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NEXT GENERATION PRODUCTS

Applying New Approach Methodologies to Compare the Biological Impact of Nicotine Pouches and Cigarette Smoke

Matthew Stevenson | Harm Reduction & Engagement

TSRC 2026 | Wednesday Afternoon - Session B, Abstract 131

Conflict of interest statement

- I am a paid employee of Imperial Brands PLC, a manufacturer of tobacco and nicotine products
- The research described within this presentation was solely funded by Imperial Brands PLC
- The views expressed in the document are those of the author and do not necessarily reflect the opinions of Imperial Brands PLC

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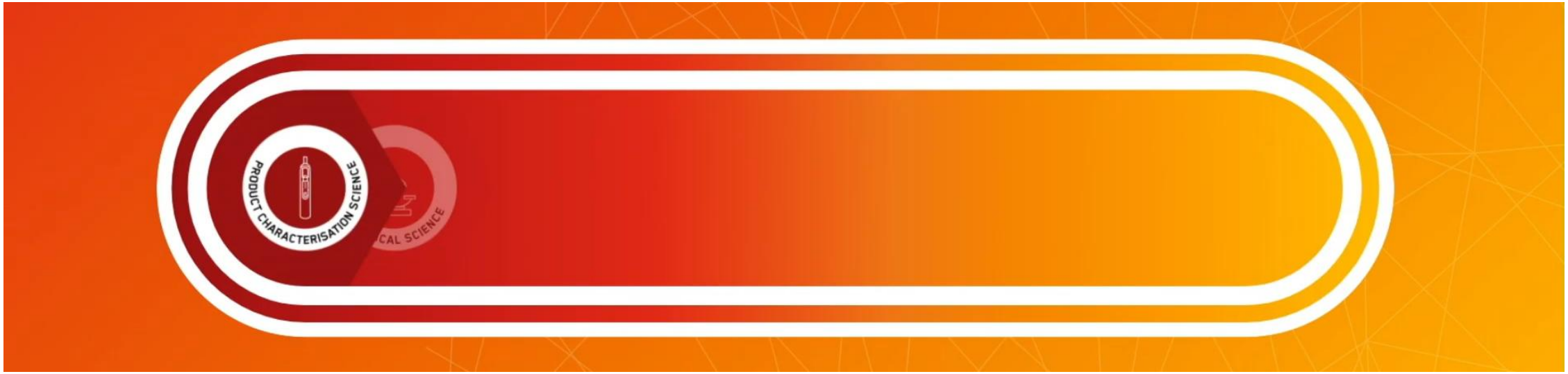
Introduction



- As a responsible manufacturer, Imperial Brands has developed a multi-stage and multi-disciplinary Scientific Assessment Framework (SAF)
- The SAF supports evidence-based assessment of product stewardship and substantiates the tobacco harm reduction potential of our next generation products

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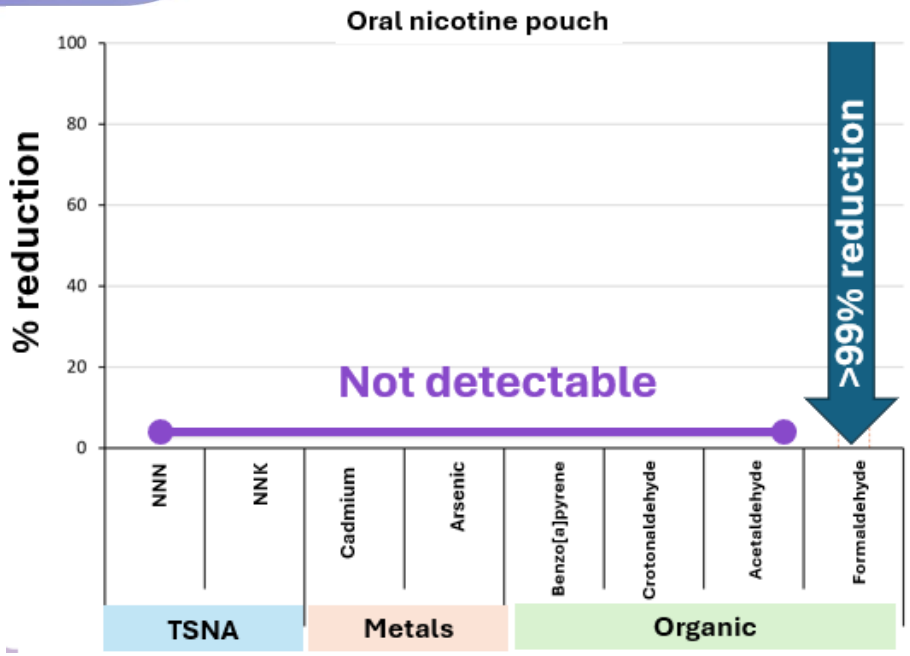
Introduction to Oral Nicotine Pouches

- Oral Nicotine Pouches (ONP) contain a plant fibre-based substrate or dry powder with high-purity nicotine
- Many toxicants present in cigarette smoke are not produced
- Nicotine is absorbed through the gums



Percentage reduction of toxicants in a representative ONP compared to a reference cigarette[1]

As ONPs don't contain or burn tobacco leaf, research demonstrates they contain significantly fewer and substantially lower levels of harmful chemicals compared to snus or cigarette smoke.

[1]Edwards *et al*. (2026) 'Chemical characterisation of Tobacco-Free Nicotine pouches reveals marked reductions in toxicant levels when compared to combustible cigarettes', Poster presentation, Society of Toxicology (SOT) 2026 Annual Meeting and ToxExpo, 25 March 2026, Abstract 5210, Poster K774.

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Oral Nicotine Pouches and *In Vitro* toxicology

- **Previous studies have demonstrated:**
  - **Marked reductions in cytotoxicity**

    (in some instances, 200-fold lower than cigarette smoke extracts)
  - **No evidence of genotoxicity.**

- **Why use New Approach Methodologies[2,3]?**
  - **Use more physiologically more relevant models.**
  - Provide **deeper mechanistic understanding**, beyond traditional testing.
  - **Assess multiple biological pathways simultaneously.**

[2]Macmillan, D. S., Holcomb, P., & Sullivan, K. M. (2026). Lost in NAMs-lation: A review of animal-free science definitions. ALTEX, 43(2), 373–379. https://doi.org/10.14573/altex.2512221,

[3]LaFollette, M., Roper, C., & Thompson-Iritani, S. (2026). Never-ending acronym madness: Proposing a harmonized definition for NAMs. NAM Journal, 2, 100085. https://doi.org/10.1016/j.namj.2026.100085

Study Objective

Primary Objective

To determine whether nicotine pouch extracts induce measurable biological effects and compare these responses with those caused by cigarette smoke in a range of physiologically relevant NAMs.

Key Questions

- Do nicotine pouch extracts cause cytotoxicity in human oral cells?
- Do they impair cellular function?
- Do they induce oxidative stress?
- Are observed effects mechanistically similar to cigarette smoke?

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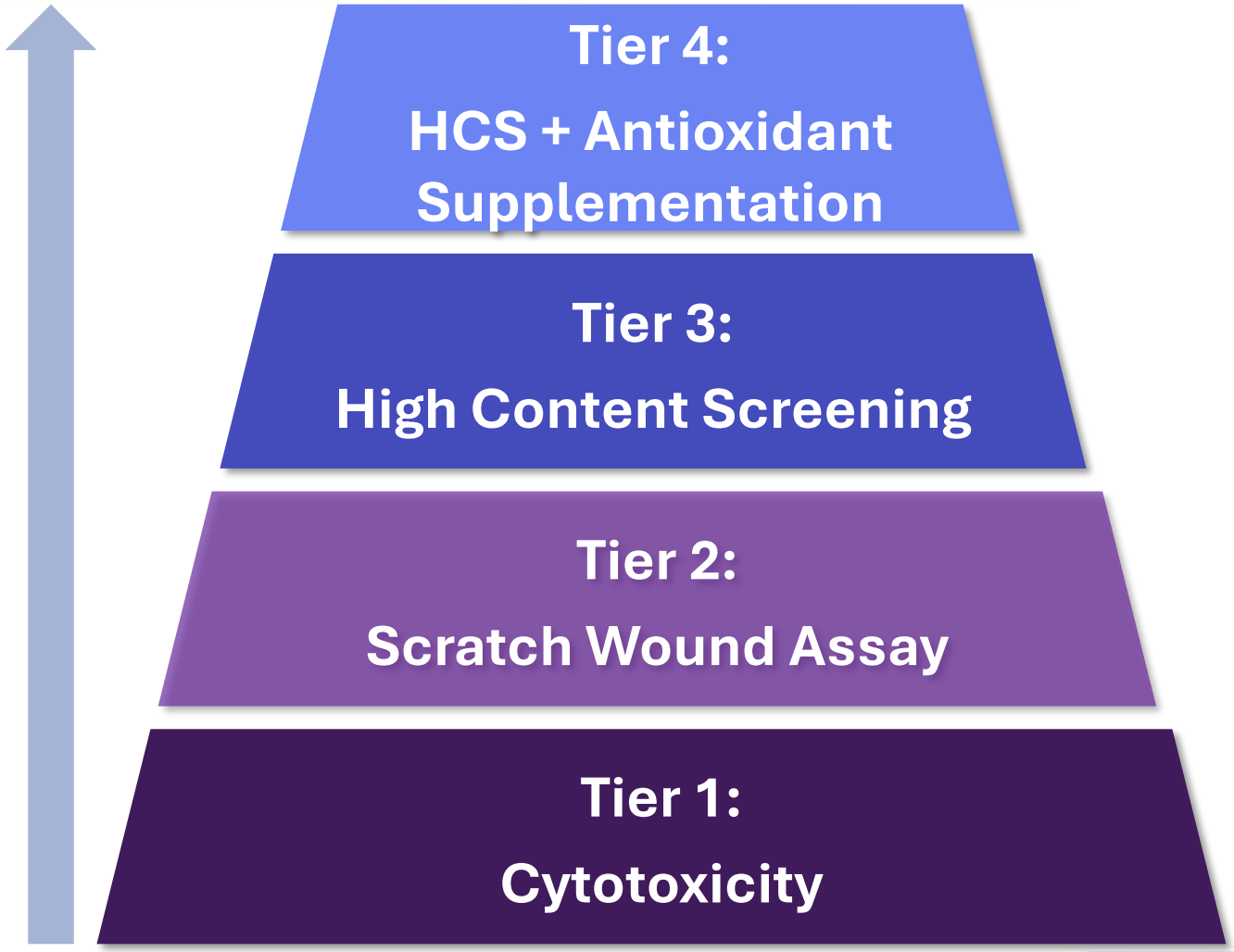
Experimental Strategy



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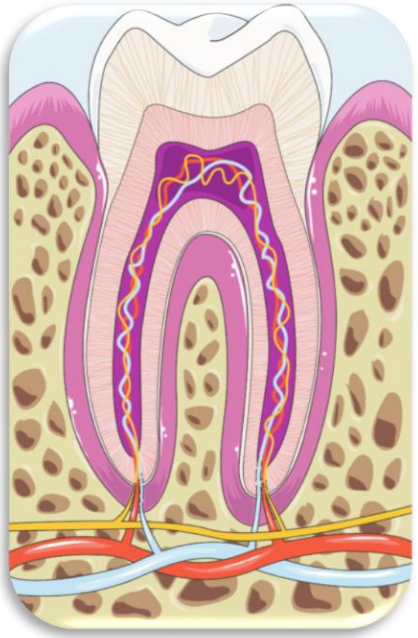
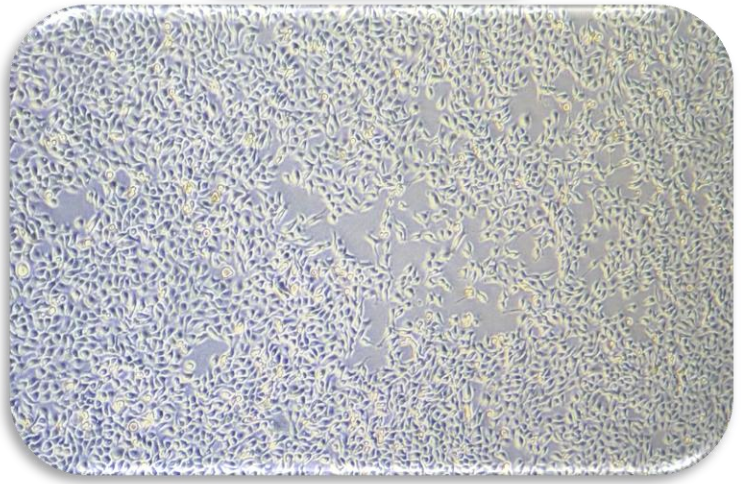
Background on cell types

- Previously published studies on ONPs have relied on either animal based or human cells that do not reflect the primary route of exposure.
- Whilst these cells have proven to be sensitive, there is a need for human based cells for the oral route.
- Whilst there are limited oral cells available, we have identified and internally validated the human gingival squamous carcinoma cell line (Ca9-22).
- To complement these cells, we use Human Coronary Arterial Endothelial Cells (HCAEC), as a downstream cell type.

1.





Microscopic images of Ca9-22 cells

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[1]Image(s) provided by Servier Medical Art (https://smart.servier.com), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

In Vitro Methodology

Cytotoxicity assessment

Product Extract Preparation: ONP & Smoke bubbled extracts prepared in Earl's minimum essential medium (EMEM)
Cell Exposure: Human gingival squamous carcinoma (**Ca9-22**) exposed to product extracts for 65 h
Functional Assay: Neutral red uptake in 96 MWP in serum free medium over 65h incubation. Outcomes were compared on a concentration required to induce 20% (EC20) and 50% cytotoxicity (EC50) basis.

Scratch wound assay

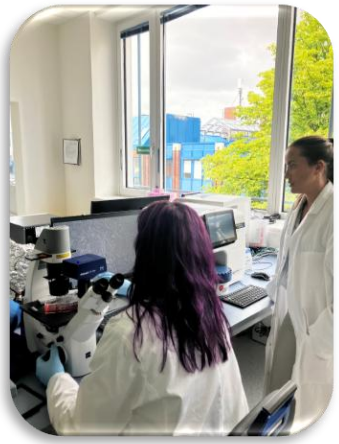
Product Extract Preparation: ONP & Smoke bubbled extracts prepared in EGM-MV2 medium
Cell Exposure: Primary human coronary artery endothelial cells (**HCAECs**) were seeded into 96-well ImageLock plates and grown to confluence. A standardized 700-800 µm scratch wound was created before exposure to increasing concentrations of ONP, TPM, or sbMED extracts for **24 h**.
Functional Assay: Cell migration was monitored every **2 h** using the **IncuCyte S3 live-cell imaging system**, with 8 technical replicates per concentration and 3 independent experimental runs. Migration was quantified as Relative Wound Density (RWD), and the time to 50% wound closure (RWD50) was calculated. Minimum Effective Concentrations (MECs) were determined using predefined statistical criteria and nonlinear regression analysis.

High Content Screening (HCS):

Product Extract Preparation: ONP & Smoke bubbled extracts prepared in EGM-MV2 medium
Cell Exposure: Primary human coronary artery endothelial cells (**HCAECs**) exposed to product extracts for 4 or 24 h
Biomarker Assessment: High-content imaging used to quantify markers of oxidative stress **(SRXN1**), stress signalling **(p-c-JUN, NFκB),** and DNA damage **(γH2AX).** Primary antibodies (1:250–1:500) targeted p-c-JUN, SRXN1, NFκB, and γH2AX. Fluorophore-conjugated secondary antibodies (640nm and 740nm) minimised autofluorescence. Nuclei were counterstained with DAPI.
Functional Assays: Intracellular glutathione (GSH) and **caspase 3/7 activity** measured to assess antioxidant status and apoptosis. Caspase 3/7 activity was assessed using CellEvent Green Detection Reagent (C-10423). Intracellular GSH was stained with monochlorobimane (mBCL).
Imaging Platform: Fluorescent immunostaining and quantitative analysis performed using the X-finity High Content Screener (Thermo Fisher Scientific).

HCS with Antioxidant supplementation

As above but with the addition of antioxidant **N-acetylcysteine (NAC, 1 mM)**.



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Test Material Preparation

Nicotine Pouch Extracts

- Commercial European-market nicotine pouch (5.8mg nicotine/pouch)
- Extracted based on - Biological evaluation of medical devices ISO 10993-12:2021:

6 g of ONP were covered with 20 mL media/PBS as extraction medium in a 50 mL tube (~ 300 mg/mL),
Agitation at 600 rpm for 1 hour,
Centrifugation and filtration through 0.45 and 0.2 µm sterile filters,
Aliquots of 500 µL per extract were frozen at -80°C.

Three independent extracts for ONP were generated. Nicotine was used as a marker for evaluating extraction efficiency.

1R6F Reference Cigarette Smoke Preparations:

- Smoke extracts in PBS
- Smoke extracts in culture medium
- Total particulate matter (TPM)

56 puffs (8cigs x 7puffs) per 1R6F using ISO 20778, Vitrocell with 3 impingers (10ml Medium or PBS each). Pooled solution concentration for 1R6F = 1.87 puffs / ml

ISO 20778 with 15 cigarettes smoked through one Cambridge filter pad (CFP). The TPM fraction was extracted from the CFP with DMSO and yielded in an average TPM concentration of 34.75+/- 0.5 mg/ml (n= 3 runs).

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Tier 1: Oral Cell Cytotoxicity Results

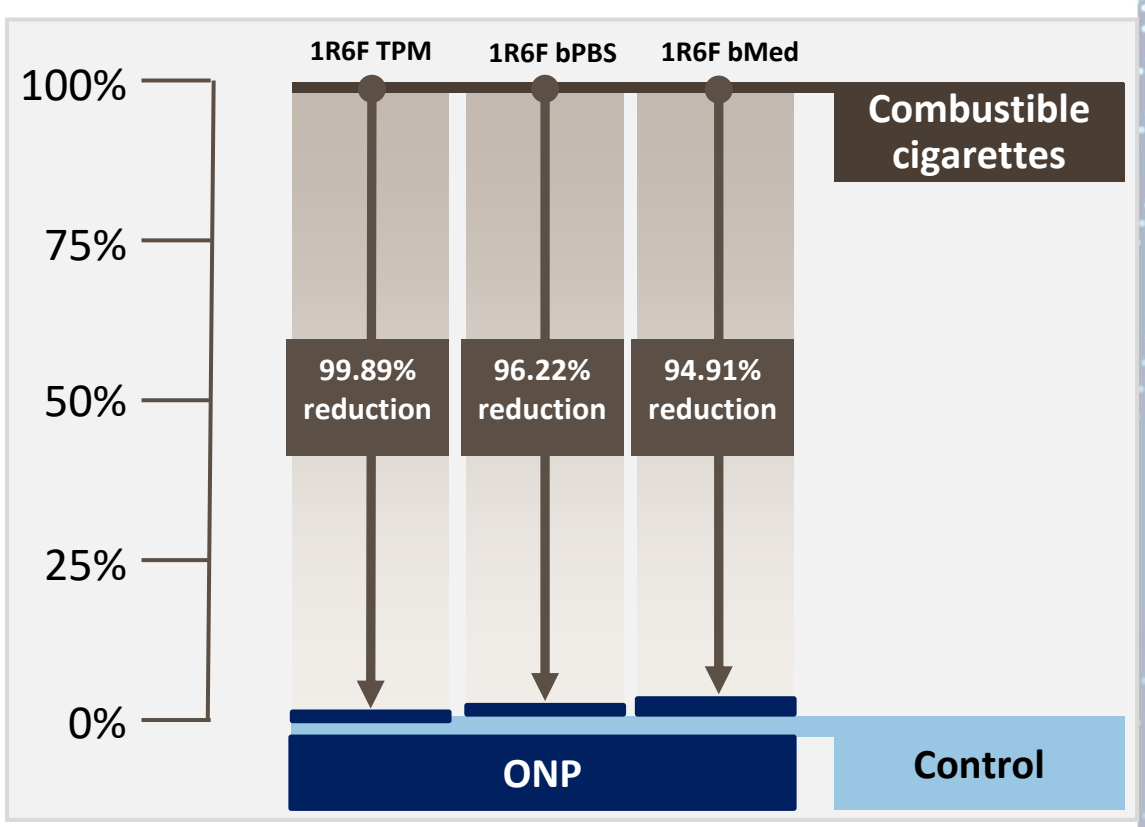
Cigarette Smoke Extracts:

- Significant cytotoxicity observed across all extracts
- EC_{20} ranged from 0.023 – 1.081µg/mL nicotine basis
- EC_{50} ranged from 0.054 – 1.897µg/mL nicotine basis

Nicotine Pouch Extract:

- Minimal cytotoxicity observed
- EC_{20} 21.23µg/mL nicotine basis
- EC_{50} (calculated*) 99.48µg/mL nicotine basis

*did not reach an EC_{50} for up to 10mg/ml extract



Comparison of the % reduction in cytotoxicity for ONP vs the different smoke extracts. Data calculated on the achieved EC_{20} values.

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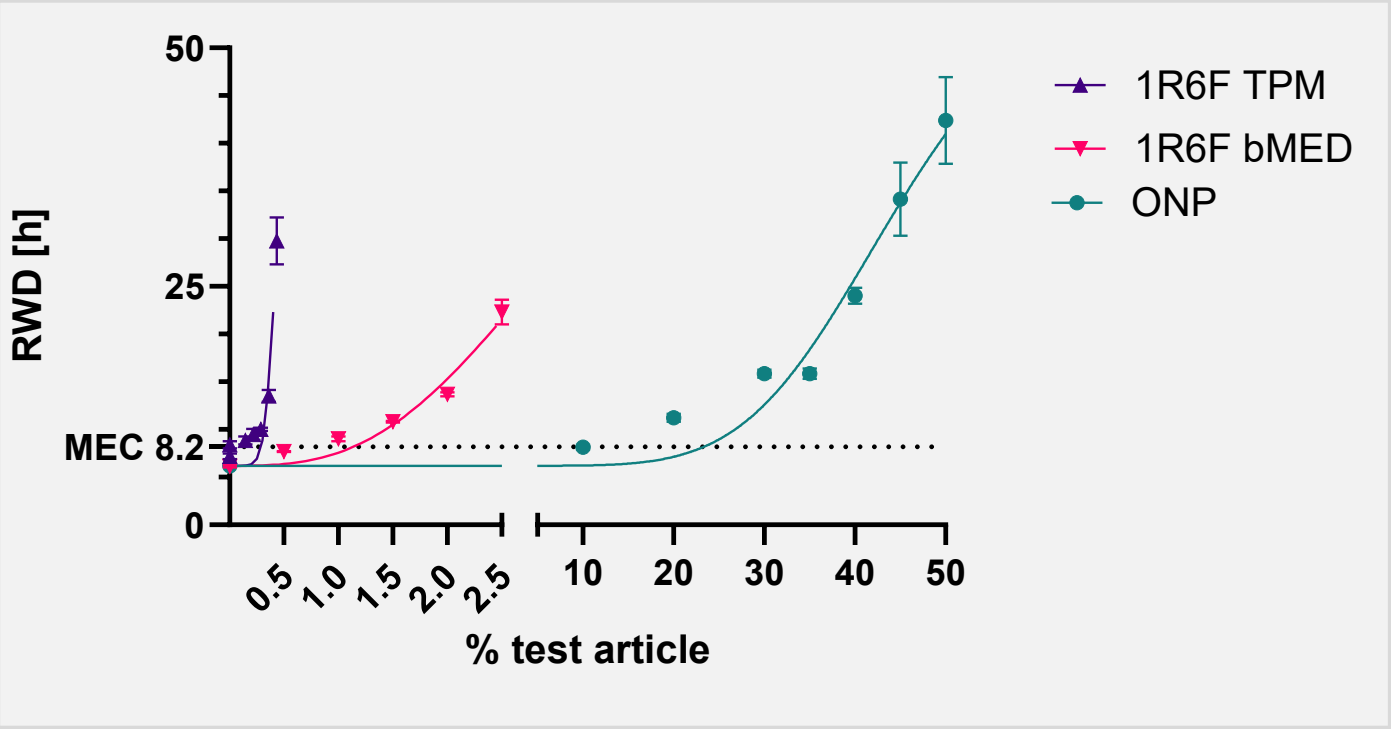
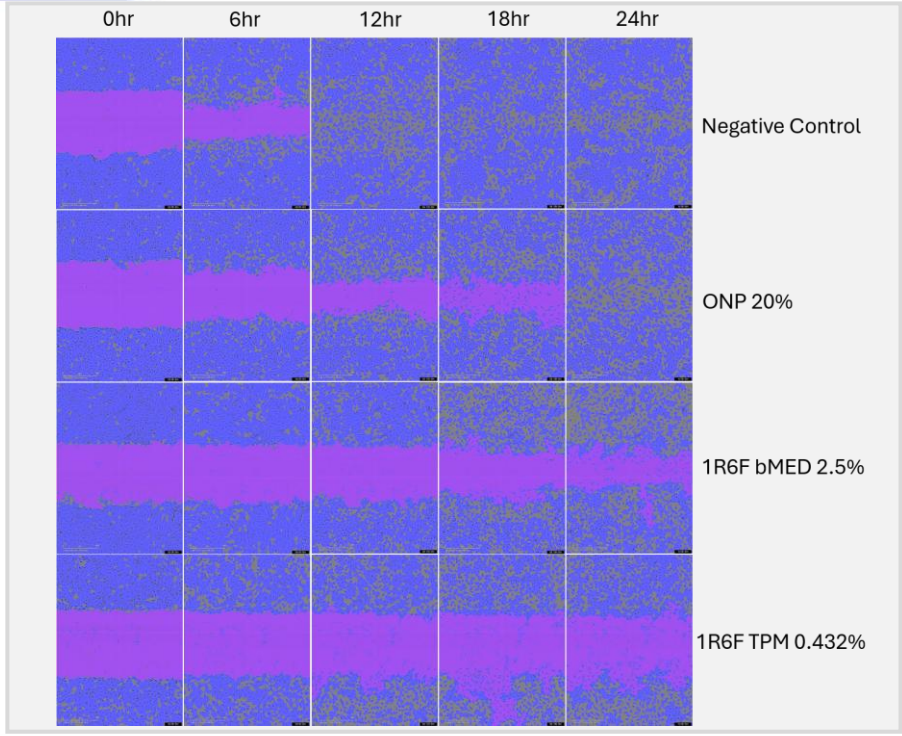
Tier 2: Scratch Wound Assay Results





RWD = Relative Wound Density, MEC = Minimum Effective Concentration

Cigarette Smoke Extracts (TPM and bMed)

Significant inhibition of endothelial migration from 5.393 - 5.661 µg/mL nicotine basis

Nicotine Pouch Extracts

Effects only observed at approximately 94-fold higher concentrations (504.9 µg/mL nicotine basis) than reference cigarette extracts

Tier 3: High-Content Screening Results

Cigarette Smoke Extracts:

Strong dose-dependent responses in:
- Oxidative stress
- DNA damage
- Cellular stress responses

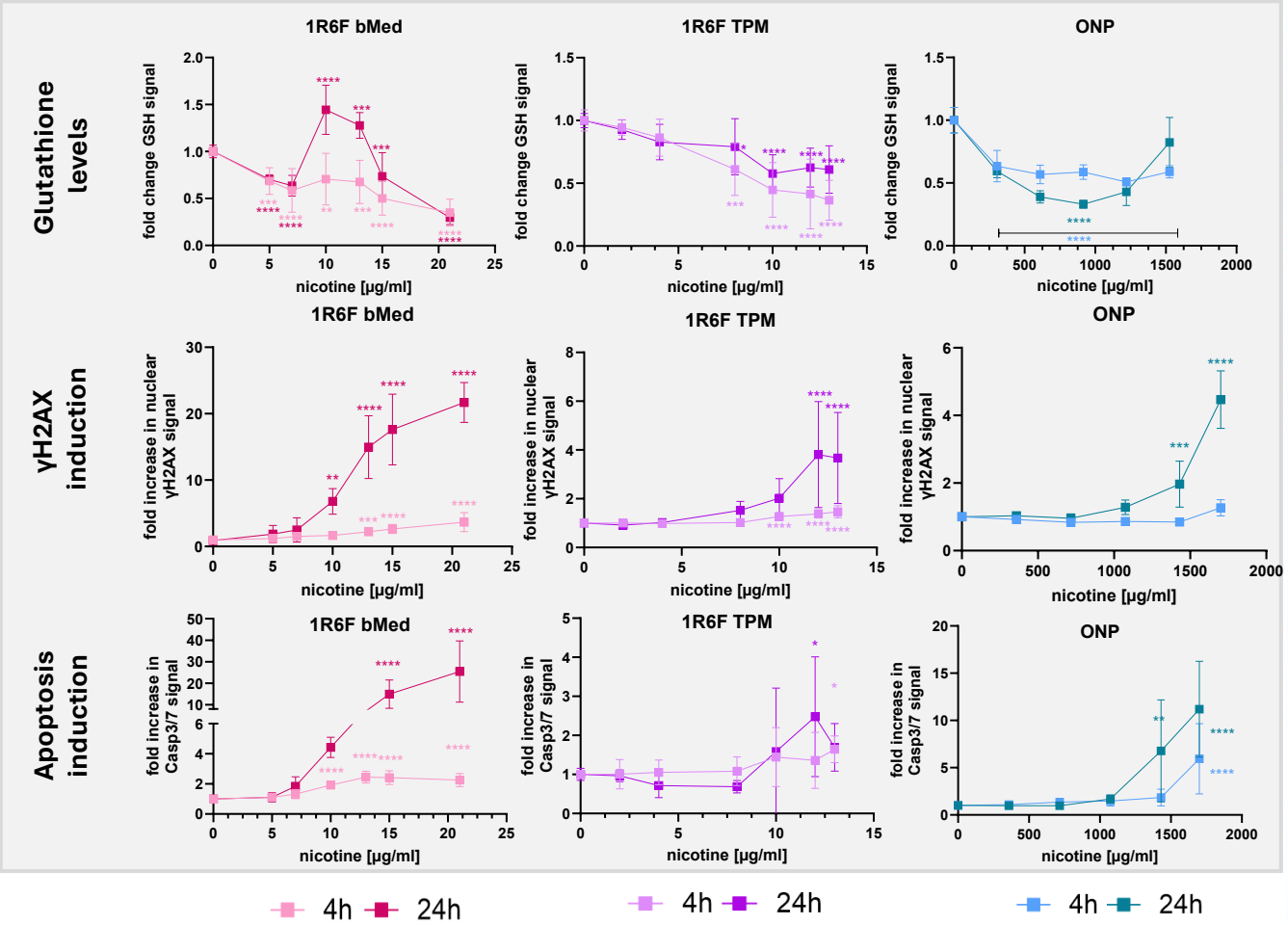
Minimal Effect Concentrations from 1.69 - 9.69µg/ml 1R6F bMed / TPM

Nicotine Pouch Extracts:

Minimal biological activity at high exposures

Minimal Effect Concentrations from 536 – 1,300µg/ml nicotine ONP extracts

55 – 769-fold reductions in toxicity for ONP vs 1R6F Cigarette extracts



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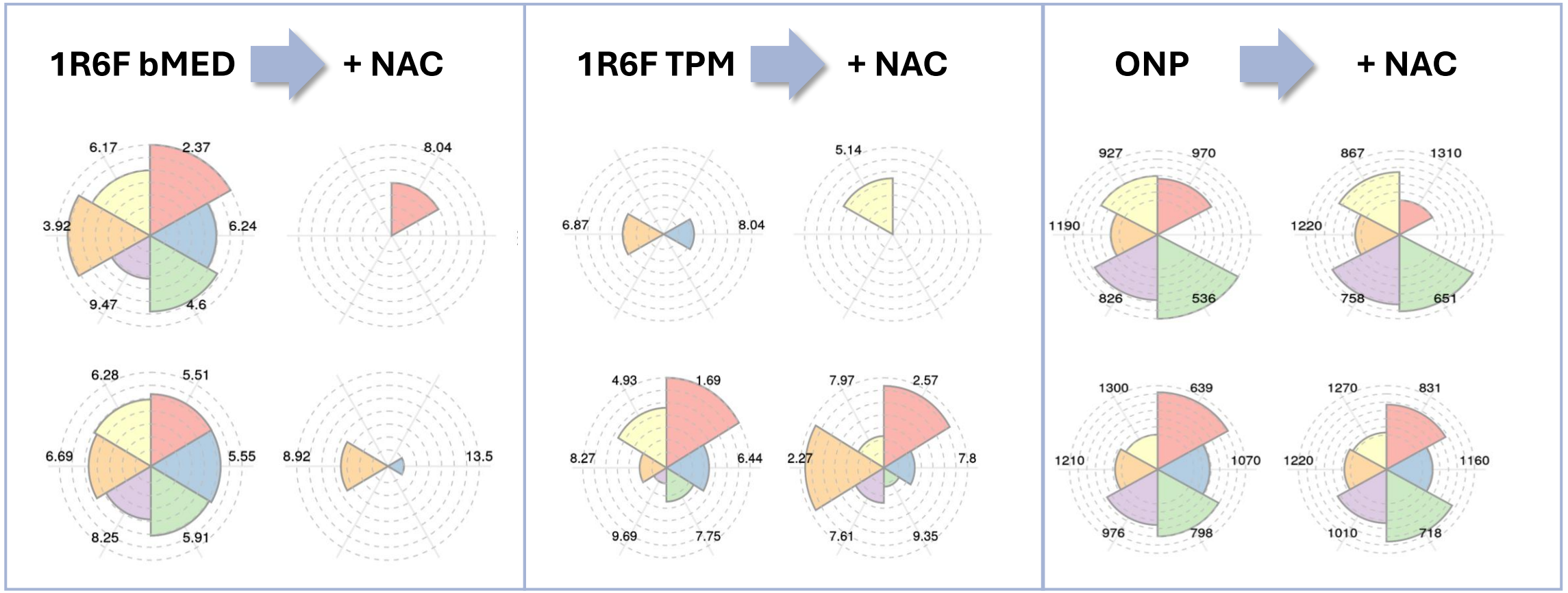
Tier 4: HCS with NAC Supplementation

Cell count (Cell death)
NfKB (Inflammatory response)
p-c-jun (Cellular Stress)
GH2AX (DNA Double Strand Breaks)
nuc size (Apoptosis)
SRXN1 (Oxidative Stress)

GladiaTox Pie charts showing MEC values and cellular responses to various extracts.

Segment size reflects effect strength - larger segments indicate lower Minimum Effect Concentrations (µg/mL).



Tier 4: HCS with NAC Supplementation

ONP Extract			1R6F TPM			1R6F bMED		
% extract	Nicotine (µg/ml)	Osmolarity	µg/ml	Nicotine (µg/ml)	Osmolarity	% extract	Nicotine (µg/ml)	Osmolarity
0	0	0.296	0	0	0.413	0	0	0.385
20	330	0.438	40	2	0.416	2	5	0.373
40	659	0.581	80	4	0.416	3	7	0.364
60	989	0.716	150	8	0.41	4	10	0.367
80	1318	0.865	200	10	0.412	5	13	0.365
			240	12	0.415	6	15	0.362
95	1565	0.973	260	13	0.405	8	21	0.366

Cigarette Smoke Extracts:

- NAC attenuated 1R6F bMED effects suggesting ROS-driven mechanisms

Nicotine Pouch Extracts:

- Effects not linked to oxidative stress pathways
- Observed responses likely associated with osmotic stress effects

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Conclusions

- Cigarette smoke extracts induced significant biological effects across all tested endpoints at low test concentrations.
- Nicotine pouch extracts showed substantially reduced biological activity across all endpoints.
- No evidence of oxidative stress-driven toxicity for nicotine pouch extracts in contrast to cigarette smoke extracts.
- NAMs provided valuable mechanistic insight beyond conventional toxicity testing.
- Findings support the reduced biological impact of nicotine pouches relative to cigarettes.

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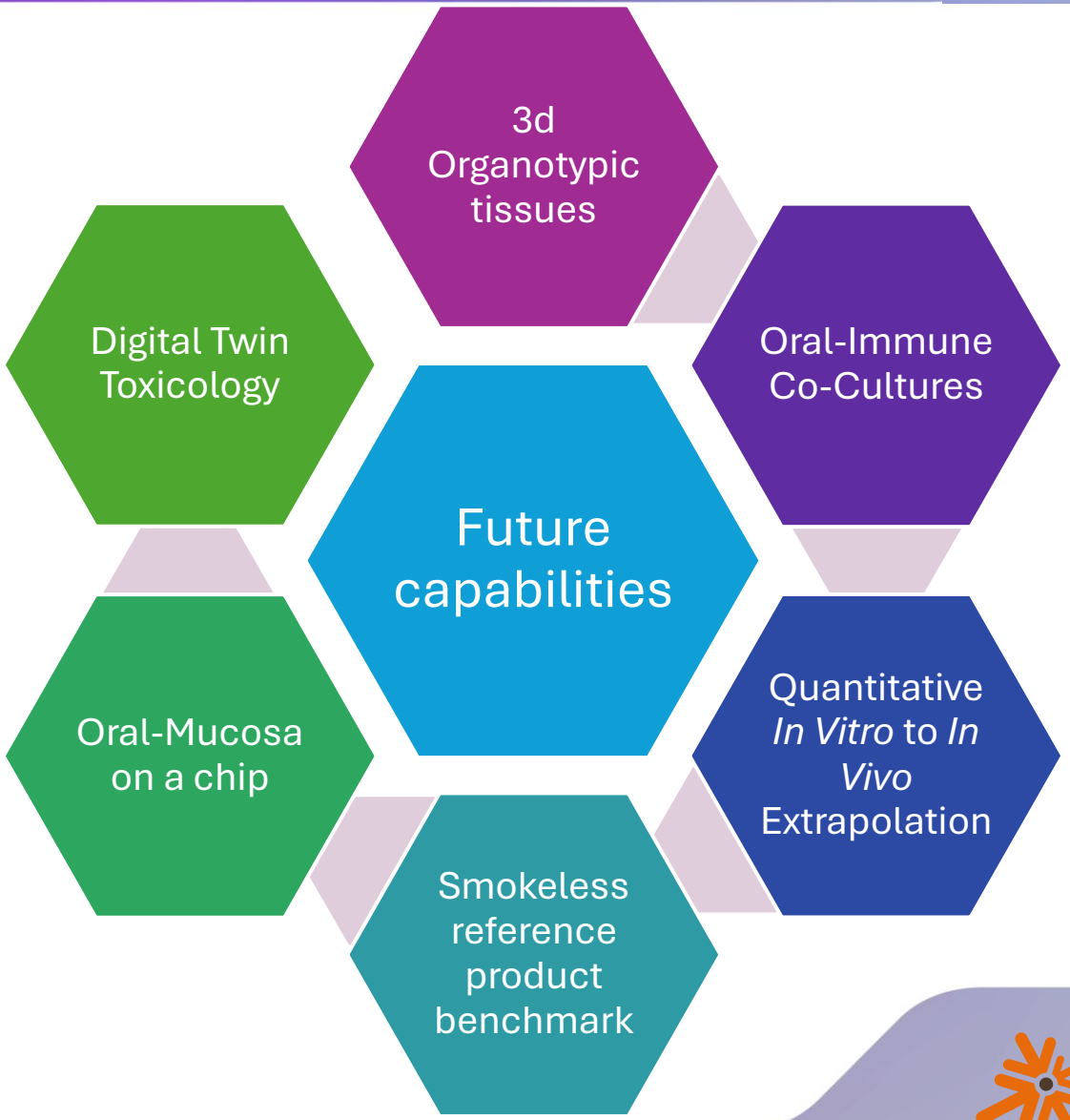
Future research: Growing mechanistic understanding and human relevance



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Thank you for your attention!