



NEXT GENERATION PRODUCTS



Reduced in vitro toxicity of Tobacco Free
Nicotine Pouches when compared with both
snus and cigarettes

Liam Simms

IIVS workshop 11;

12th-13th November 2025

Snus has potential as THR product

Oral Tobacco-based product

Many toxicants present in cigarette smoke are not produced

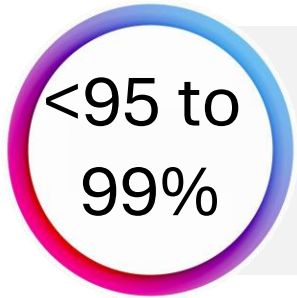
Nicotine is absorbed through the gums



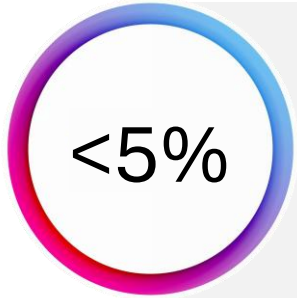
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The “Swedish experience”



Less harmful than cigarettes¹



Smoking rates in Sweden which is the **lowest** in Europe²
(24% in Germany/ 14% Denmark)³

Low rate of smoking in Sweden is widely attributed to availability of snus⁴



Snus use in Sweden

Combined snus and cigarette use is comparable to the average smoking rate in Europe³

¹Gartner CE et al., Assessment of Swedish snus for tobacco harm reduction: An epidemiological modelling study. Lancet 2007;369:2010-4 ;

²SmokeFreeSweden: <https://smokefreesweden.org/2024/11/13/breaking-news-swedes-first-in-world-to-become-smoke-free-its-a-lesson-for-the-world/> ; ³Eurobarometer <https://europa.eu/eurobarometer/surveys/detail/2995> ; ⁴Ramström, et al., (2016) Patterns of smoking and

snus use in Sweden: implications for public health. *International journal of environmental research and public health*, 13(11), p.1110.



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TFNP - a new category of oral nicotine products

Tobacco-Free Nicotine Pouches (TFNP)

No tobacco. Plant fibre-based substrate or dry powder with high-purity nicotine

Many toxicants present in cigarette smoke are not produced

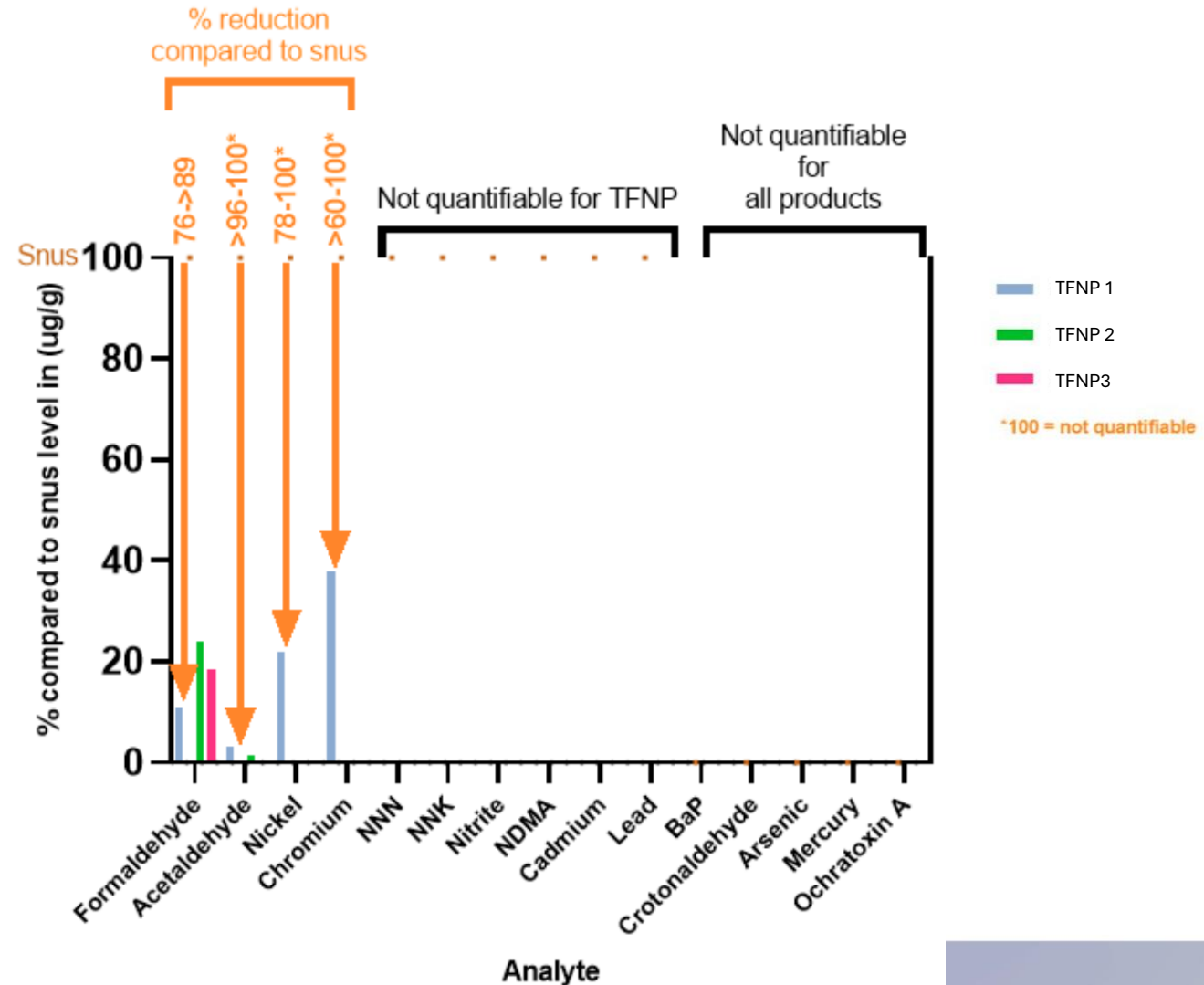
Nicotine is absorbed through the gums

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



Lower analytes (GothiaTek®), seen in TFNP when compared to snus

- In a comparison to snus, TFNPs products were 60-100% lower for the analytes formaldehyde; acetaldehyde nickel and chromium
- In the TFNPs NNN; NNK, nitrite NDMA, cadmium and lead were not quantifiable
- Both snus and TFNP did not contain any measurable BaP; crotonaldehyde; arsenic, mercury or Ochratoxin A



Reduced toxicity of Tobacco Free Nicotine Pouches when compared with both snus and Cigarettes

Data from 3 studies comparing snus and TFNP to each other and to 1R6F Reference cigarette

Study 1: TFNPs, snus and 1R6F reference cigarette using CORESTA battery

Study 2: Effects of flavours and nicotine on TFNP biological activity

Study 3: Use of High Content Screening (HCS) to compare TFNPs, Snus and 1R6F

Two further studies *in vitro* studies comparing snus and TFNPS

Study 4: Bishop et al., (2022) BAT products

Study 5: Yu et al., (2024) BAT products



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Description of test products (Study 1)



	<i>Product type</i>	<i>Coding</i>	<i>Primary substrate</i>	<i>Product nicotine content</i>
Skruf Superwhite Fresh #2	TFNP	TFNP #2	Plant fibers	5.8 mg/pouch
Skruf Superwhite Fresh #3	TFNP	TFNP #3	Plant fibers	10.1 mg/pouch
Skruf Slim Fresh White portion	Scandinavian Snus	Snus	Tobacco leaf	10.9 mg/pouch
1R6F Reference cigarette	Combustible cigarette	1R6F	Tobacco leaf	0.7 mg/cigarette ^a



Extract generation - TFNP and snus

TFNPs and snus were extracted based on - Biological evaluation of medical devices ISO 10993-12 (same method for Studies 1-3)

In brief,

1. 6 g of TFNPs or snus were covered with 20 mL phosphate-buffered saline (PBS) as extraction medium in a 50 mL tube (~ 300 mg/mL)
2. Agitation at 600 rpm for 1 hour.
3. After centrifugation and filtration through 0.45 and 0.2 µm sterile filters
4. Aliquots of 500 µL per extract were frozen at -80°C.

Three independent extracts for each TFNP test article were generated.

Nicotine was used as a marker for evaluating extraction efficiency. The average extraction efficiency in this study was 66%–75%.



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1R6F TPM generation and nicotine concentration's of extracts

Total particle matter (TPM) of 1R6F cigarettes were conditioned at 22°C and 60% relative humidity according to the ISO standard 3402 after conditioning the cigarettes were smoked according to the ISO 3308 (*35 ml puff volume/ 2 sec puff duration/ 60 sec interval/ bell shape puff profile, no ventilation block*)

Cigarette Smoke Condensate collected on a Cambridge filter



H₂O and nicotine analysis

Extraction of CSC in DMSO for in vitro tests → TPM

Table 1 Nicotine content of TPM in DMSO, TNFP, and snus PBS Extracts

Products:	<i>mg nicotine/mL solvent (±SD; n=3)</i>
TFNP #2 PBS *	1.70 ± 0.07 mg/mL PBS
TFNP #3 PBS *	2.51 ± 0.15 mg/mL PBS
Snus PBS *	2.59 ± 0.10 mg/mL PBS
1R6F TPM	1.35 mg/mL DMSO

* Contained the same flavour



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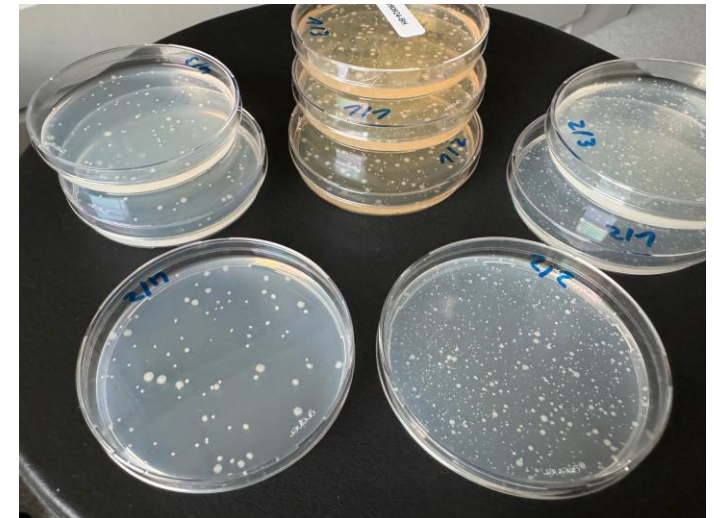
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In vitro testing battery

TPM, Snus and TFNPs extracts were tested in three assays (CORESTA battery) in BioToxLab IMB

- **NRU** - neutral red uptake *in 96 multiwell plate in serum free medium over 65h incubation*
 - BEAS-2B (ECACC;Cat.No.:95102433), human bronchial epithelial cell (lung)
 - HepG2 (ATCC,Cat.No.:HB8065), human hepatoma (liver)
- **IVM** - *in vitro* micronucleus *in 24 MWP*
 - V79 (ECACC;Cat.No.:86041102), hamster lung fibroblast
- **Ames test**
 - *Salmonella typhimurium* (+/- S9) TA98; TA100; TA102; TA1535; and TA1537

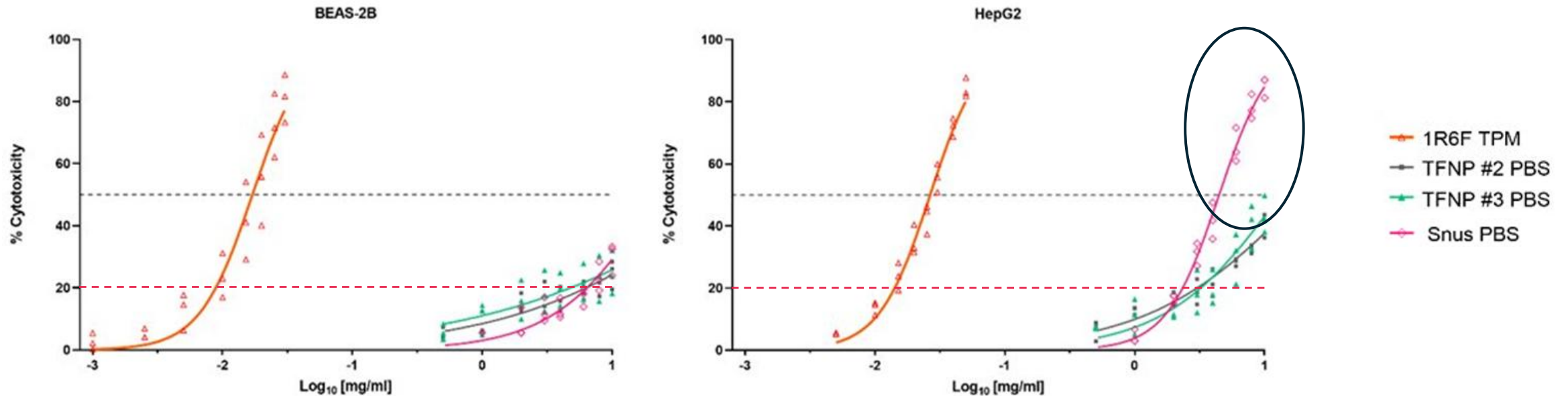
All methods are ISO17025 accredited



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NRU – cytotoxicity results (mg/mL)



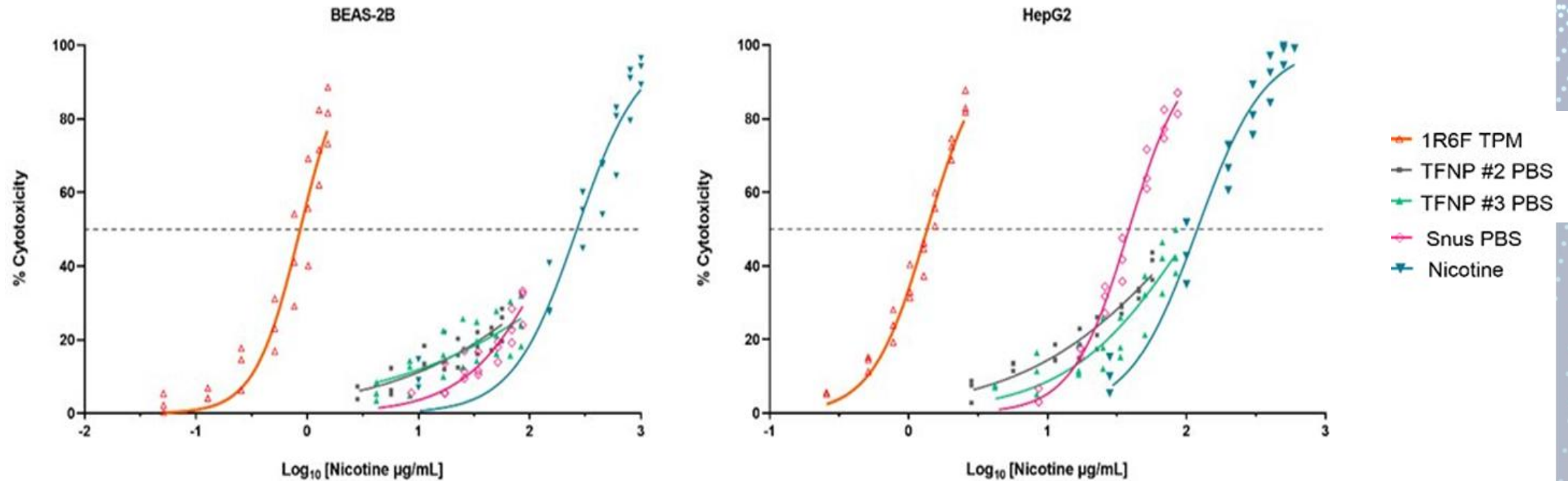
- All extracts showed statistically significant cytotoxicity, >20% cytotoxicity.
- In the EC₂₀, 1R6F TPM was >150 times more cytotoxic than TFNPs, and snus, in both cell lines (Table 1).
- HepG2 cells (liver cells) detect cytotoxicity at a lower concentration than BEAS-2B cells for all the test items. TFNPs appear less cytotoxic than Snus with HepG2, snus obtaining an EC₅₀ whereas TFNPs did not induce an EC₅₀

Table 2: Relative potency

	Relative potency (EC ₂₀ mg/mL) against 1R6F TPM	
	BEAS-2B	HepG2
TFNP #2 PBS	790	216
TFNP #3 PBS	597	230
Snus PBS	798	167



NRU - Comparison to nicotine concentration ($\mu\text{g}/\text{mL}$)



The cytotoxicity induced by increasing concentrations of TFNPs and snus extracts in the NRU assay was related to the nicotine cytotoxicity.

- The cytotoxicity data from 1R6F TPM, particularly with HepG2 cells, suggest that the tobacco-free products outperform SNUS in the harm reduction profile.
- The corresponding nicotine concentration for the top dose were: 57 (TFNP #2), 84 (TFNP #3), and 86 (Snus) μg nicotine/mL, these nicotine concentrations were similar to the nicotine levels found in the saliva of moist snuff users (73 μg nicotine/mL *).

* Hoffmann D, Adams JD. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* 1981;41(Pt 1):4305-4308.
Zanetti F, et al. Assessment of a 72-hour repeated exposure to Swedish snus extract ... *Food Chem Toxicol* 2019;125:252-270.



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Ames mutagenicity results negative for snus and TFNPs (TA98 and TA100 ± S9)

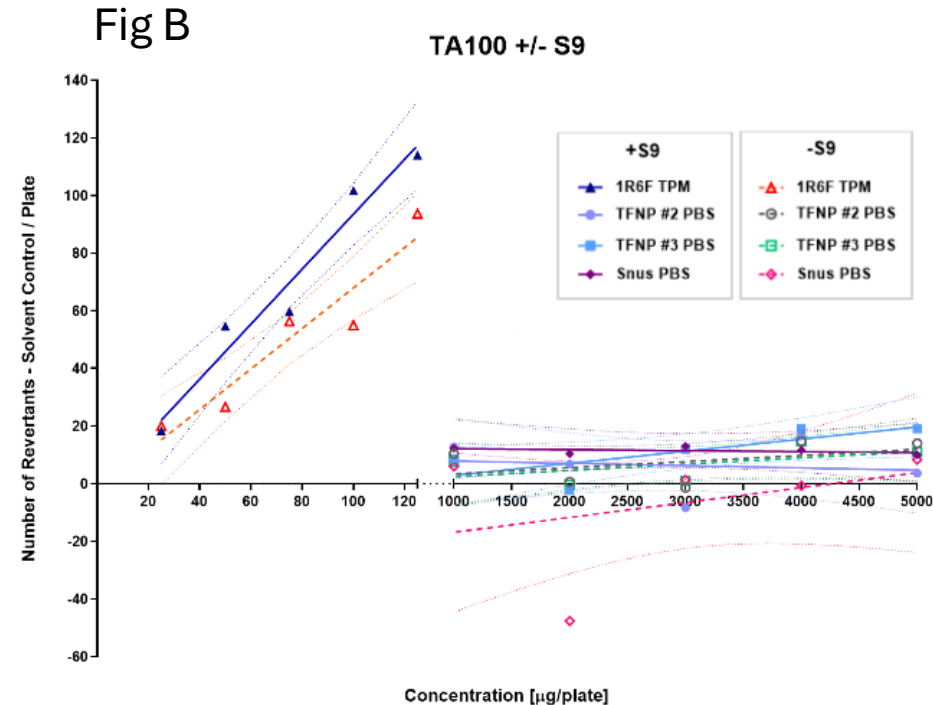
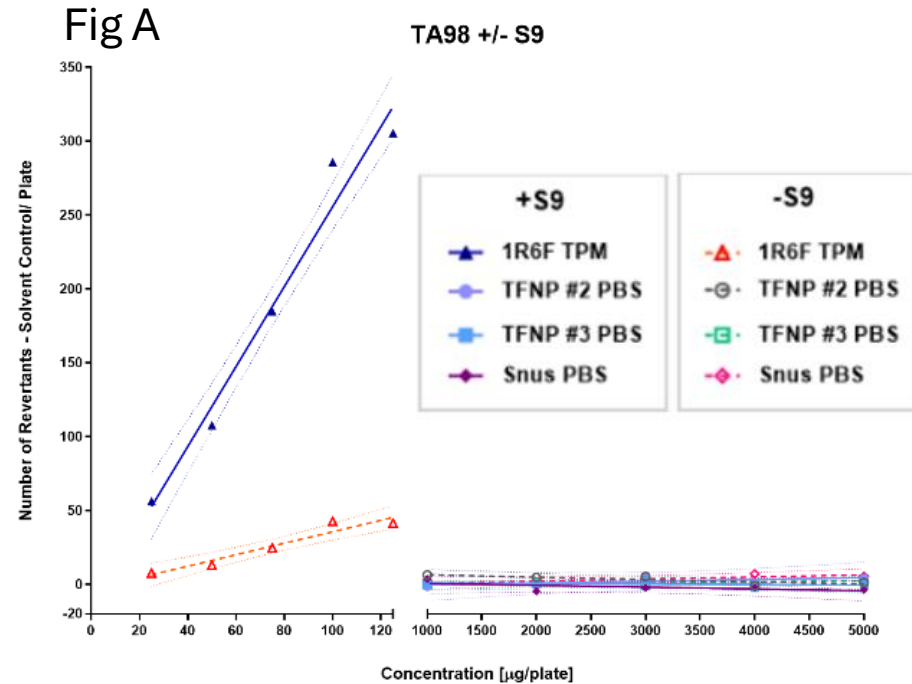
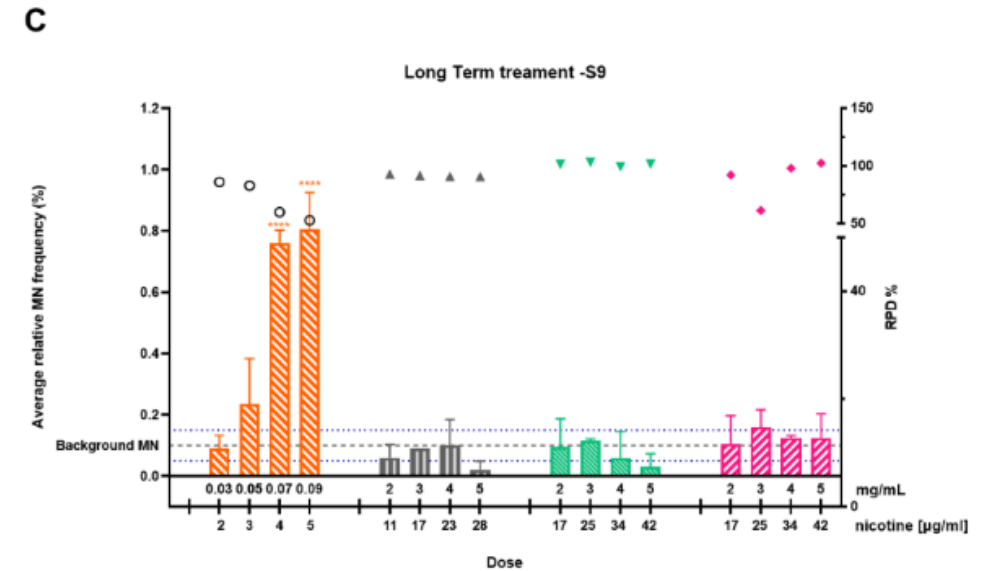
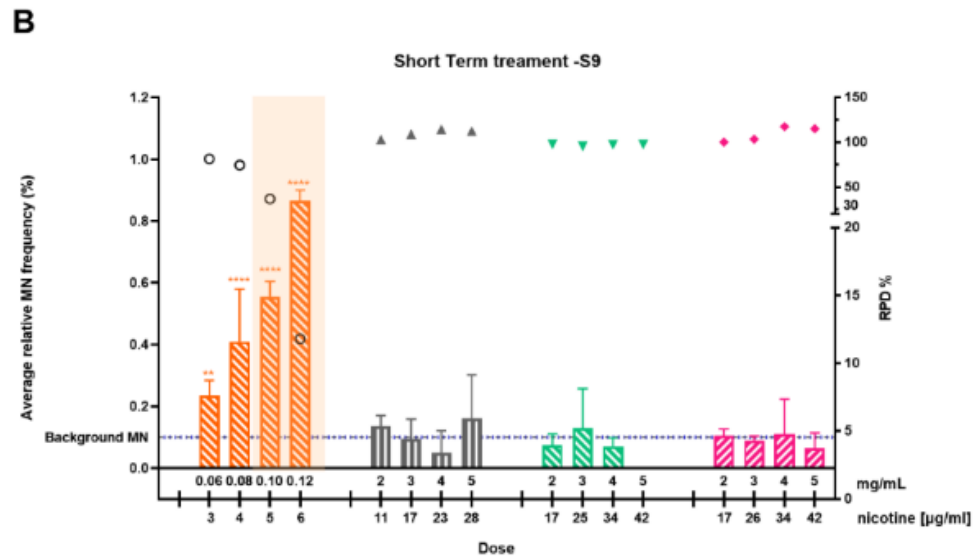
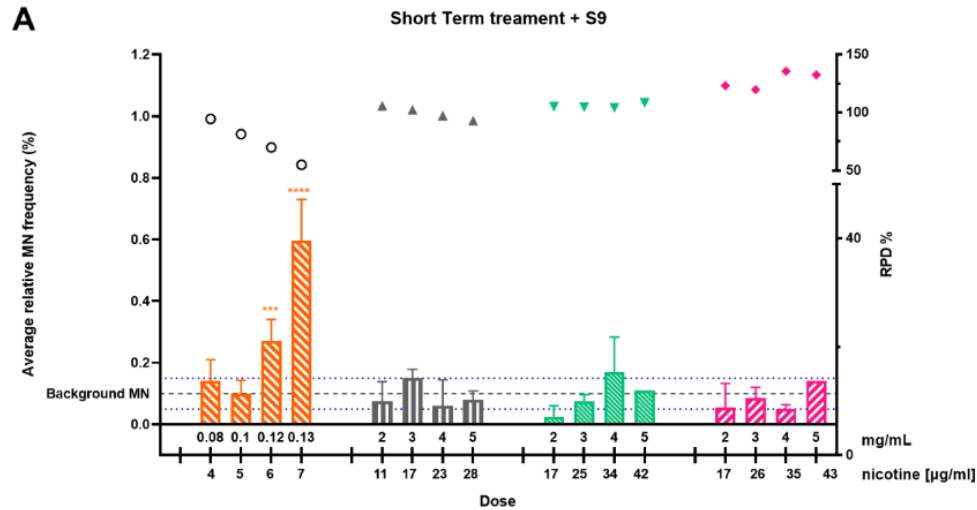


Figure A: TA98 (left) and Figure B TA100 (right) both +/-metabolic activation after exposure to 1R6F TPM, snus, and two TFNP extracts in the Ames assay. Dotted lines represent the 95% confidence intervals about the slope

No dose-dependent or statistically significant increases in revertant frequencies were observed for either TFNPs and snus extracts in any of the five *S. typhimurium* strains (TA98, TA100, TA102, TA1535, and TA1537) with or without S9 metabolic activation, when compared with the negative controls.

IVM – genotoxicity negative for Snus and TFNP



The diagrams shows the IVM assay for (A) short-term treatment +S9, (B) short-term treatment -S9 and (C) long-term treatment -S9, in V79 cells.

1R6F TPM had a strong genotoxic response in all treatment conditions. **No genotoxicity or cytotoxicity for TFNP or snus at highest dose**

IVM, in vitro micronucleus; RPD, relative population doubling; SD, standard deviation;
 $**p \leq 0.01$, $***p \leq 0.005$, $****p \leq 0.0001$

A comparison of 21 TFNP pouches in the CORESTA Battery (Study 2)



Twenty-one flavoured Tobacco free nicotine pouch extracts were tested in the CORESTA battery and compared to 1R6F

Methods were same as before (Study 1):

- **Pouch Extraction** (ISO 10993-12:2021):
TFNP pouches were extracted in PBS (300 mg/mL), shaken 1 hr at RT, filtered & frozen.
- **NRU Assay** (BEAS-2B & HepG2):
TFNP extracts (0.5–21 mg/mL) vs. 1R6F TPM (0.005–0.05 mg/mL); negative & positive controls.
- **Ames Assay** (OECD 471):
5 *Salmonella* strains ($\pm$ S9); TFNP extracts (1–5 mg/plate); controls included.
- **IVM Assay** (OECD 487):
V79 cells short-term $\pm$ S9 & long-term -S9; TFNP extracts (2–5 mg/mL), 1R6F TPM (0.03–0.14 mg/mL).
- **Statistical Analysis:**
Chi-Square & Cochran-Armitage trend test for significance

Table 3: Zone flavours tested

Test Article	Nicotine strength (mg nicotine/pouch)	Flavour direction
TFNP #1	4.08	Menthol
TFNP #2	6.03	Menthol
TFNP #3	7.22	Mint/Lime
TFNP #4	9.63	Red Berry/Vanilla
TFNP #5	9.63	Watermelon
TFNP #6	9.63	Orange
TFNP #7	9.63	Peach
TFNP #8	9.63	Liquorice
TFNP #9	10	Citrus
TFNP #10	11.89	Mint
TFNP #11	12	Citrus/Mint
TFNP #12	12.03	Menthol
TFNP #13	14	Berry/Vanilla
TFNP #14	14.45	Menthol
TFNP #15	14.54	Menthol
TFNP #16	15.05	Berry
TFNP #17	16	Menthol
TFNP #18	16	Sriracha/Lime
TFNP #19	17.65	Red Berry/Vanilla
TFNP #20	18	Menthol
TFNP #21	20	Menthol
1R6F	N/A	N/A

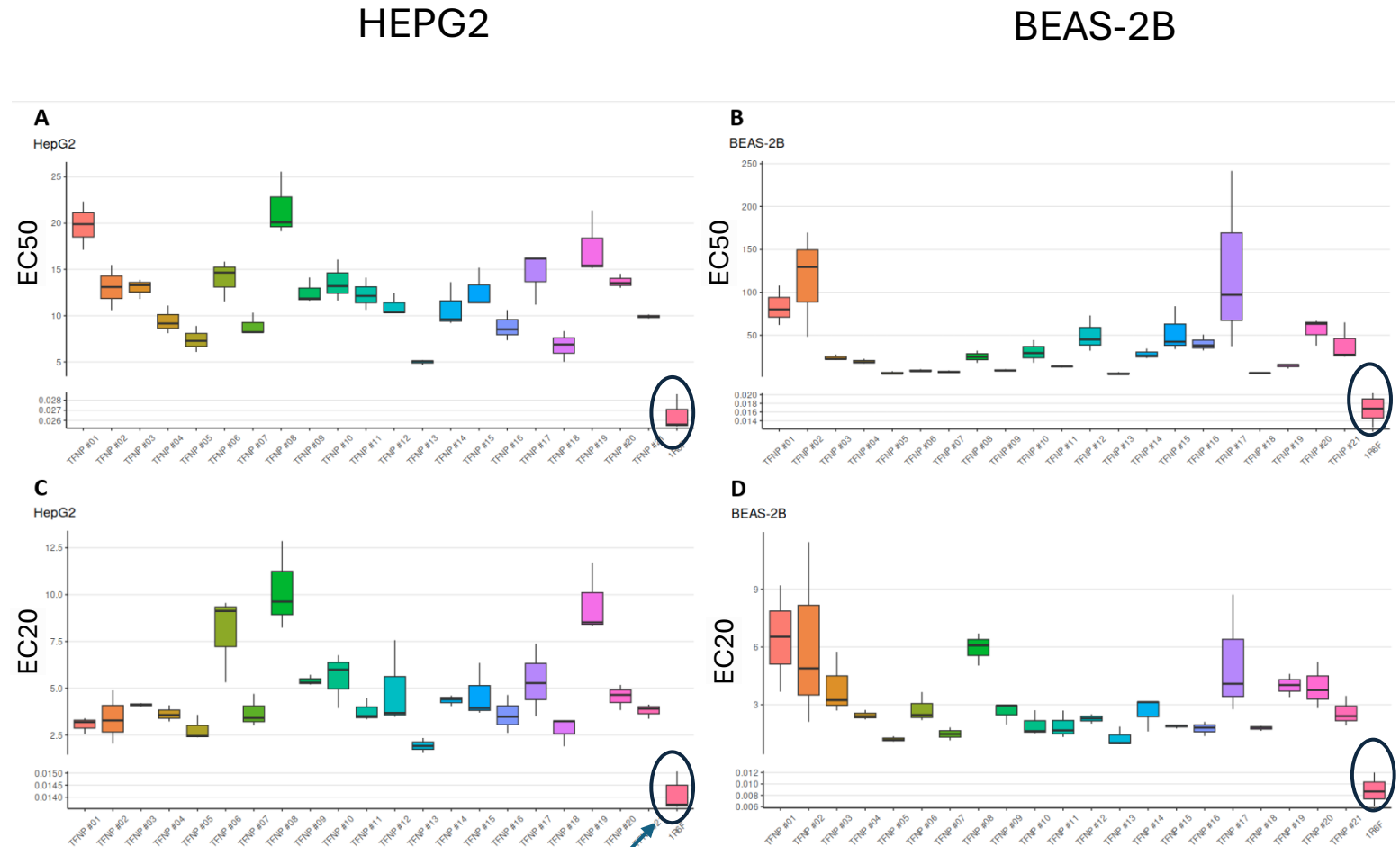


NRU results: No effect of flavours or nicotine content on cytotoxicity

12/21 TFNP extracts did not induce EC50 values (Figures A and B) in the **BEAS-2B** cell line while **8/21** TFNP extracts did not induce EC50 values in the **HepG2** cell line (Extrapolated EC50 values have been included).

- EC20 (Figures C and D) values obtained for the **TFNP extracts** ranged between **32 and 618 times less cytotoxic** than the **1R6F cigarette TPM** when calculated on a per nicotine basis.

- Variation in the EC20 values was observed between TFNP products, but no correlation was observed with nicotine content or flavour. This variance was less pronounced for BEAS-2B.



1R6F



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Negative AMES and IVM, no effects of nicotine or flavours for TFNPs

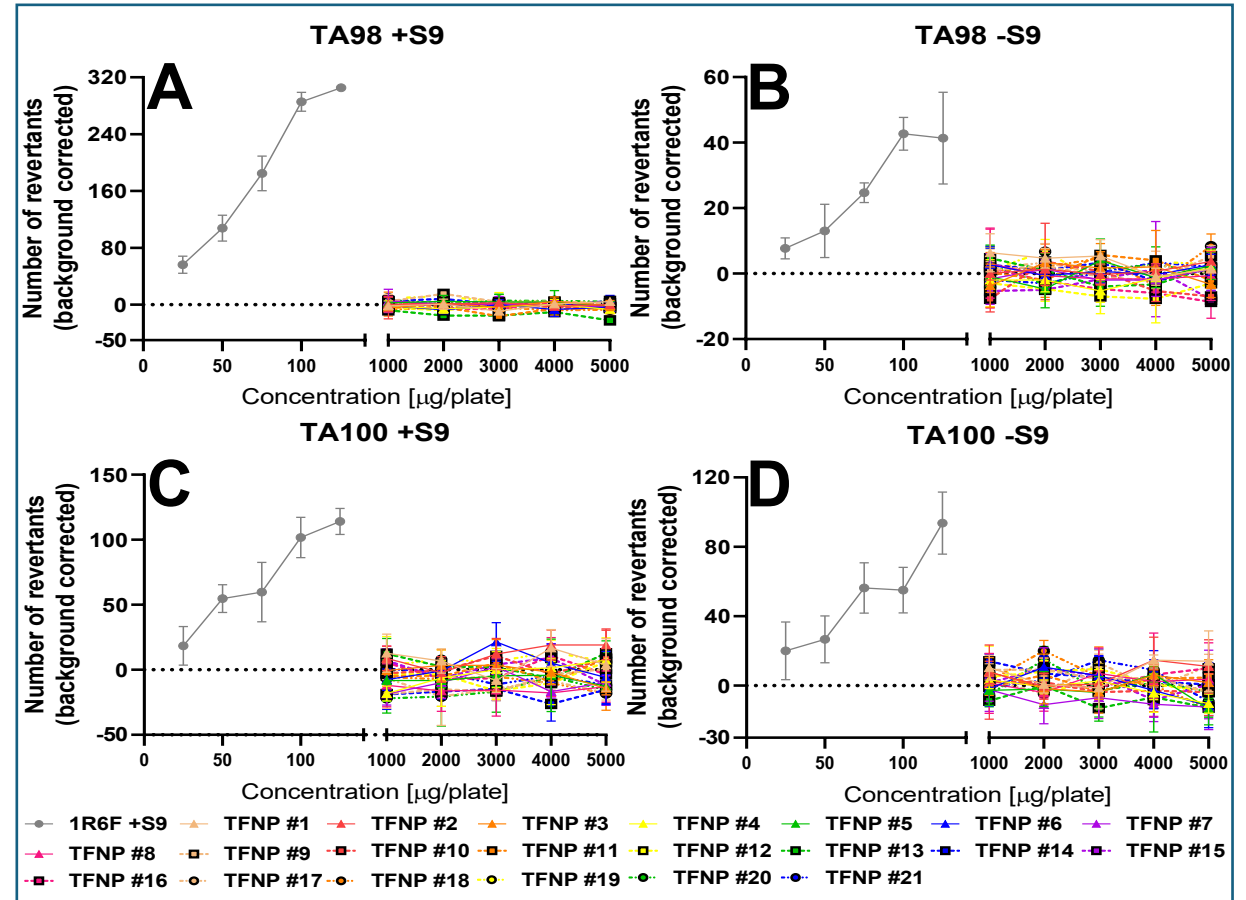
AMES

- **All TFNP extracts were negative in all of the 5 test strains.**
- 1R6F TPM caused a significant reproducible, dose-dependent increases in the number of revertants in TA98 (+/-S9), TA100 (+/-S9) and TA1537 (+S9).
- The 1R6F TPM was classified as mutagenic under the test conditions.

IVM results (Results not shown)

- **No TFNP extracts increased micronucleus frequencies** (n=3) when compared to the negative controls (short +/- S9; long -S9) not genotoxic in the IVM assay (maximum concentration of 5000 µg/ml).
- 1R6F was mutagenic in the assay

1R6F vs TFNP in TA98 and TA 100 +/- S9



HCS study with snus; TFNP and 1R6F in human coronary artery endothelial cells (HCAECs) $\pm$ NAC (Study 3)

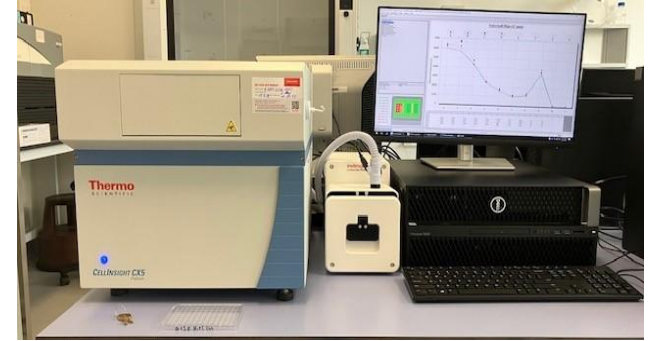
METHODS

Extract Preparation:

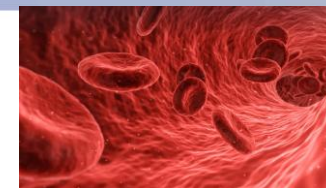
- Snus & TFNP extracts $\rightarrow$ EGM-MV2 medium; nicotine quantified; stored at -70°C
- **TPM**: 1R6F cigarettes $\rightarrow$ Cambridge pads; extracted with DMSO
- **SbMed**: (1R6F)Smoke bubbled through medium (1R6F only) ; nicotine + 8 carbonyls measured; stored at -70°C

Cell Culture & Exposure:

- Primary HCAECs (male 55, non smoker) were seeded in collagen-coated 96-well plates
- Exposed 4 or 24 h $\pm$ NAC (final concentration 1mM/well)
- Pure nicotine (20–1500 $\mu\text{g/mL}$) used to look for nicotine-specific effects (Data not shown)



HCS analysis of HCAECs exposed to TFNP; snus and 1R6F, No effects of NAC for snus and TFNP



HCAECs: Human coronary artery endothelial cells, lining blood vessels

Exposure: Snus, TFNP, and 1R6F extracts $\pm$ antioxidant NAC.

Results:

- **MECs:** Larger segments = lower MEC (higher potency); lower values. Expressed as $\mu\text{g/ml}$ of nicotine

- **p-c-JUN:** Orange segments; key signaling protein for growth, differentiation, cell death.

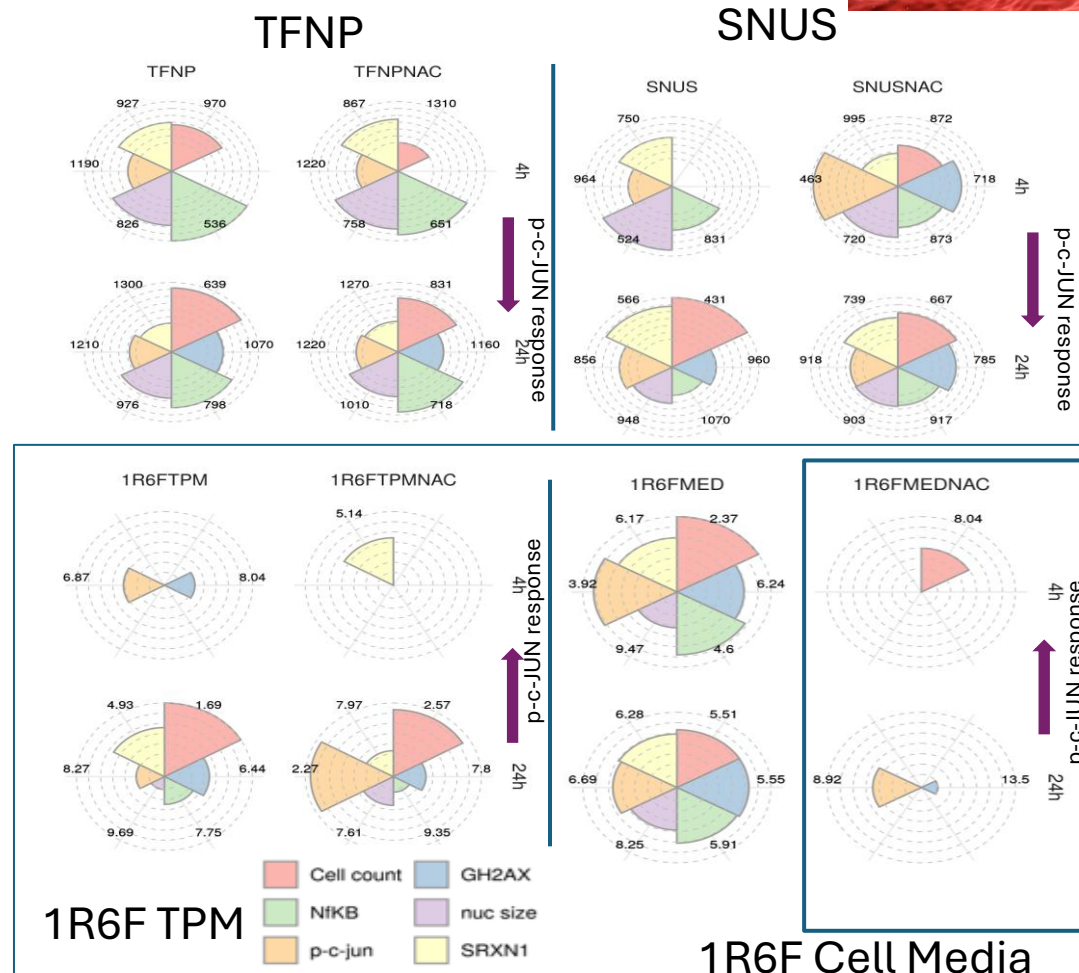
- **TFNP/Snus decreased** $\downarrow$ **p-c-JUN response** (4 and 24 hours)

- **Smoke/TPM increasing** $\uparrow$ **p-c-JUN response** (4 and 24 hours)

With NAC Effect:

- Minimal impact on Snus/TFNP

- **Strong mitigation of SbMed effects** $\rightarrow$ **oxidative stress is the major driver** (blue box)



Trelles Sticklen et al., (2025) High content screening of tobacco free nicotine pouch extracts shows marked reductions in toxicity when compared to cigarette smoke extracts CORESTA 2025; Smoke bubbled medium (SbMed); NAC N-acetyl cysteine; MEC Minimum effective concentration first concentration out of control values; TPM total particulate material;
<https://github.com/philipmorrisintl/GladiatoX?tab=readme-ov-file>



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Studies 4 and 5, show comparable *in vitro* evidence for the reduced harm potential of TFNPS vs snus from other authors



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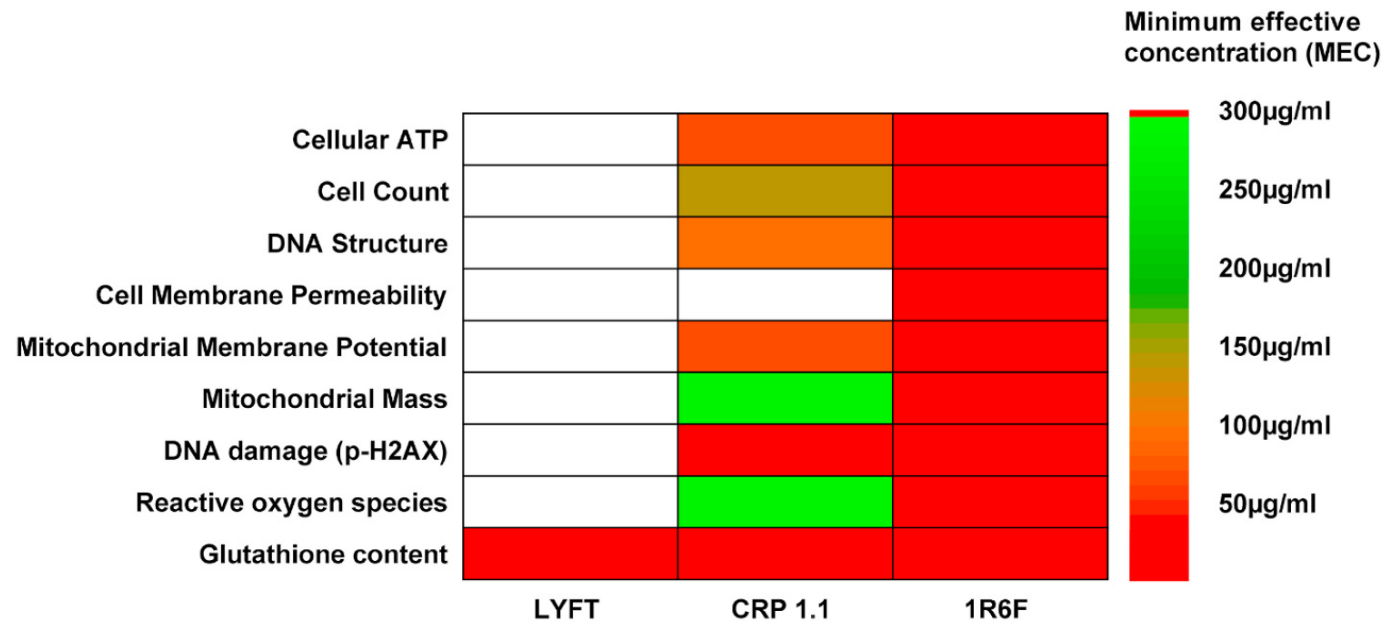
SCIENCE

Study 4: Bishop et al., (2020) reported minimal effects of TFNP vs Snus and Combustibles when using HCS

Bishop et al, (2020) used HCS to compare Lyft®(TFNP) vs CRP 1.1. (snus, CORESTA reference product) and combustibles extracts to H292 cells.

For the HCS endpoints in the heatmap below the TFNP only exceeded the MEC (1.5x control value) for GSH, with Snus affecting multiple cellular organelle markers.

From Bishop et al .(2020) A Heatmap for HCS data illustrating the minimum effective concentration (MEC), or lowest concentration that induced a 1.5-fold increase above the media control and presented as nicotine concentration for each product type in this study. The white squares indicate none of the concentrations tested exceeded the MEC value.



Study 5: Yu et al., (2024) reported snus extract exposure (but not TFNP) lead to increased cell stress responses and cytokine induction.

- Yu et al.,(2024) reported the similar results with the CORESTA battery, essentially no difference in response between snus (CRP1.1) and TFNP extracts (LYFT) in NRU, AMES and MLA assay.
- 30 mins of exposure to snus (CRP1.1),extract , increased the activity of c-JUN, p38 MAPK, p53 (involved in the cellular stress pathways). **TFNP extracts there was no significant increase in phosphorylation of the panel of proteins (not shown).**
- Snus, CRP1.1 extracts also induced the production of inflammatory mediators such as IL-1 α , IL5, IL6, IL8 after 48hr exposure time. **For the TFNP extracts there was no secretion of proinflammatory cytokines (see below)**
- These inflammatory markers may also be correlated with oral lesions seen following snus use but not generally reported for TFNP usage (Alizadehgharib S et al. (2022)



(Taken from Yu et al., 2024, a heatmap showing an increase in cytokines seen after 48 hours of continuous exposure for cells exposed Snus (CRP1.1) extract vs 4 TFNPs (extracts 3x LYFT and NDSP).



Alizadehgharib, S., et.al., 2022. The effect of a non-tobacco-based nicotine pouch on mucosal lesions caused by Swedish smokeless tobacco (snus). *European Journal of Oral Sciences*, 130(4), p.e12885.

Supporting additional literature of reduced activity of TFNPs vs snus reported by other authors

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RESEARCH ARTICLE

OPEN ACCESS

Chemical characterization of tobacco-free “modern” oral nicotine pouches and their position on the toxicant and risk continuums

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ABSTRACT

As compared with cigarette smoking, use of Swedish snus is associated with significantly fewer health risks. Nicotine pouches (NPs), a new form of oral nicotine product, are smokeless and tobacco-free, containing a nicotine-containing cellulose matrix inside a fiber pouch. NPs are similar in appearance to

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In vitro evaluation of mutagenic, cytotoxic, genotoxic and oral irritation potential of nicotine pouch products

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A screening approach for the evaluation of tobacco-free ‘modern oral’ nicotine products using Real Time Cell Analysis

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ABSTRACT

In many regulated industries there is an increasing pressure to provide timely and robust risk assessment data to support product launches. Real-time cell analysis (RTCA) is a tool that allows for the fast and relatively labour-free cytotoxic assessment of test compounds, compared to traditional methods. Here, we propose an application for the RTCA platform to provide a screening approach, to evaluate the cytotoxic potential of tobacco-free nicotine pouches, also termed modern oral product (MOP), to determine the contribution of differing nicotine



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Mutation Research - Genetic Toxicology and Environmental Mutagenesis

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Multi-endpoint *in vitro* toxicological assessment of snus and tobacco-free nicotine pouch extracts

Fan Yu^a, Emma Bishop^a, Fabio Miazzi^a, Rhian Evans^a, David Smart^a, Damien Breheny^a, David Thorne^a

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ORIGINAL ARTICLE

The effect of a non-tobacco-based nicotine pouch on mucosal lesions caused by Swedish smokeless tobacco (snus)

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Abstract

Oral mucosal lesions are commonly found in Swedish smokeless tobacco (snus) users



Food and Chemical Toxicology 145 (2020) 111713

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An approach for the extract generation and toxicological assessment of tobacco-free ‘modern’ oral nicotine pouches

Bishop E, East N, Bozhilova S, Santopietro S, Smart D, Taylor M, Meredith S, Baxter A, Breheny D, Thorne D^a, Gaca M

British American Tobacco, R&D, Southampton, Hampshire, SO15 8TL, UK

Cureus

Open Access Review Article

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Tobacco-Free Nicotine Pouches and Their Potential Contribution to Tobacco Harm Reduction: A Scoping Review

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Abstract



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Conclusions

- Snus is considered to be the first harm reduction product when compared to combusted tobacco; the removal of the tobacco and replacement with plant fibers with pharmaceutical grade nicotine in TFNPs leads to a greater harm reduction potential.
- A reduction in GothiaTek analytes is clearly seen when comparing snus and TFNPs, this reduction in toxicants translates into further reductions in, *in vitro* toxicity
- TFNP extracts demonstrated no response in the *in vitro* genotoxicity assays in IVM and mutagenicity Ames tests under the assessed conditions.
- The cytotoxicity data from TFNPs, particularly with HepG2 cells, suggest that the tobacco-free products could outperform snus in the potential harm reduction profile
- Further mechanistic studies add to the weight of evidence of the reduced activity of TFNPs when compared to traditional snus in terms of biological responses and this may support the general reported finding of reduced formation of oral lesions in TFNP users
- The results presented add to the growing body of scientific evidence supporting a positive role for TFNPs in THR strategies, beyond that of snus; however, further chemical characterisation, preclinical, clinical, and perception and behavior studies are required for a fuller scientific substantiation



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