SYSTEMS TOXICOLOGY ASSESSMENT OF A 72-HOUR REPEATED EXPOSURE TO SWEDISH SNUS EXTRACT AND TOTAL PARTICULATE MATTER FROM 3R4F CIGARETTE SMOKE ON GINGIVAL ORGANOTYPIC CULTURES

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Introduction and Objectives

Introduction. Swedish snus is a smokeless tobacco product that contains reduced levels of harmful compounds compared with cigarette smoke. In Sweden, where snus use exceeds smoking among men, relatively low rates of major smoking-related diseases have been recorded. To understand how snus use could support current tobacco harm reduction strategies, the mechanisms of toxicity must be investigated. Currently published studies lack a clear consensus on the effects of snus use on oral health, mainly due to confounding factors in epidemiological data **[1-2]**. *In vitro* studies, performed by administering snus extract to cell cultures, have demonstrated weak adverse effects of snus at clinically relevant, and even higher, concentrations **[3-5]**.

Objective. This study aimed to determine, using a systems toxicology approach, the biological impact of a repeated exposure of human gingival epithelial organotypic cultures to extracts from both a commercial and a reference snus and that of exposure to total particulate matter (TPM) from cigarette smoke over a period of 72 hours.

Methods

Treatments. CORESTA Reference Product (**CRP1.1**; Tobacco Analytical Services Laboratory, North Carolina State University, Raleigh, NC, USA) and General Classic White (**GCW**; Swedish Match, Stocholm, Sweden) snus were extracted (1 h) and diluted in phosphate-buffered saline (PBS). **TPM** was generated from 3R4F cigarettes (University of Kentucky, Lexington, KY, USA), extracted in 100% ethanol (ETOH) with the standard Cambridge filter method, and diluted in PBS + 2% ethanol.

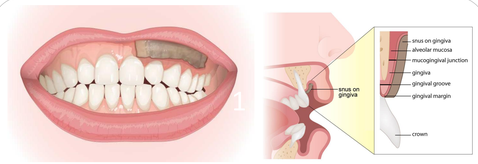
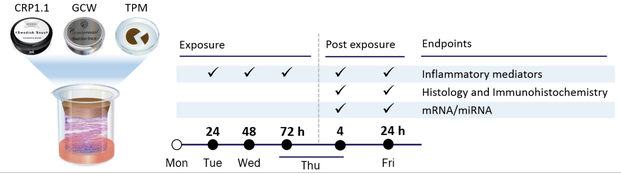


Illustration of localization of the pouch in the mouth of snus users.

Human gingival organotypic epithelial cultures (EpiGingival™). The cultures were derived from a human 46-year-old male donor, non-smoker (MatTek corp., Ashland, MA, USA).

mg nicotine/L PBS			
SHAM	0 (PBS)	0 (PBS)	0 (PBS-ETOH)
LOW	14.8	13.5	12.7
MEDIUM	59.1	67.6	50.7
HIGH	738.6*	811.0*	-
	CRP1.1	GCW	TPM



Study design. Three independent preparations of CRP1.1, GCW, and TPM were done in the week before exposure. In the week of exposure, the organotypic cultures were covered with 100 µL of PBS on the apical side ± snus/TPM. After the 72-hour exposure, the cultures were covered by PBS without snus extracts or TPM for 4 and 24 hours (post exposure). * Nicotine concentrations relevant for user's exposure **[9]**.

Inflammatory mediator analysis. The concentrations of released inflammatory mediators were measured in the basolateral medium using a Luminex®-based technology (Luminex, Austin, TX, USA).

Histology and immunohistochemistry. Morphology of the cultures was evaluated in hematoxylin & eosin-stained tissue sections.

mRNA/miRNA data processing and analysis. Transcriptomics data were analyzed in the context of hierarchically structured network models describing the molecular mechanisms underlying essential biological processes **[6]**. The effects of exposure were quantified in terms of "network perturbation amplitudes" (NPA) **[7]**. The NPA values were assigned to the various networks, which were categorized into four categories: cell proliferation (CPR), cellular stress (CST), cell fate (CFA), and inflammatory process network (IPN). An aggregation of the NPA values is termed the "Biological Impact Factor" (BIF), which provides a high-level quantification of the mechanistic impact of the exposure **[8]**. Differentially expressed miRNAs were determined using standard approaches (adjusted *p*-values < 0.05), and their potential target genes were identified using an integrative approach based on a public miRNA-mRNA interaction knowledgebase [http://multimir.ucdenver.edu/].

Results

	CRP1.1	GCW	TPM
Nicotine	1477.11 ± 26.75	1351.73 ± 63.26	2533.39 ± 130.48
NNN	29.28 ± 0.36	40.26 ± 0.50	459.67 ± 10.24
NNK	7.88 ± 0.08	11.99 ± 0.11	343.52 ± 7.12
NAT	24.09 ± 0.28	27.61 ± 0.21	389.80 ± 7.01
NAB	0.99 ± 0.03	1.45 ± 0.06	33.16 ± 0.48

Table 1. Summary of chemical analyses for nicotine and tobacco-specific nitrosamines (TSNA) in the tobacco product preparations (100% concentrated). Nicotine concentrations were measured using liquid chromatography (LC) high-resolution accurate-mass mass spectrometry (MS) (MicroTOF QII, Bruker Daltonik GmbH, Bremen, Germany). TSNAs were analyzed using LC/MS on an Ultimate 3000 ultra-high performance LC system coupled to a Q-Exactive MS (Thermo Fisher Scientific, Santa Clara, CA, USA). Nicotine is expressed in mg/L, TSNAs, ng/L ± SEM. NNN: N'-nitrosonornicotine; NAT: N'-nitrosoanatabine; NAB: N'-nitrosoanabasine; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; N=9.

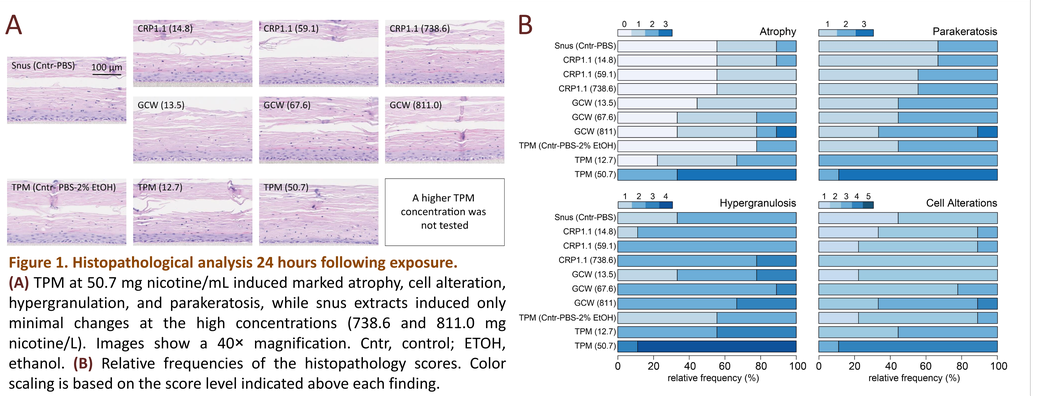


Figure 1. Histopathological analysis 24 hours following exposure.
(A) TPM at 50.7 mg nicotine/mL induced marked atrophy, cell alteration, hypergranulation, and parakeratosis, while snus extracts induced only minimal changes at the high concentrations (738.6 and 811.0 mg nicotine/L). Images show a 40× magnification. Cntr, control; ETOH, ethanol. **(B)** Relative frequencies of the histopathology scores. Color scaling is based on the score level indicated above each finding.

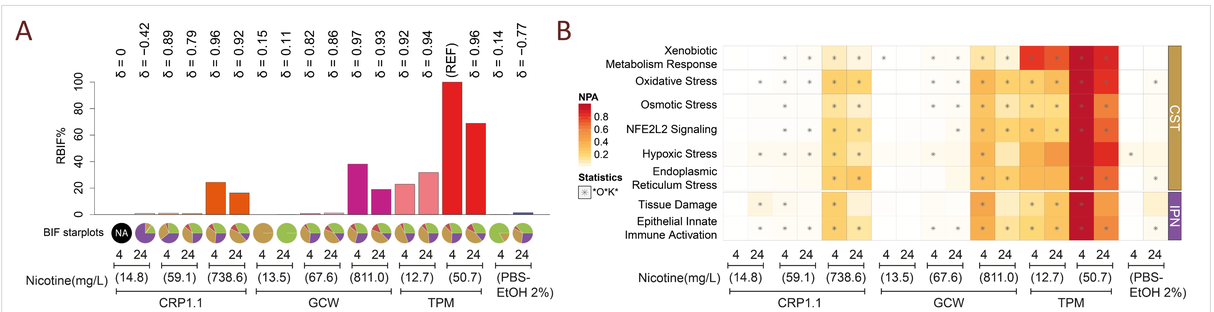


Figure 2. Analysis of the BIF and NPA of selected networks.
The BIF score **(A)** was higher for TPM-exposed cultures than for the snus-exposed counterparts; NA, not applicable; starplot color legend: Red, CPR; Green, CFA; Violet, IPN; yellow, CST. NPA scores of selected networks **(B)** evidenced similar effects between high snus extract concentrations and TPM low concentration (12.7 mg/L), except for the *Xenobiotic Metabolism Response* network.

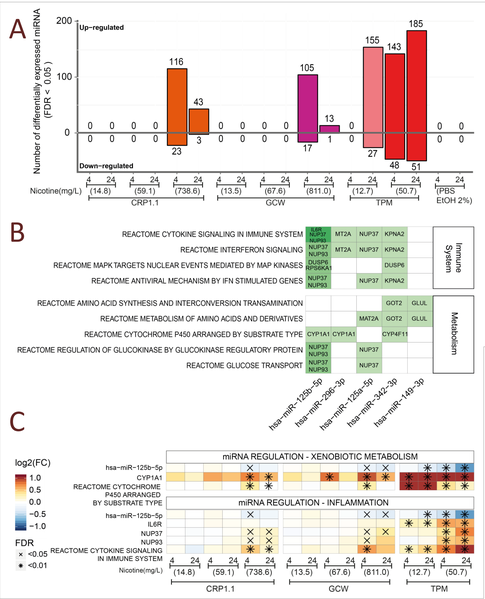


Figure 3. Results of the inference of relevant miRNAs with biological functions.
TPM exposure generally induced a higher impact on miRNA expression than snus extracts **(A)**. An integrated computational analysis pipeline revealed 10 high-confidence miRNAs that were likely downregulated up to 143 target mRNAs corresponding to 57 significantly enriched Reactome pathways. Five of these miRNAs are displayed, regulating target mRNAs included in networks belonging to *Immune System* and *Metabolism* **(B)**. miR-125b-5p was inferred to be involved in regulation of the inflammatory response and cytochrome P450 signaling pathways **(C)**. FC, fold change; FDR, false discovery rate.

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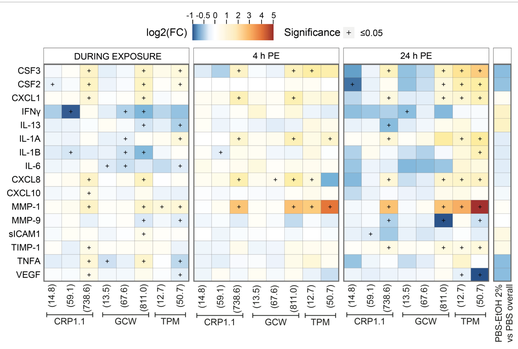

Figure 4. Inflammatory mediator analysis.
At the low and medium concentrations, snus extracts exerted a limited effect on the secretion of inflammatory mediators. The high concentrations instead elicited a more marked effect. In general, the impact of snus extracts on the secretion of inflammatory mediators tended to decrease or remain stable with the duration PE, whereas the effects of TPM further increased over time.

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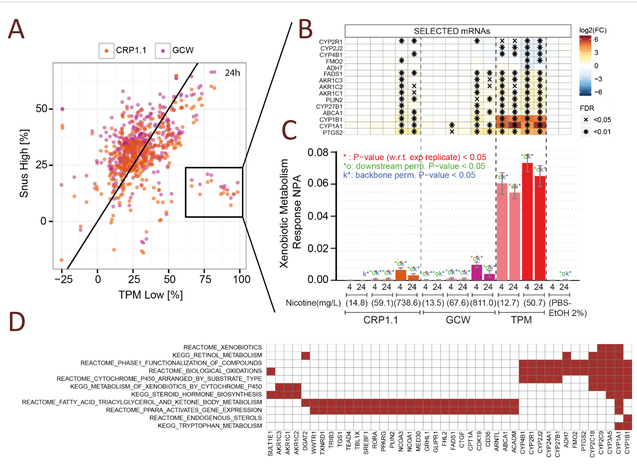

Figure 5. Differential regulation of mRNA involved in xenobiotic metabolism.
Comparing the 24 hours post-exposure impact of the high snus extracts concentrations with that of the low TPM for a large gene set collection **[10] (A)**, we identified a subset of genes showing a different response following snus or TPM exposure **(B)**, dominantly associated with *Xenobiotic Metabolism Response* **(C and D)**.

Conclusions

- At concentrations relevant for snus use in humans, snus extracts minimally damaged the gingival cultures.
- Swedish snus extract induced an inflammatory response in gingival cells that tended to revert upon exposure cessation, while the inflammatory response induced by TPM exacerbated with time.
- Swedish snus extract contain a lower concentration of TSNAs comparted with the TPM from cigarettes at a similar nicotine concentration, suggesting lower chemical exposure of the oral tissues.
- The minimal activation of the drug metabolism molecular pathways in gingival cells exposed to Swedish snus extracts further suggests the low exposure to chemicals.
- A consistently lower number of miRNAs was altered in gingival cultures by snus extract exposure compared with exposure to TPM. Mir-125b-5p might be related to pathological conditions *in vivo*.