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Characterizing the genotoxic potential of e-cigarette components in vitro

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Outline

1.	2.	3.	4.
Nicotine-induced “genotoxicity”	Comprehensive in vitro genotoxicity assessment	Non-flavored e-liquid-induced “genotoxicity”	Collection of e-cig aerosols for in vitro assessment
Key concepts:			
- Mode-of-action - Lysosomo-tropism	- Multiplexed genotoxicity endpoints	- Extreme culture conditions - DNA damage response	- Critical review of literature
Final conclusions			

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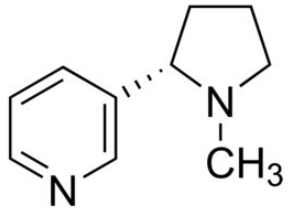
1. Nicotine-induced “genotoxicity”

Assessment of nicotine's genotoxic potential to address the paucity of modern-day data

- **(-)-Nicotine** was evaluated in a state-of-the-art battery of in vitro genetic toxicology assays (under GLP conditions) as part of safety assessment of e-cigarette chemical components.

- Mammalian genotoxicity: flow cytometry-based *in vitro* micronucleus (**MN**) assay (MicroFlow®, Litron Laboratories, USA).

(-)-Nicotine:



- Test system: Chinese hamster ovary-Wolff Bloom Litton (**CHO-WBL**) cell line (provenance: Merck Research Laboratories, USA).

- Results: concentrations **≤3.95 mM** had **no effect** on background levels of MN after 24 h treatment, but **≥4.93 mM**, tandem increases in **MN** and **hypodiploid nuclei** were observed → evidence of **aneugenicity**.

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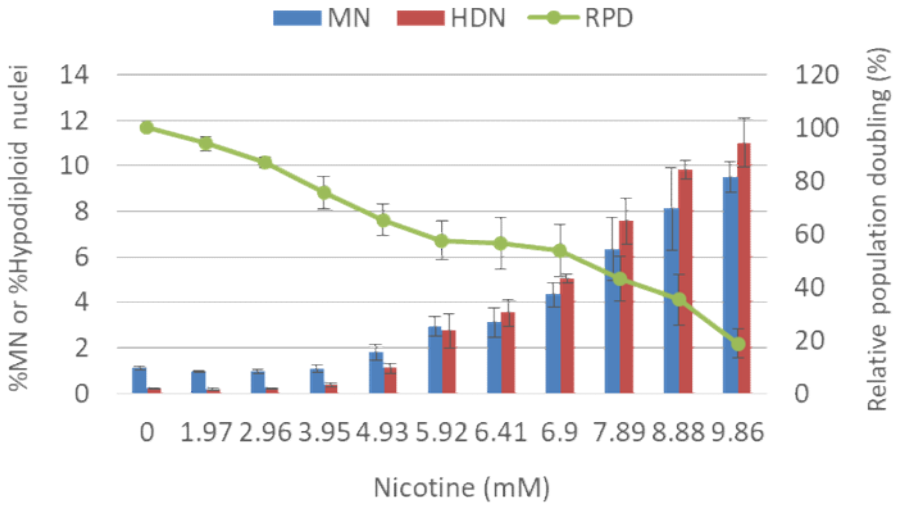
Mode-of-action analysis of MN results



Smart et al., 2019, Environ. Mol. Mutagen. 60: 778-791

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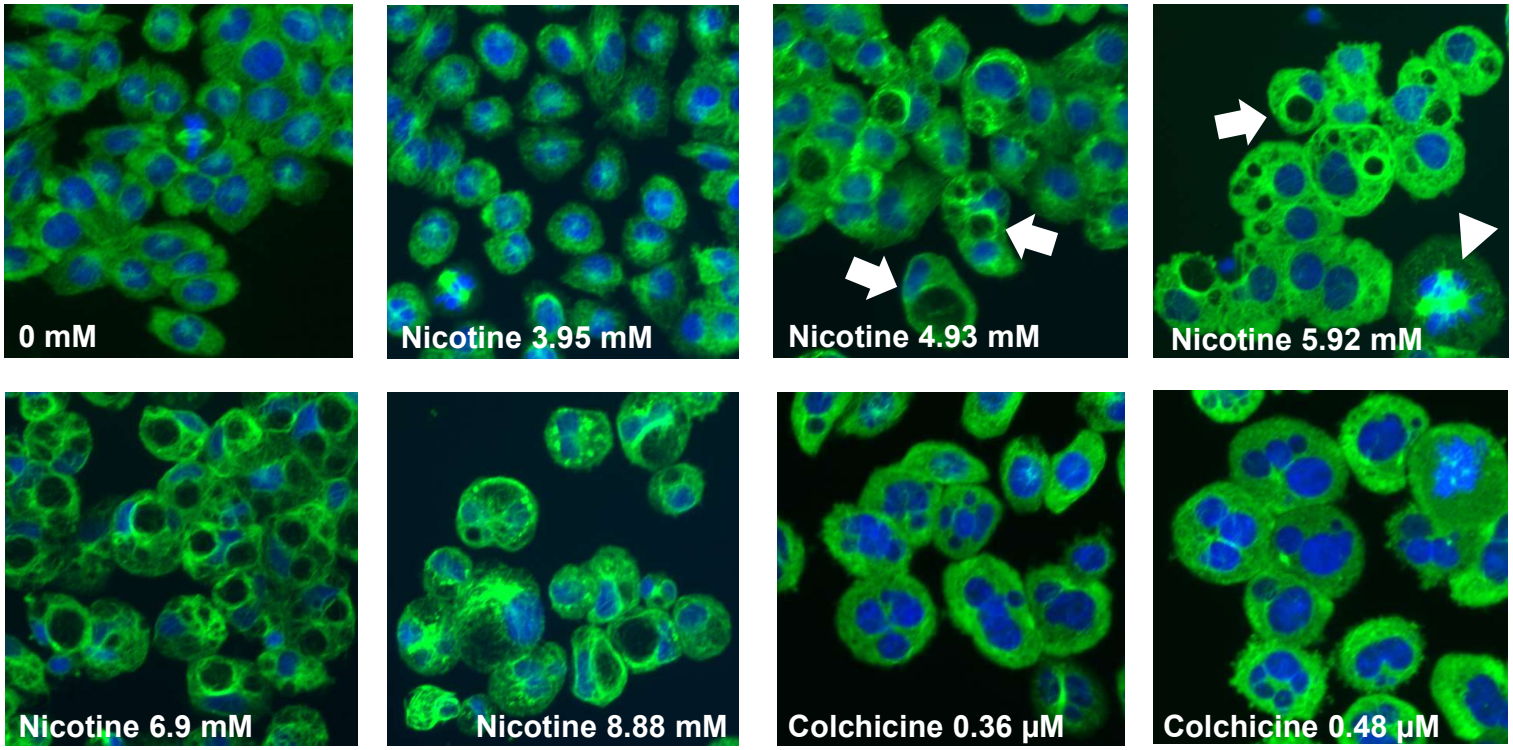
Nicotine-induced perturbation of microtubules

- α-Tubulin = Green; Nuclear DNA = Blue. Examples of **vacuolization** (⇧) and an **abnormal** spindle (△).



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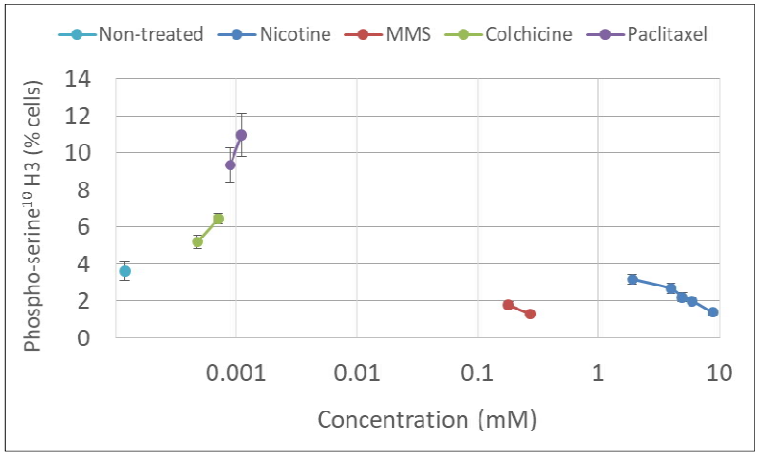
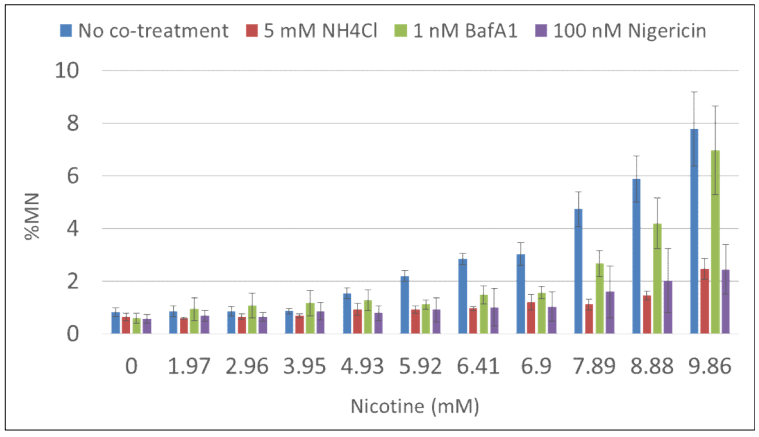
Lack of histone phosphorylation and probable lysosomotropic mode-of-action



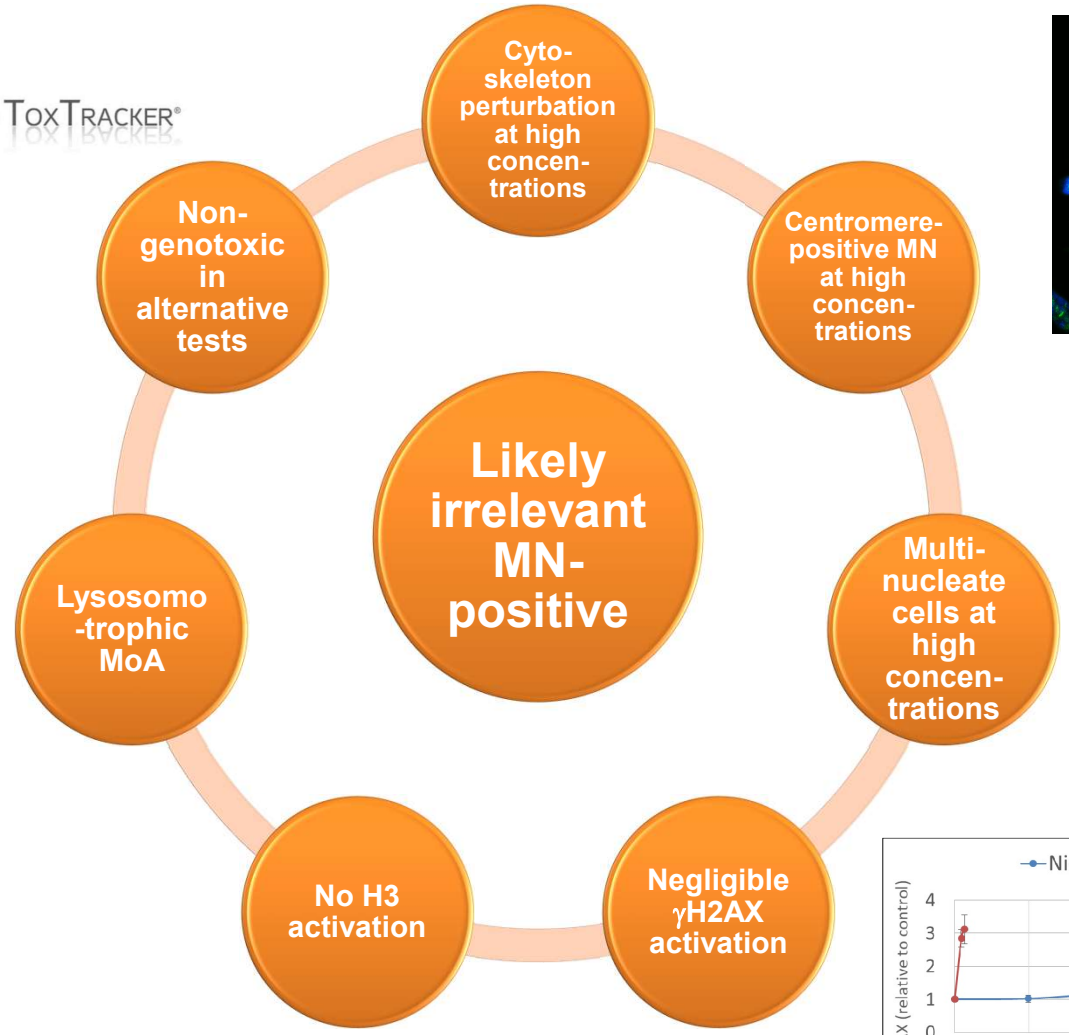
- **Phospho-serine[10] H3**: occurs in M-phase cells → marker of **aneugenicity** (measured at 4 h).
- Concentration-dependent **decrease** observed for nicotine.

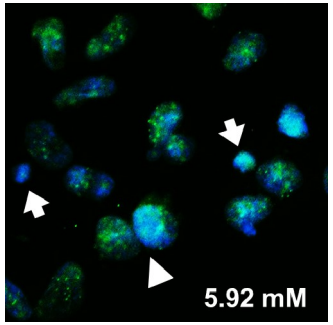


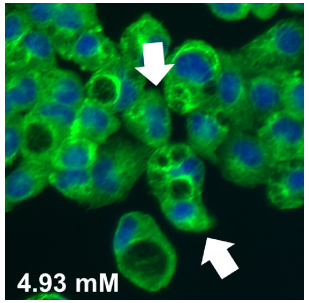
- **Lysosomotropism**: trapped chemicals accumulate in acidic cellular compartments → organelle **swelling** → **microtubule** perturbation.
- Nicotine-induced MN modulated by **increasing pH** of acidic compartments chemically.

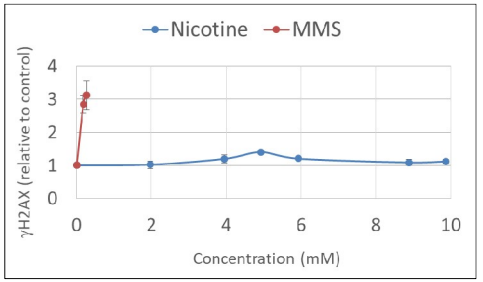
Smart et al., 2019, Environ. Mol. Mutagen. 60: 778-791

ToxTracker®









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2. Comprehensive in vitro genotoxicity assessment

Why comprehensive in vitro assessment?

- Affords:

  - Broad **understanding** of genotoxic potential.
  - **Mode-of-action** insights.
  - Fulfilment of **regulations**.
  - **3Rs** benefits.

- Lack of data **complementarity** in multi-assay assessments.

- Potential **specificity** issues in rodent cell lines.

- Hence, an **integrated** assay concept in **human** cells:

  - **Chromosome damage**.
  - **Gene mutation**.
  - **Mode-of-action**.
  - **Leverage** existing assays and technologies.

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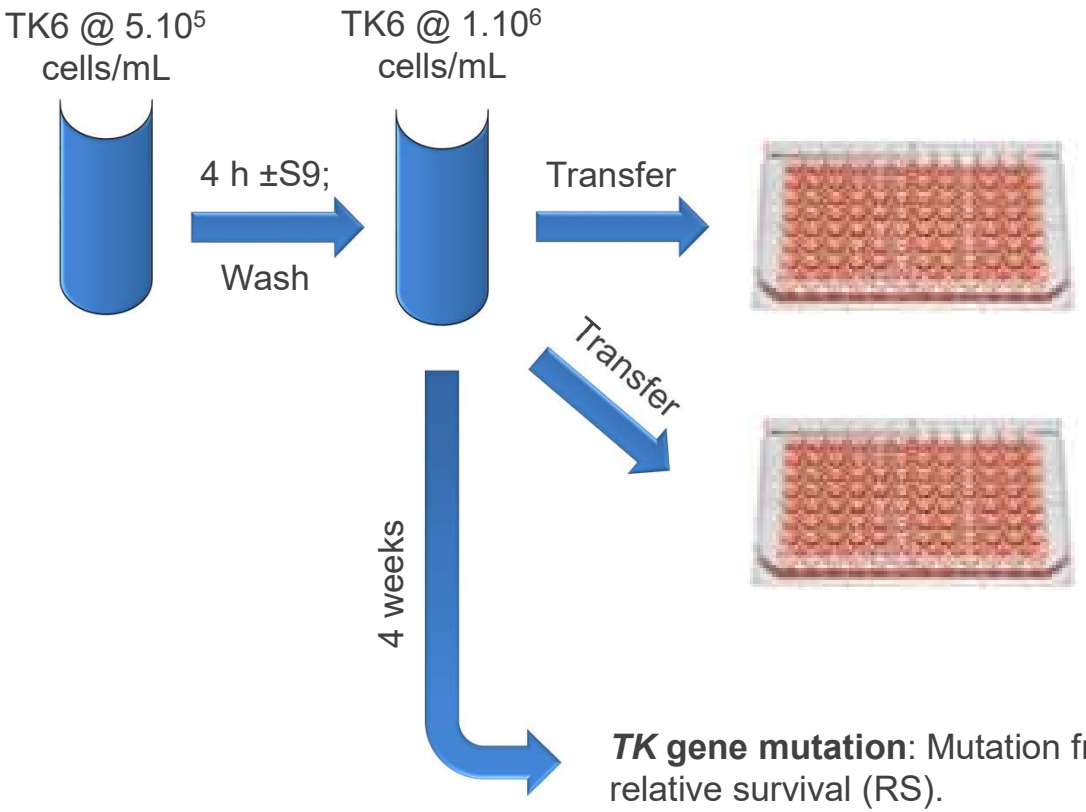
One human cell culture, multiple endpoints



MultiFlow®: Phospho-ser^{10}-H3, γH2AX, nuclear p53, cleaved-PARP, cell cycle.

MicroFlow®: MN, relative population doubling (RPD), cell cycle.

***TK* gene mutation**: Mutation frequency (MF), relative survival (RS).

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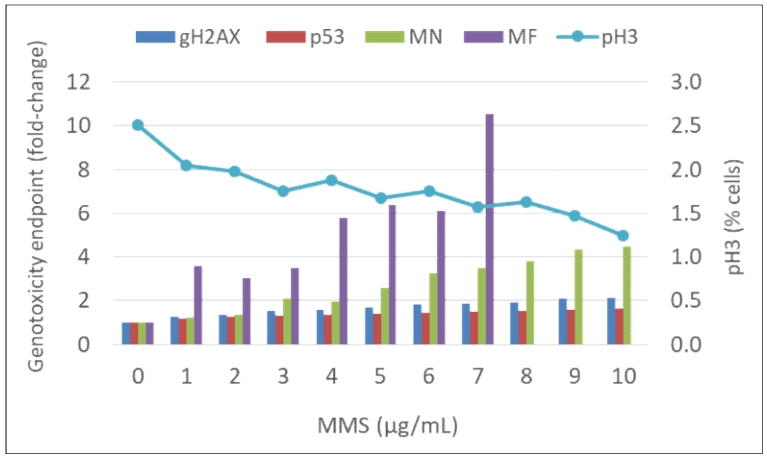
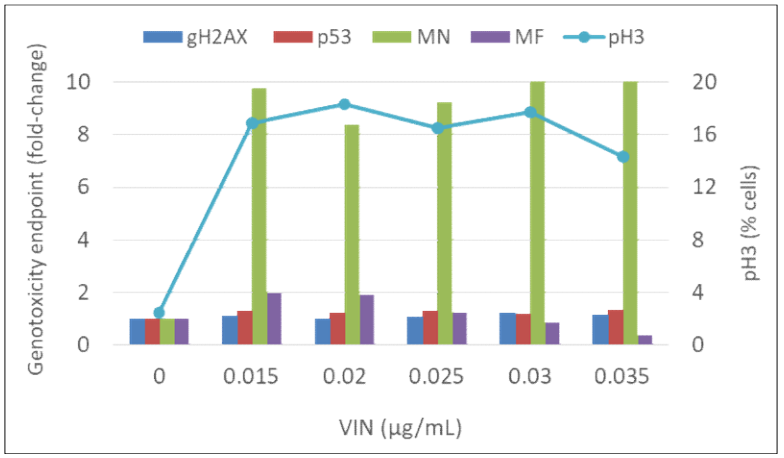
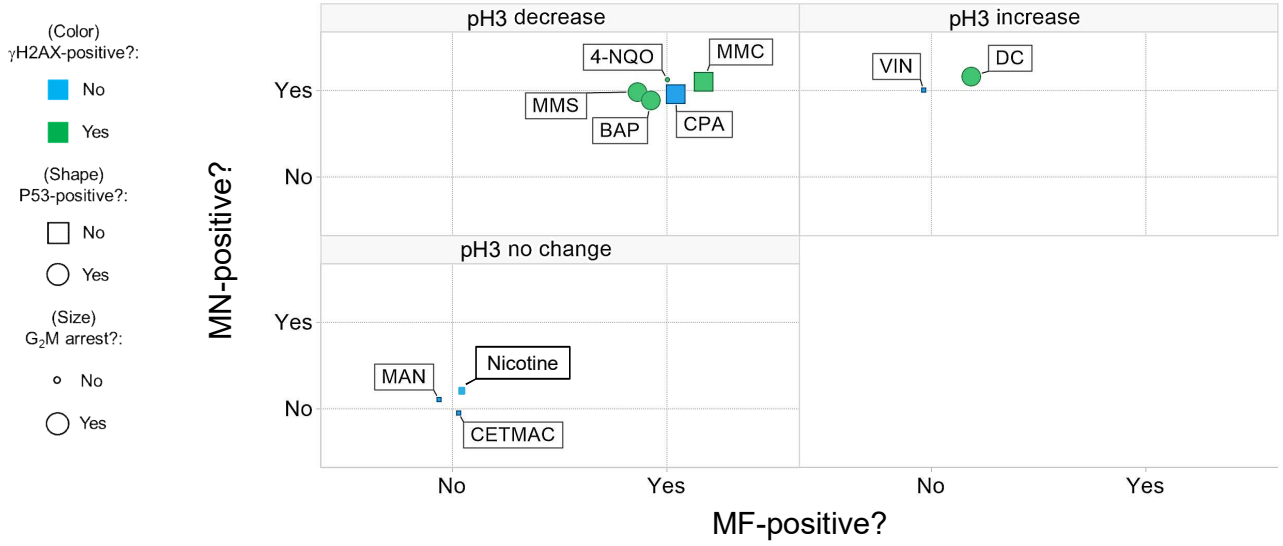
Smart et al., 2020, Mutat. Res. Gen. Tox. En. 849: 503129

Multiparametric data to inform hazard potential







4-NQO	4-Nitroquinoline 1-oxide
BAP	Benzo[a]pyrene
CETMAC	(2-Chloroethyl) trimethyl-ammonium chloride
CPA	Cyclophosphamide
DC	Sodium diclofenac
MAN	D-Mannitol
MMC	Mitomycin C
MMS	Methyl methanesulfonate
VIN	Vinblastine sulfate

Smart et al., 2020, Mutat. Res. Gen. Tox. En. 849: 503129

Comprehensive analysis accomplished

- **Successfu**l integration of the 3 genotoxicity endpoints into 1 assay without compromising the performance of any.
- Prototypical genotoxins were **readily** detected and induced response **signatures** commensurate with their mode-of-action:
  - DC revealed as potential **in vitro aneugen**.
- Non-genotoxins produced **negligible** changes in all assay endpoints.
- Possible **important** role to play in product assessment.
- Although not amenable to **screening**.

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3. Non-flavored e-liquid-induced “genotoxicity”

Establishing the "baseline" effects of neat NFELs

- **Non-flavored e-liquids** (NFELs) are the foundations for flavored e-liquid development.

- Contain varying levels of **propylene glycol** (PG), **vegetable glycerin** (VG) and **nicotine**.

- **Why** characterize these effects in the in vitro MN assay?

  - To serve as a **point of reference** for future MN studies on the aerosols of flavored e-liquids.
  - To shed light on the impact of **extreme culture conditions**.

	PG content (%)	VG content (%)	Water (%)	Nicotine (20 mg/mL)
NFEL-A	70	20	10	✓
NFEL-B	40	40	20	✓
NFEL-C	20	73	7	✓
NFEL-D	100	0	0	✓
NFEL-E	0	100	0	✓
NFEL-F	70	20	10	×

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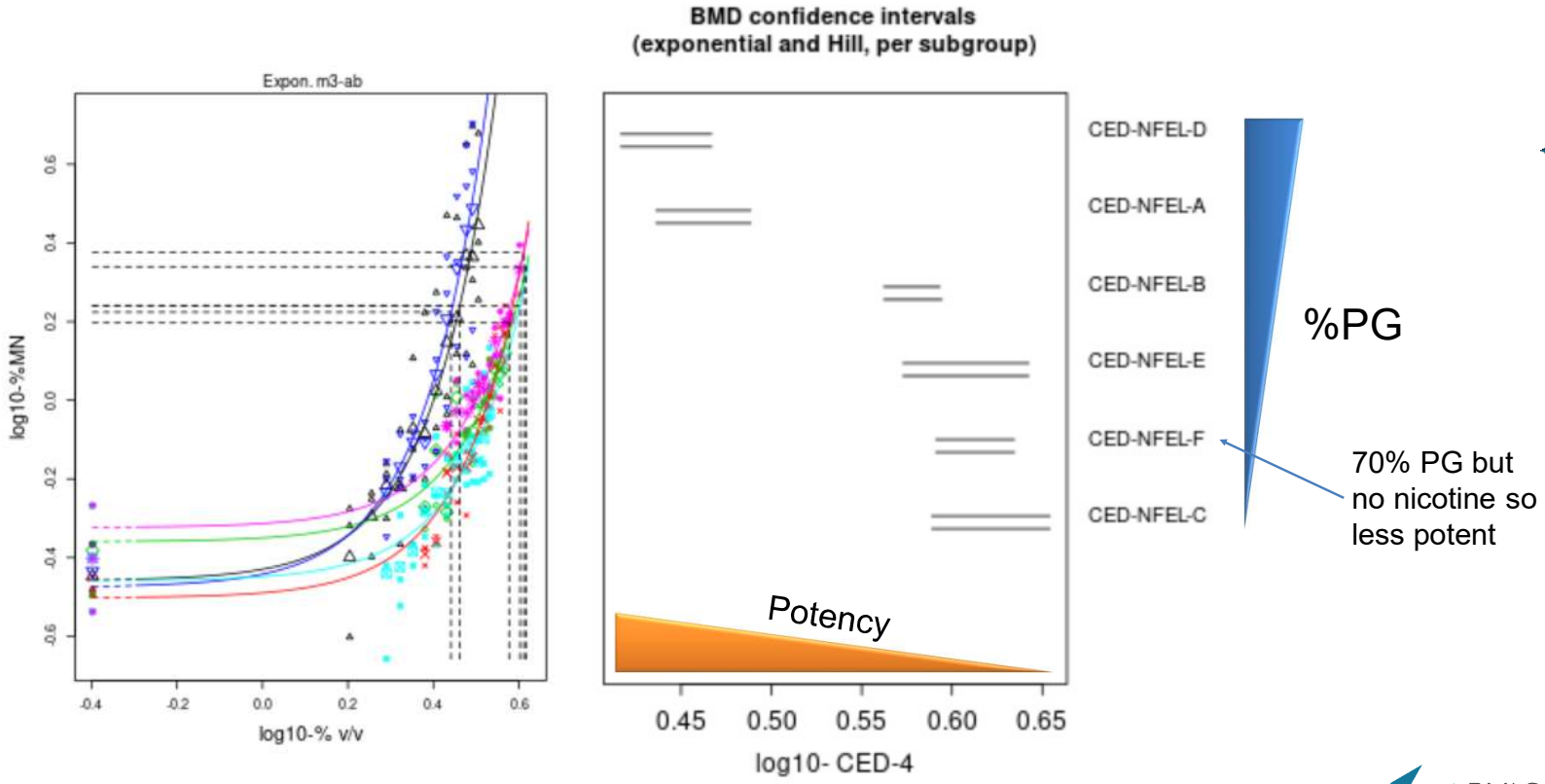
PG-predominate NFELs were more potent MN inducers after 24 h exposure in CHO-WBL cells



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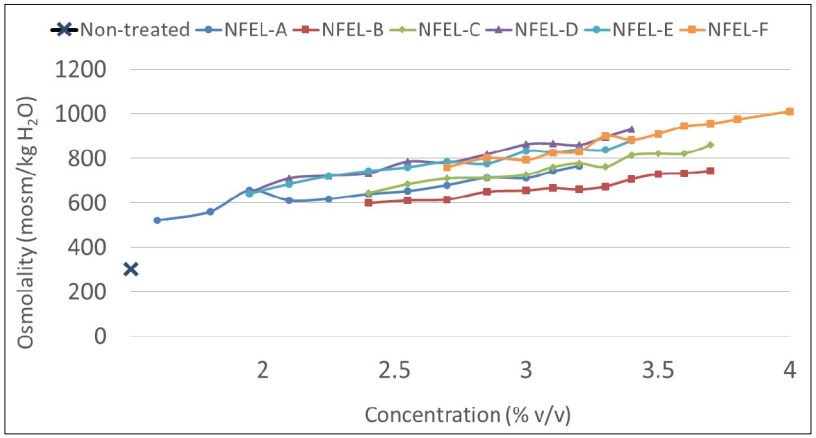
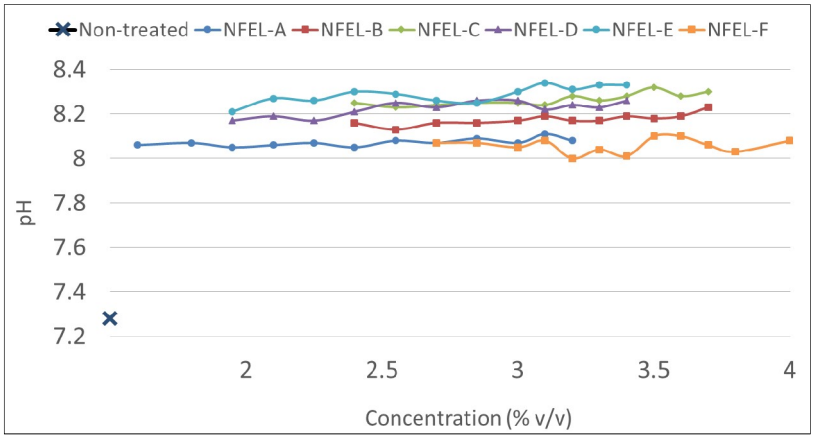
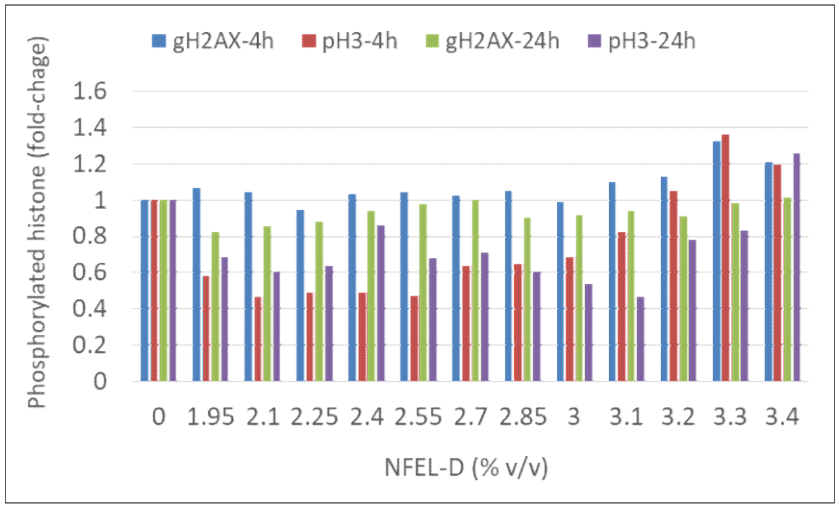
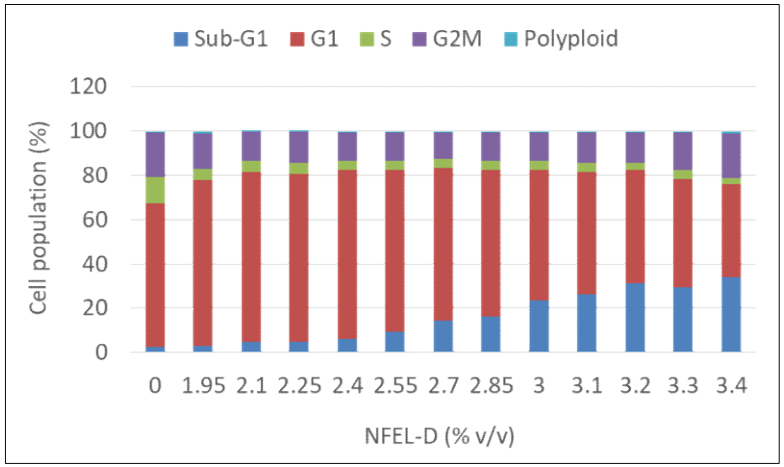
All NFELs induced extreme culture conditions but none triggered the DNA damage response (DDR)



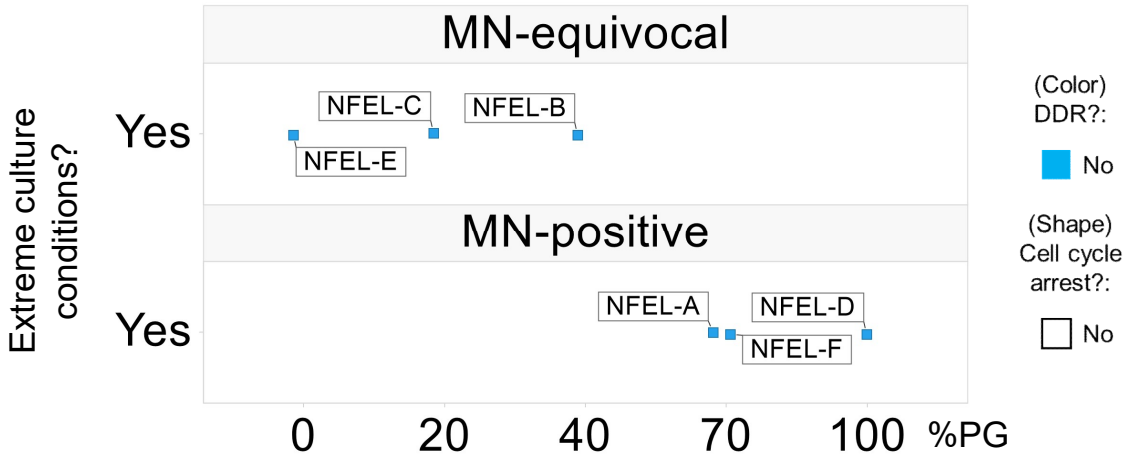






Smart et al., 2019, Tox. Res. App. 3: 1-9

Holistic approach improves final interpretation



- Further evidence of **likely irrelevant** MN-positive results with PG-predominate NFELs in CHO cells:

  - **Fundamental DDR** to genuine genotoxins is **not** triggered.
  - Extreme culture conditions are **not** implicated.

- In future aerosol studies, **divergences** from this reference dataset might indicate a possible **genotoxic hazard**.

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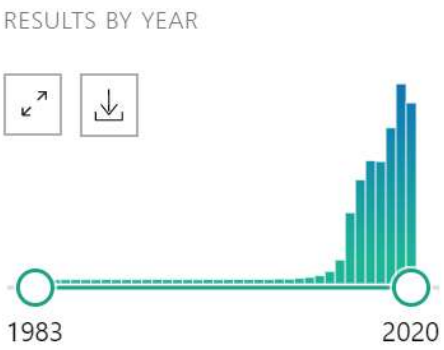
4. Collection of e-cig aerosol for in vitro assessment

In vitro assessment of e-cig aerosols

- **Expanding** research domain.
- Utility in **hazard identification**.
- To-date, **no** standardized aerosol collection methodology for **submerged** cell culture assays.



- **Consensus** from Institute for In Vitro Science Inc. workshop members to **map** types of methods used and published.

Compilation of a robust database

- **PubMed®** search and **keyword** triaging.
- Data from **47 publications** used in analysis.

Smart & Phillips. 2020, J. Appl. Toxicol. In press

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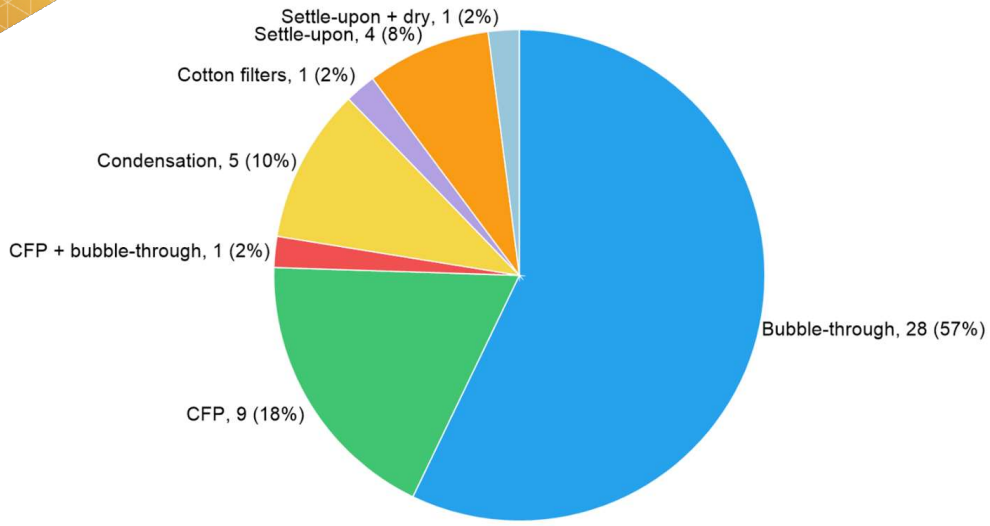
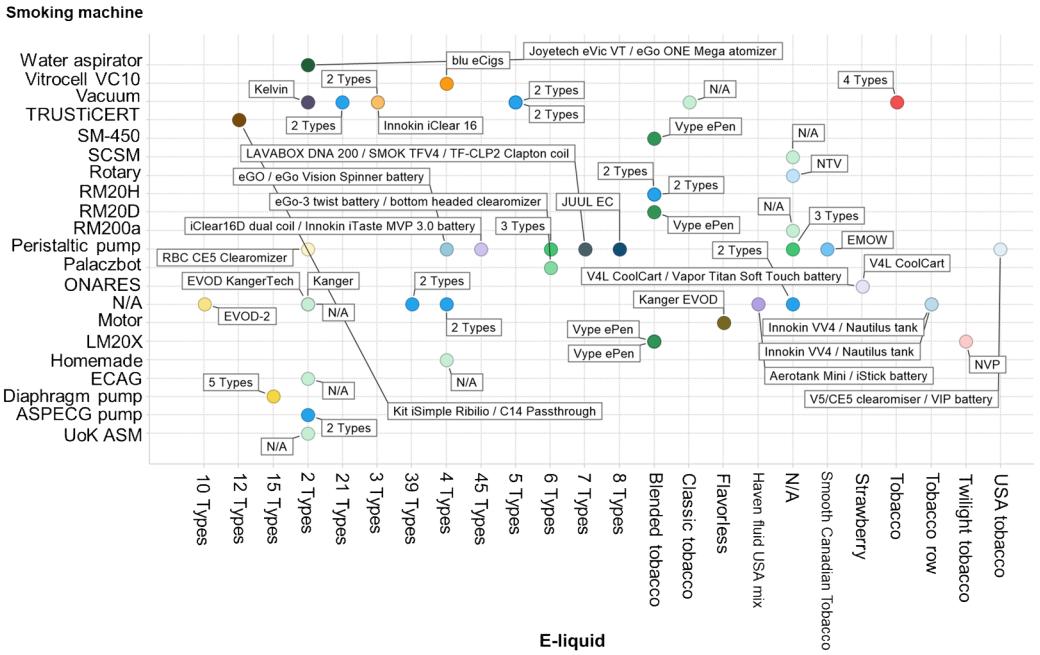
Seven broad collection methods cited and large heterogeneity among other study elements





CFP: Cambridge filter pad

Smart & Phillips. 2020, J. Appl. Toxicol. In press

Critical data gap

- **Dearth** of **chemical characterization** data on the collected aerosol fractions:

  - Are trapped fractions **representative** of their native aerosols?

Conclusions and opportunities

- **Wide-range** of aerosol collection approaches used.
- Most optimal collection method **not** currently known.
- **Improve** the value of in vitro data by:

  - Identifying the **best** collection method.
  - **Standardizing** aspects of aerosol generation & trapping.

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Smart & Phillips. 2020, J. Appl. Toxicol. In press

Final conclusions

- **Conventional** in vitro genotoxicity assessment of e-cigarette components can reveal **unexpected** findings.
- **Mode-of-action** analysis can facilitate the understanding of “traditional” genotoxicity data and mitigate any concerns over biological relevance.
- Approaches such as the integrated TK6 cell assay might provide a solution for **comprehensive** in vitro genotoxicity assessment.
- Further research is required to **optimize** the collection of e-cigarette aerosols for evaluation in submerged cell culture-based assays.

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