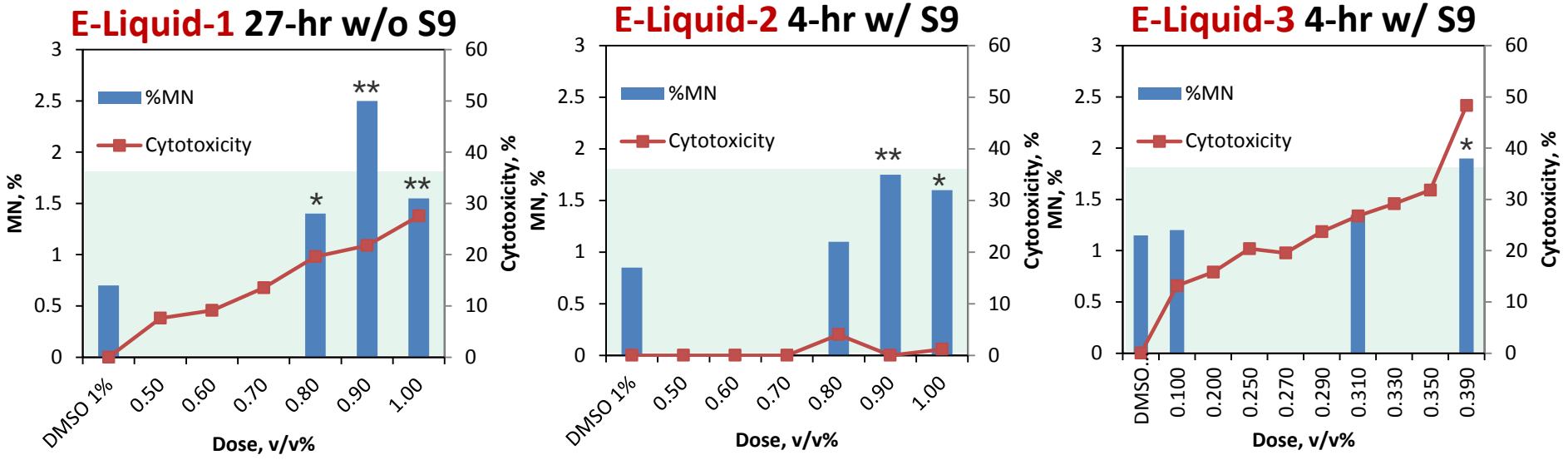
Weight of Evidence



- **Individual ingredients** - Literature review on toxicity & CMR information
- **Flavor Mixtures** - *In Vivo*: 90-day rat inhalation studies, focusing on respiratory tract
- **Product** (e-liquid & aerosols)
  - Harmful and Potentially Harmful Constituents
  - *In Vitro*: cytotoxicity & genotoxicity
    - (if necessary) *in Vivo* genotoxicity per ICH 2012

E-Liquid - *in vitro* Tox Screening

- **E-Liquids: PG/VG/Nicotine/Flavors**
  - Ames: 5 strains ➔ **Negative**
  - NRU: 3T3 fibroblasts ➔ **Not Cytotoxic**
  - MN: TK6 ➔ ***Positive***



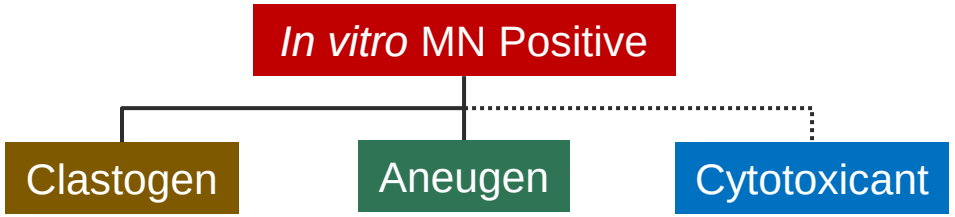
* p ≤0.05, ** p ≤0.01 using Fisher's Exact Test 1-Tailed Test

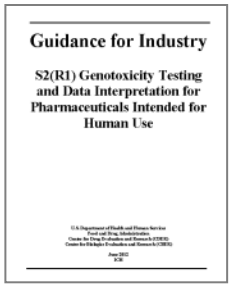
Follow-Up Considerations

Positive *In Vitro* Genotoxic (MN) Results:

- Reformulation
- Ingredient breakdown & test
- Mechanistic investigation
- In vivo genotoxicity study per ICH S2 (R1) Guidance (2012)

Standard *in vitro* testing is sensitive but not very specific (false positive)

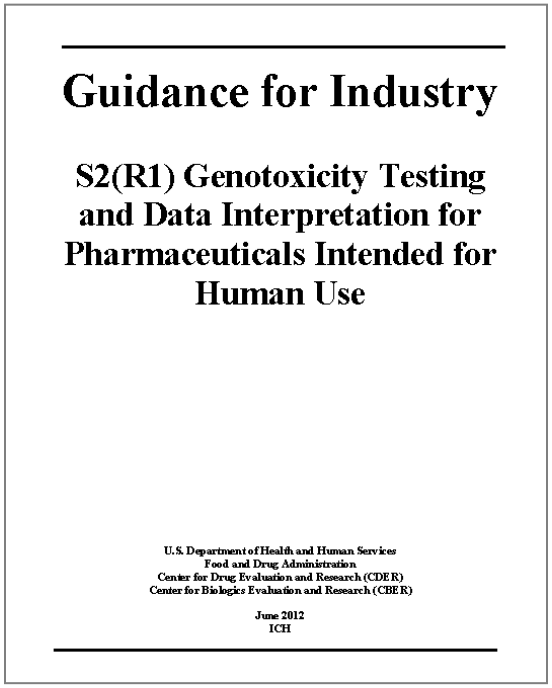



Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

"In summary, negative results in appropriate in vivo assays, with adequate justification for the endpoints measured, and demonstration of exposure are considered sufficient to demonstrate absence of significant genotoxic risk."

ICH Guidance S2(R1) Genotoxicity (2012)



Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

June 2012
ICH

"Standard Genotox Battery"

Option-1:

- *in vitro* Bacterial mutation (Ames)
- *in vitro* Chromosomal damage (MN, MLA)
  - (if negative) *in vivo* MN
  - (if positive)
    - *in vitro* mechanistic + *in vivo* MN

Option-2:

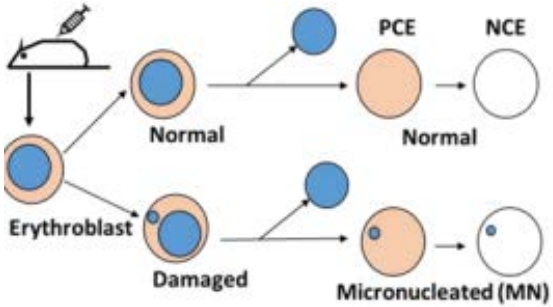
- *in vitro* Bacterial mutation (Ames)
- *In vivo* (Two) Tests (MN & Comet)

(Adapted) e-Liquids & Aerosols:

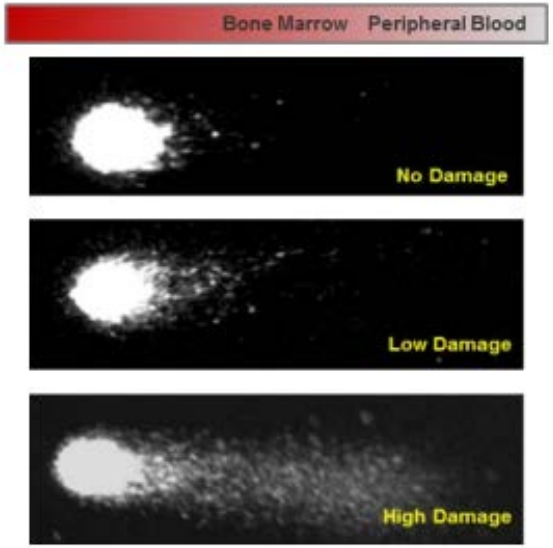
- *In vitro* Bacterial mutation (Ames)
- *In vitro* MN
  - (If MN positive) two *in vivo* (MN & Comet)

In vivo Genotoxicity Endpoints

- Bone Marrow MN (Chromosomal damage)
  - Bone marrow flushed 2-4 hrs after the last exposure; pellets smeared
  - Endpoint: %MN-PCE (micronucleated-polychromatic erythrocyte, immature erythrocyte or reticulocyte)



- Alkaline Comet (DNA breaks)
  - Detecting single or double stranded DNA breaks – tissue specific; single cell gel electrophoresis assay
  - pH>13, coiled DNA loops→ nucleoid + DNA fragments
  - Endpoint: % Tail DNA (fluorescence intensity of tail DNA)
  - Level of DNA damage is correlated to the length and amount of fragmented DNA that migrates outside the cell nucleus (Comet tail)

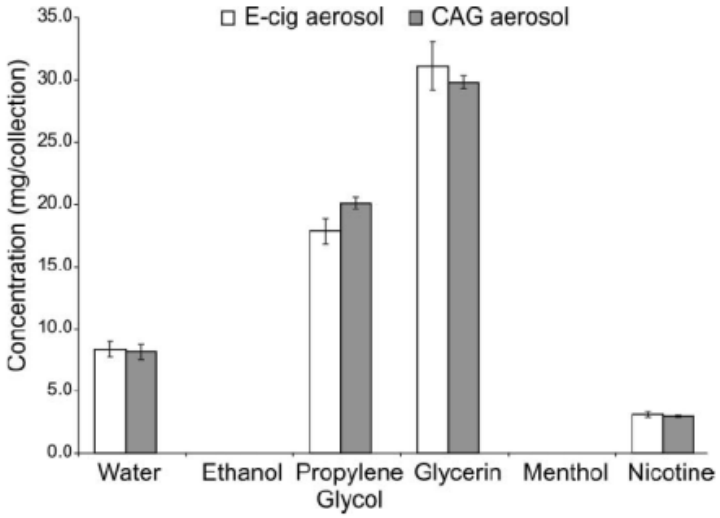


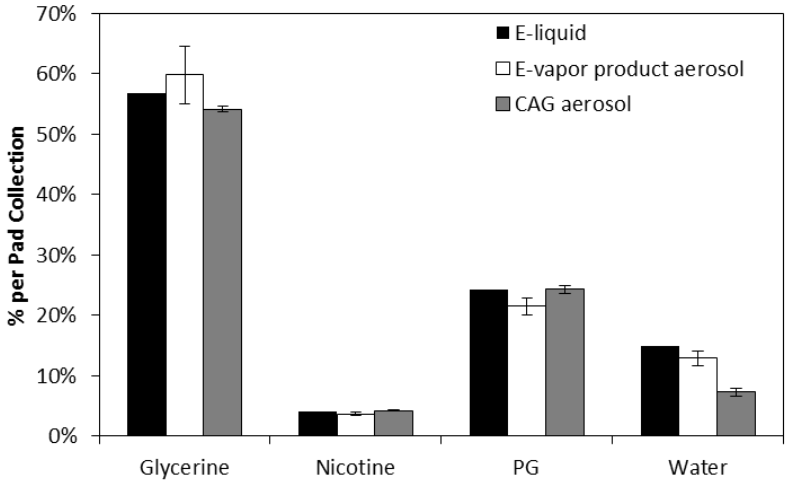
Study Design

- OECD 474: Mammalian Erythrocyte MN Test (2016)
- OECD 489: In Vivo Mammalian Alkaline Comet Assay (2016)

Topic	Suggested by ICH Guidance	Study Design Used	Note
Study duration	Single or repeated	Repeated (3-4 days)	Can be part of safety tox study
Animals, sex	Young rodents M (unless sex-specific)	Rats, M/F (~7 week at start)	The sex with reduced exposure may not be scored
Route of exposure	Clinically relevant	Nose-only inhalation	Aerosol exposures
Top dose	Max. tolerated dose (MTD)	MTD (range-finding)	Max. feasible/ possible dose
Endpoints	DNA break; cytogenetics	Comet & MN	Preferable in a single study
Target tissues	Clinical relevant; site of contact	Nasal, lung, liver; bone marrow	Exposure-relevant
Exposure confirmation	Cytotoxicity or exposure	Plasma nicotine & cotinine	Systemic exposures similar or higher than clinical
Positive controls	Not always; other route acceptable	PC for each endpoints; oral	If established, not always

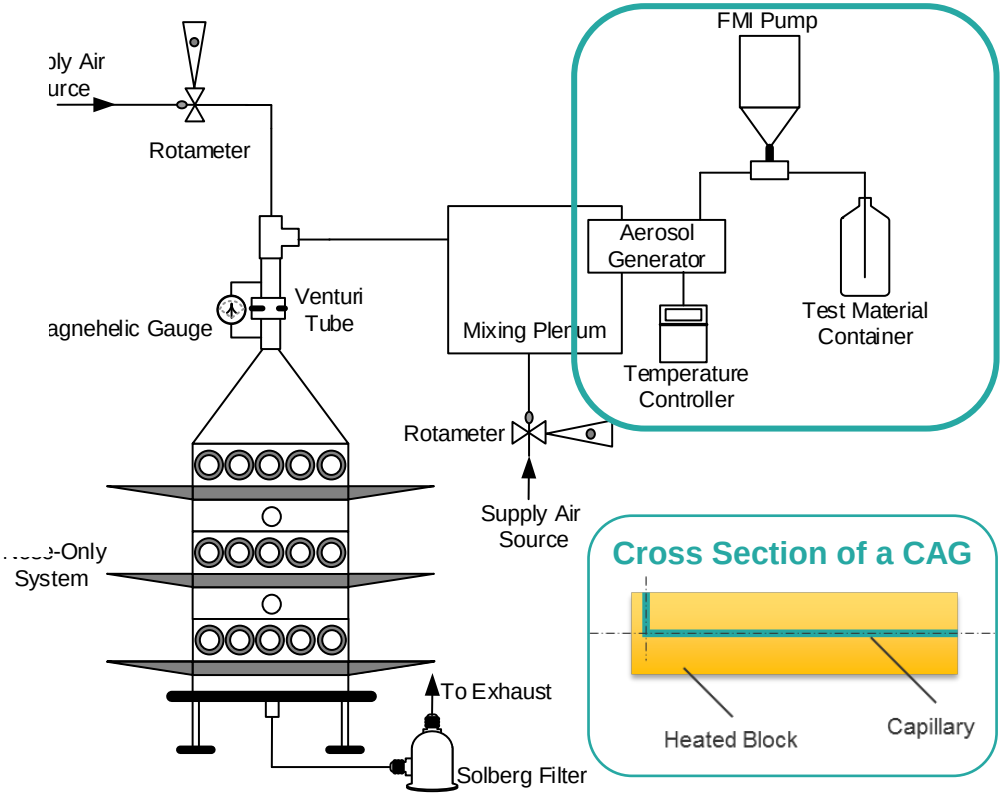
Nose-Only Exposure System





Werley et al. 2016; Zhang et al. 2017

Capillary Aerosol Generator (CAG)



Parameters Measured

- Aerosol Mass
- Particle Size
- Nicotine
- PG
- Flowrate
- Temperature & RH
- $\%O_2$

Tolerability: Maximum Tolerated Dose (MTD)

- E-Liquid-1
  - M/F rats, initial top dose at 2 mg/L TPM (~48 µg/L nicotine), up to 6 hrs per day for 3 days
  - F rats showed signs of toxicity and clinical signs (tremor, labored breathing)
  - M rats survived the top dose
  - MTD for males (2 mg/L TPM) and females (1 mg/L TPM)
- E-Liquid-2 and E-Liquid-3 (MTD, mg/L)

E-liquid-2	TA	Base Formulation
M	1.8	1.65
F	1.0	0.9

E-liquid-3	TA	Base Formulation
M	2.0	2.0
F	1.2	1.2

Definitive Study: *in vivo* MN / Comet Assay

Groups	Test Materials	Animal Number (M/F)
Negative Control	Filtered Air	6/6
Test Article (TA)	TA-Low (~¼ MTD)	6/6
	TA-Mid (~½ MTD)	6/6
	TA-High (MTD)	8/8
Reference	Base Formulation (PG/G/Nicotine, flavor free) (MTD)	8/8
Positive Control	CP 20 mg/kg/day (2 d); EMS: 200 mg/kg (1 d)	6/6

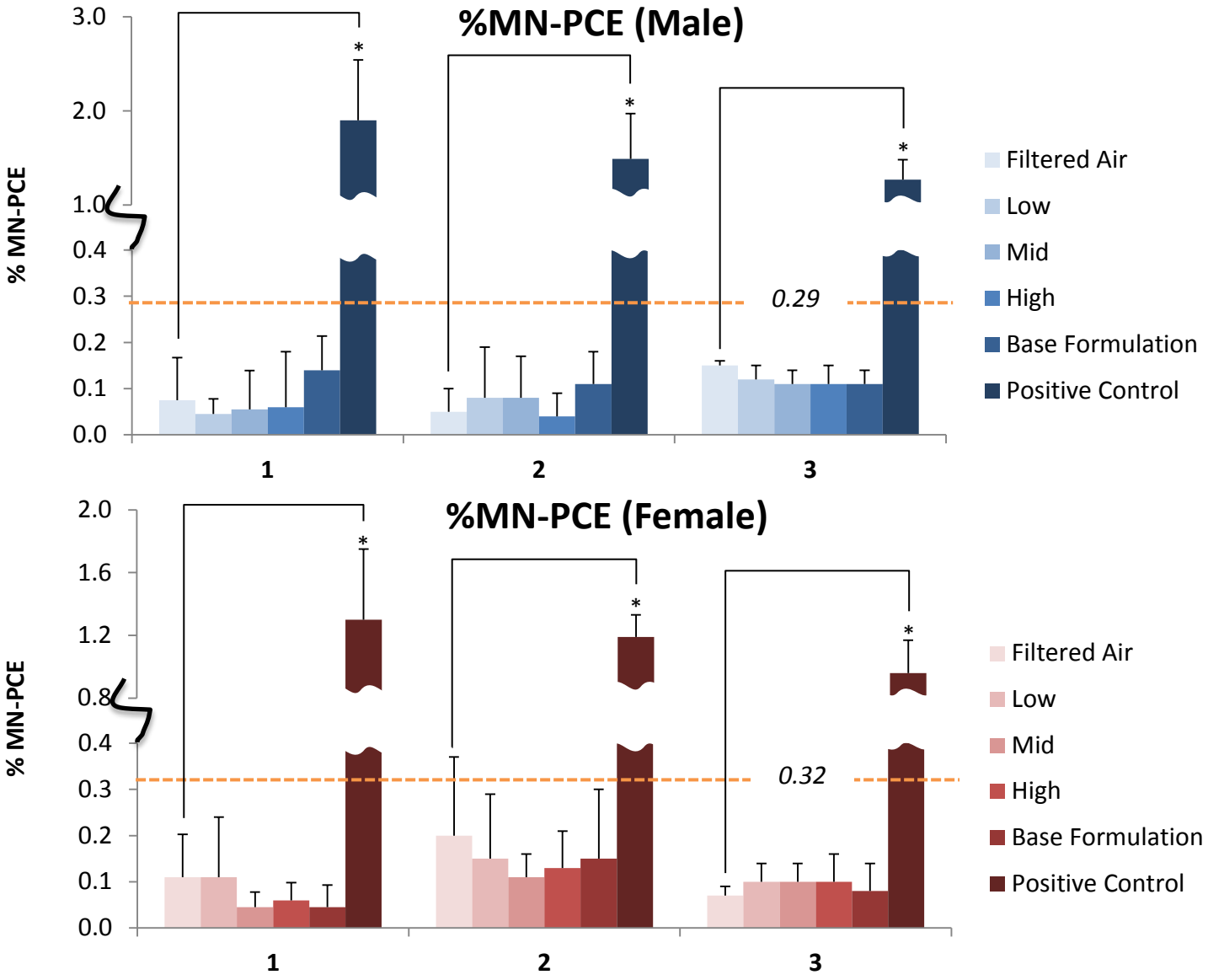
Exposure regimen

- Nose-only inhalation, up to 6 hrs/day, 4 days
- Aerosol generated by a CAG: ~ 275 ◦C
- Particle size: MMAD 0.7-1.1 µm (GSD 1.6 - 2.2)

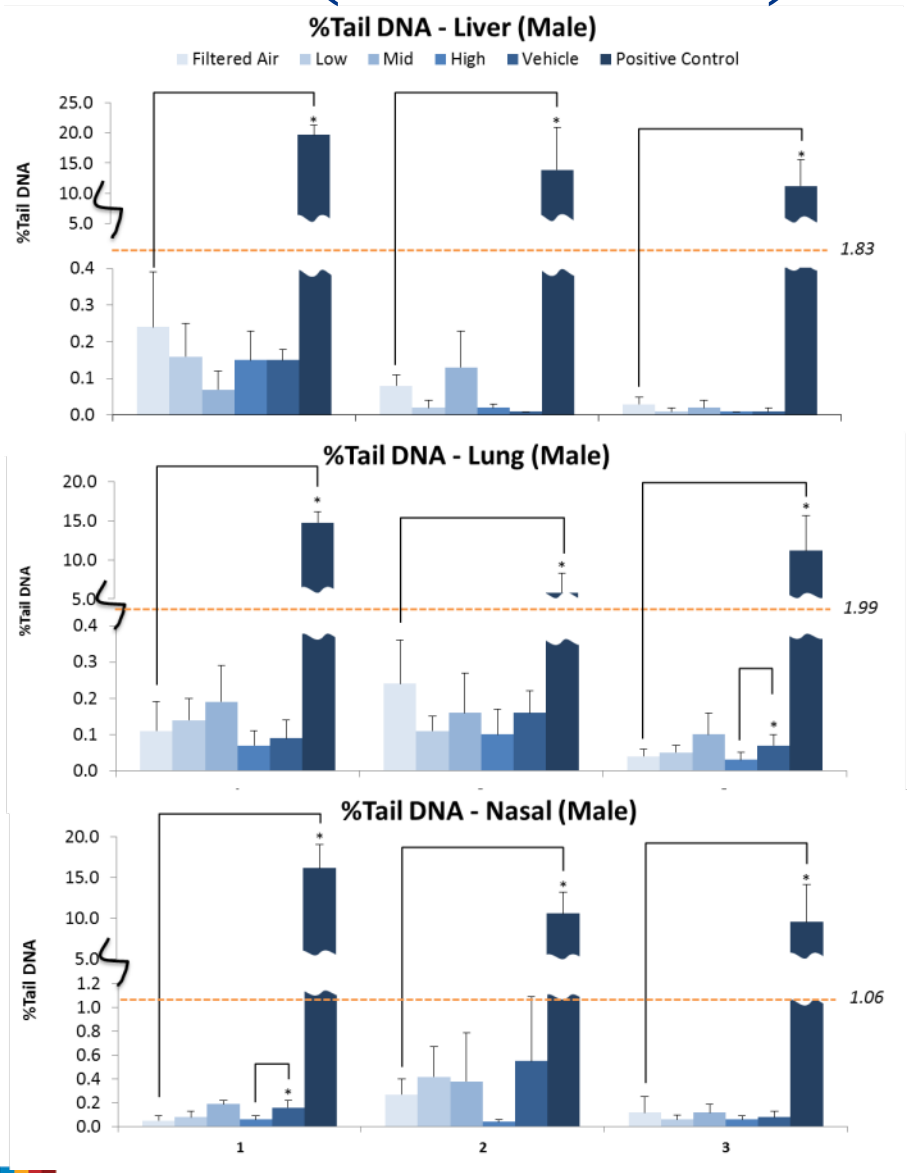
Sample collection

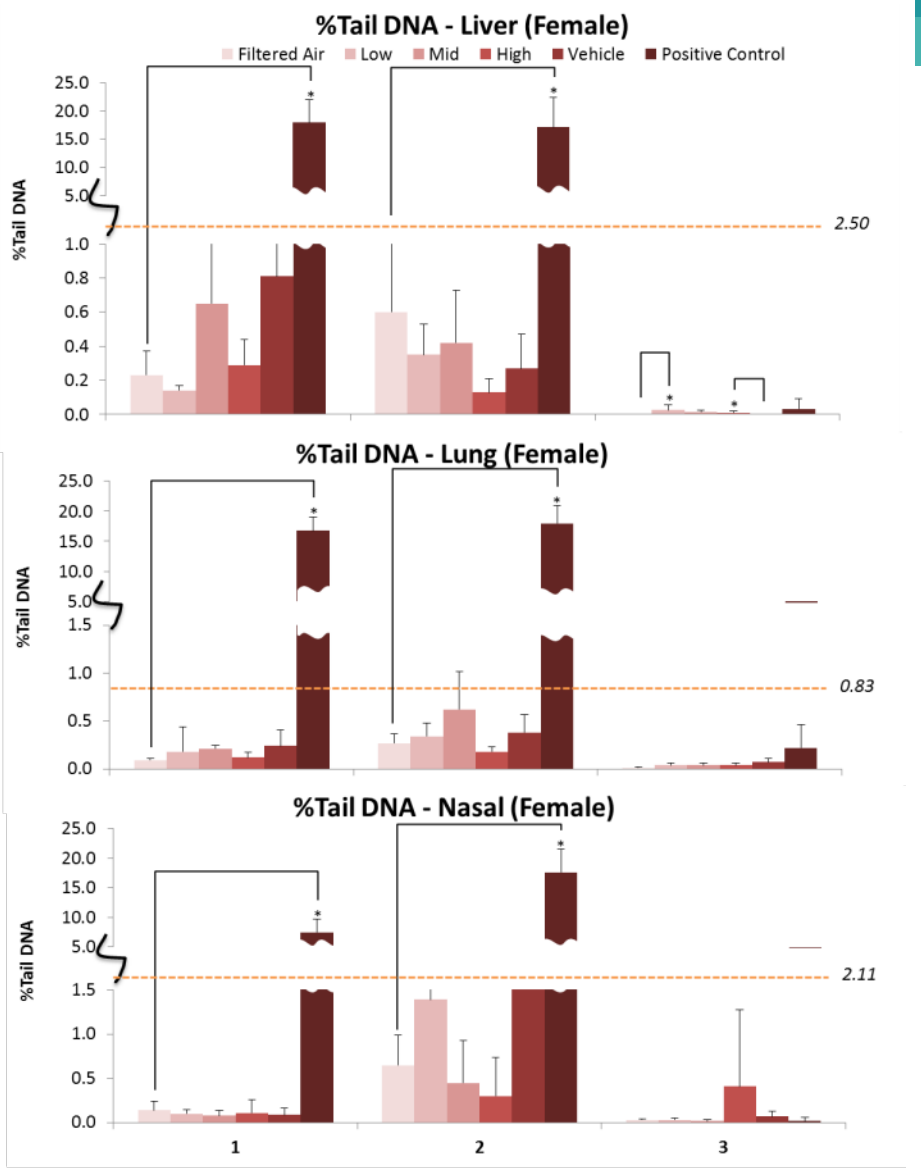
- Positive control: 2-4 hrs after EMS (18-24 hrs after the 2nd CP)
- Post-exposure plasma: nicotine and cotinine (within 5 min)
- MN: bone marrow
- Comet: nasal, liver, and lung tissue

MN (%MN-PCE)


%MN-PCE (Male)
% MN-PCE
3.0
2.0
1.0
0.4
0.3
0.2
0.1
0.0
0.29
1
2
3
Filtered Air
Low
Mid
High
Base Formulation
Positive Control
%MN-PCE (Female)
% MN-PCE
2.0
1.6
1.2
0.8
0.4
0.3
0.2
0.1
0.0
0.32
1
2
3
Filtered Air
Low
Mid
High
Base Formulation
Positive Control

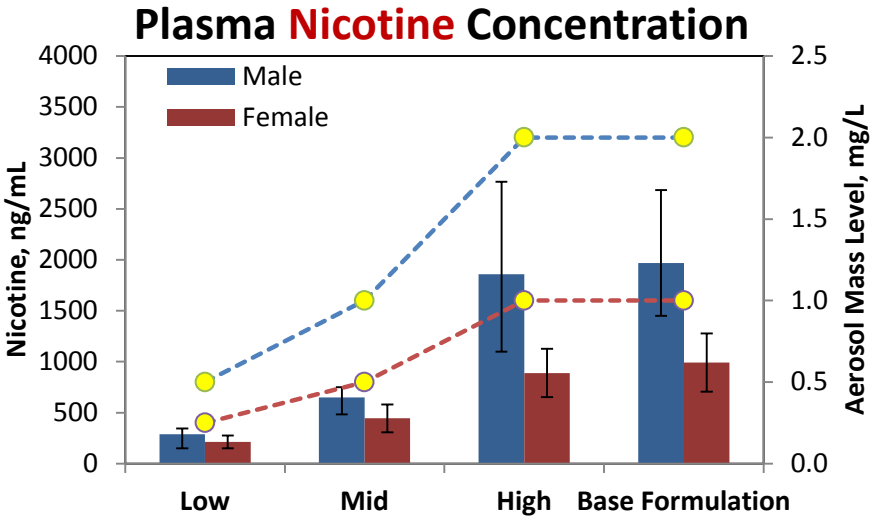
Comet (%Tail DNA)

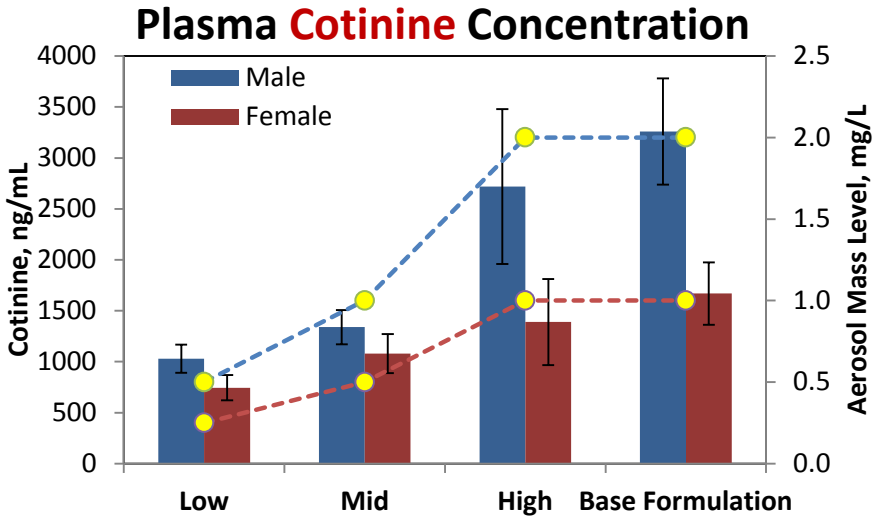




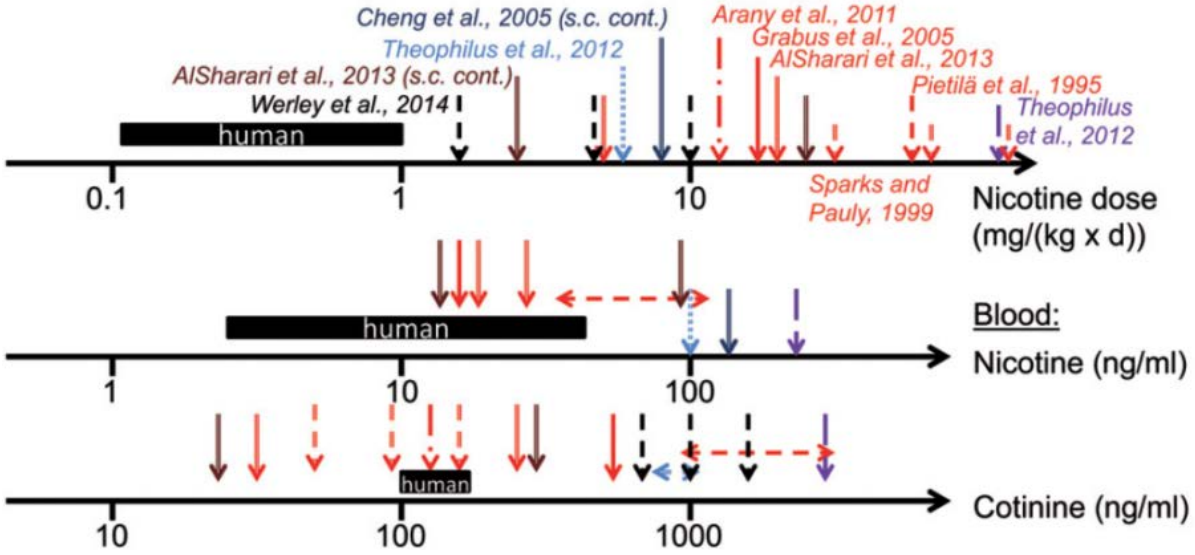
Biomarkers of Exposure – Plasma levels

E-Liquid-1



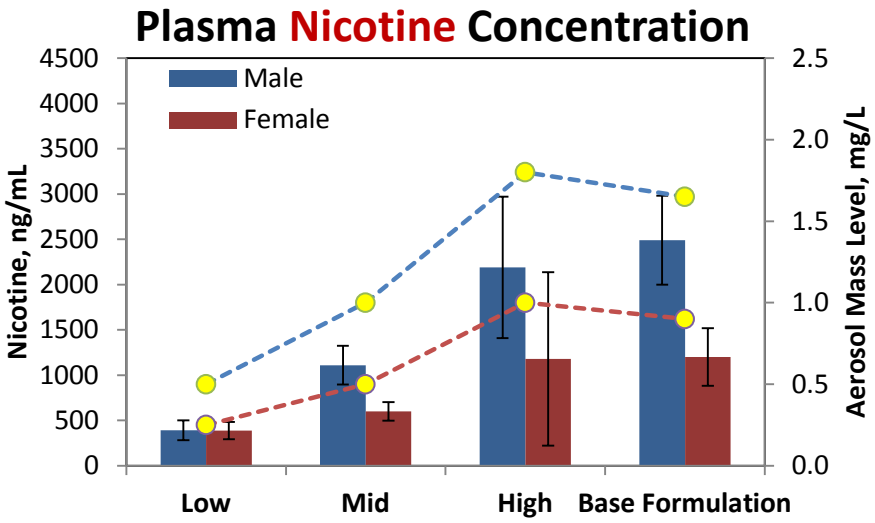


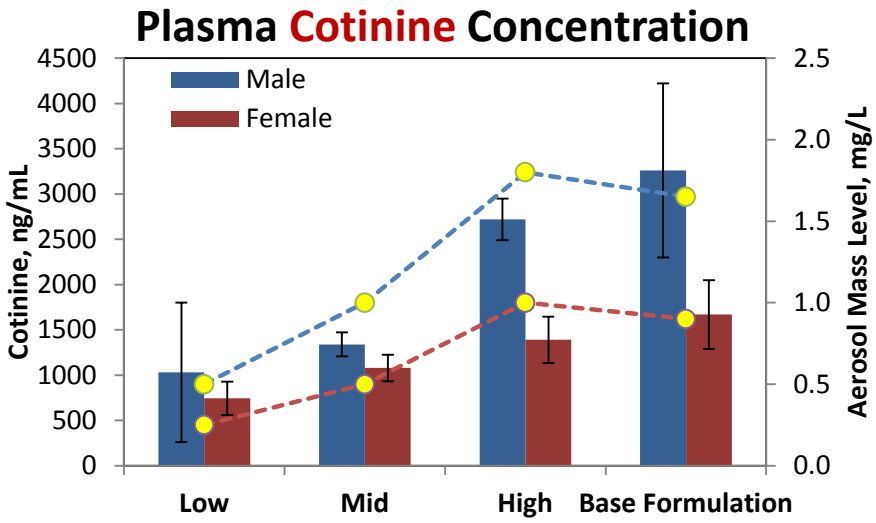
Compared to human & rodent data (*Haussmann, 2016*)



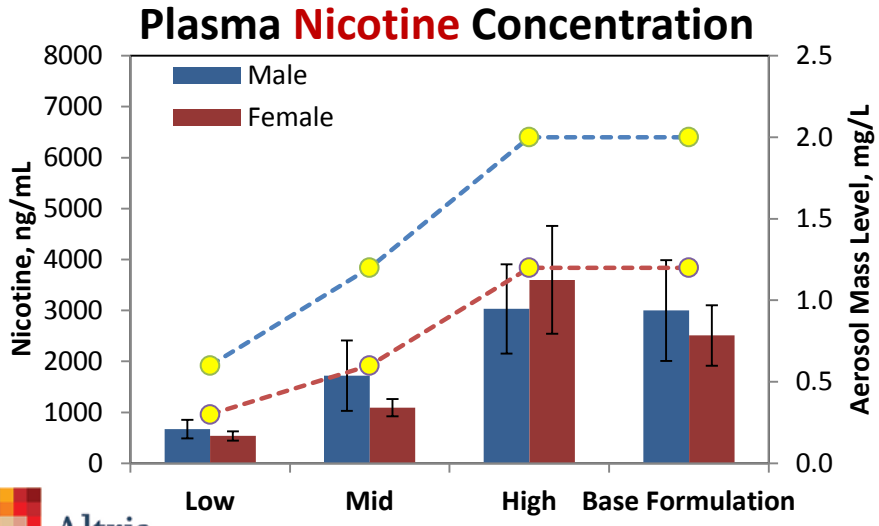
Biomarkers of Exposure – Plasma levels

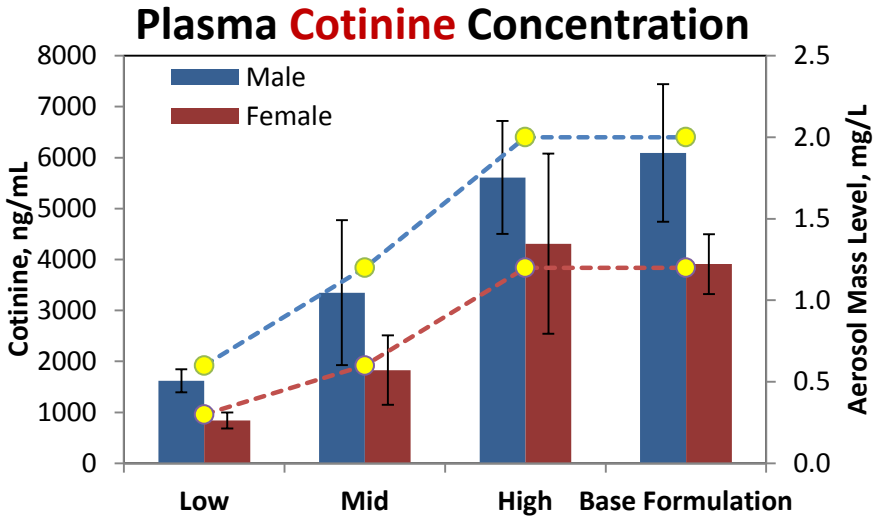
E-Liquid-2





E-Liquid-3





Summary

- Three ENDS e-liquids were tested in combined *in vivo* genotoxicity studies via inhalation according to ICH S2(R1) guidance, as a follow-up of positive *in vitro* MN results.

- Exposure concentrations were set to the MTD, based on mortality and abnormal clinical signs. Males groups were found to be able to tolerate higher TPM (total particulate matter, aerosol mass) exposure levels.

- There was no increase in two genotoxicity endpoints (MN and Comet) in all three e-liquids and their base formulations, compared to the negative control (filtered air). The plasma nicotine and cotinine levels increased with increasing TPM exposure concentration in the three studies.

- **In summary, under the tested conditions, negative results in the combined *in vivo* assays, with the examined target tissues and the markers of exposure, demonstrated absence of significant genotoxic risk.**

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- Erica McCaman
- Archana Akalkotar
- Michelle Moy
- W^{m} Clue Nethero

Reference

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Thank You!

Questions?