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Chronic Toxicity and Lung Tumorigenesis in A/J Mice Following Lifetime Exposure to Aerosol from the Tobacco Heating System 2.2 in Comparison with Exposure to 3R4F Reference Cigarette Smoke

APTox Porto
7 February 2019

The research described in this presentation was sponsored by Philip Morris International.

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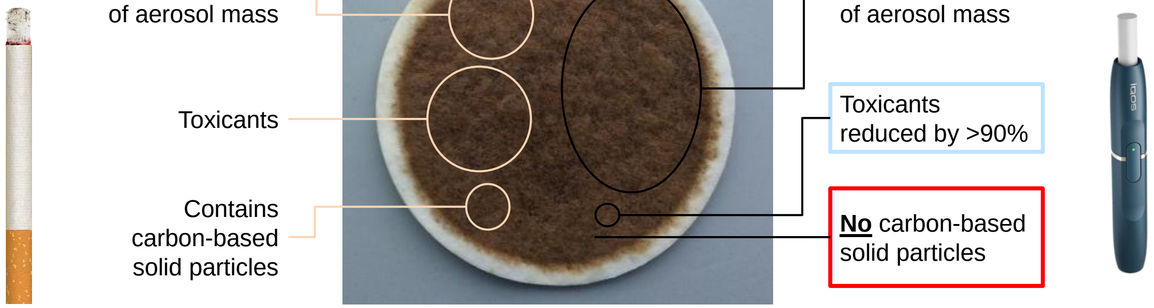
INTRODUCTION

Study Objective

To assess the impact of lifetime exposure of A/J mice to Tobacco Heating System (THS) 2.2 aerosol, compared with that of 3R4F reference cigarette smoke, on:

- Systemic toxicity
- Development of lung inflammation and emphysema
- Lung tumor incidence and multiplicity

Cigarette Smoke vs. Heat-not-Burn Aerosol



Smoke and aerosol were collected on a Cambridge filter pad using the Health Canada Intense smoking regime

- Approximately 6,000 chemical constituents have been identified in cigarette smoke
- Some of these constituents are categorized as harmful and potentially harmful (HPHC), and many of the HPHCs are formed during combustion (burning) of the tobacco

- Lower temperatures reduce constituents in the aerosol
- Nicotine is transferred via distillation

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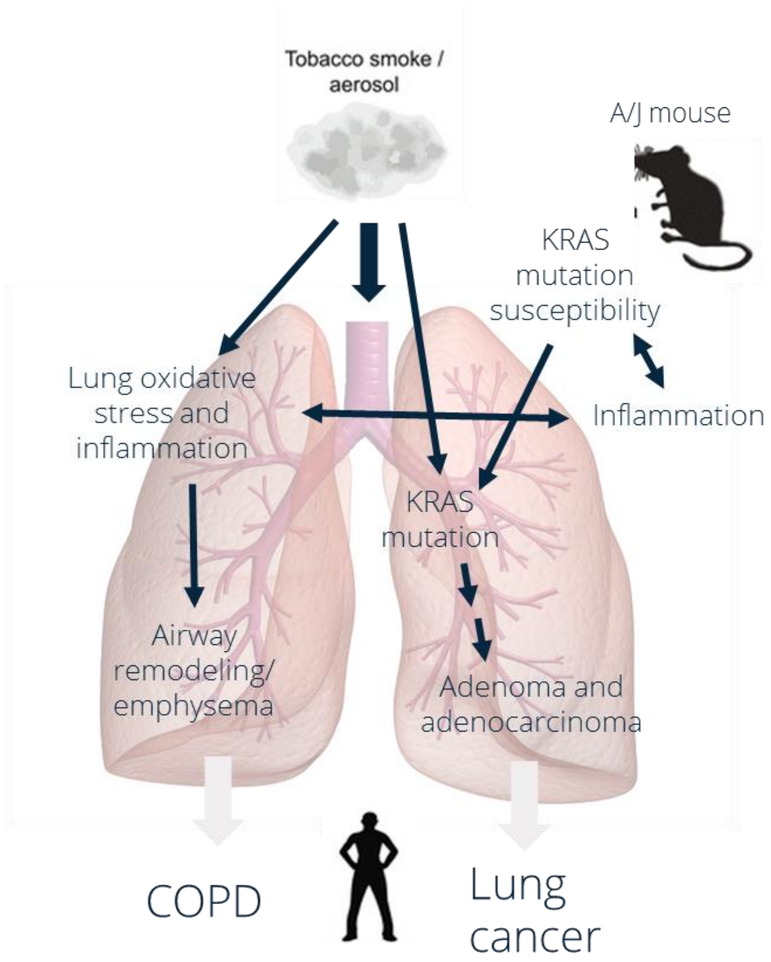
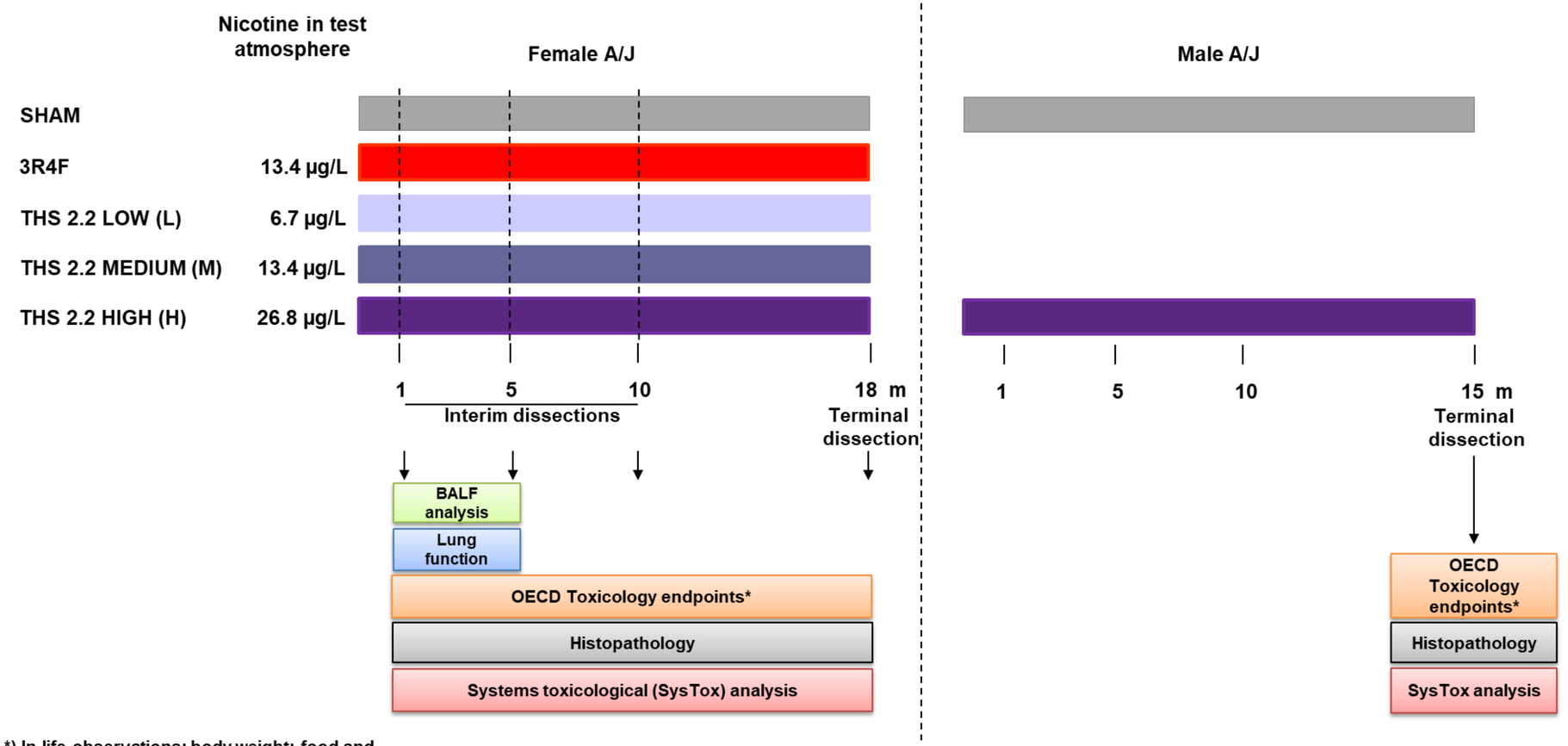
Why A/J Mice?



- Highly susceptible to lung tumor induction (Coggins, 1998)
- Develops a mainstream cigarette smoke concentration-dependent lung tumor response after an inhalation period of 15 to 18 months (Stinn et al., 2005; Stinn et al., 2010)
- Lung tumor susceptibility in A/J mice related to *Kras* mutation or increased transcription, similar to what is seen in in some smokers' lung cancer
- No other suitable model for cigarette smoke-induced lung tumorigenesis (Coggins, 2010)

Coggins CR (1998) Toxicol Pathol. 1998 May-Jun;26(3):307-14; discussion 315. doi: 10.1177/019262339802600301; Stinn et al. (2005) Inhal Toxicol. 2005 May;17(6):263-76. doi: 10.1080/08958370590922544; Stinn et al. (2010) Toxicology. 2010 Sep 10;275(1-3):10-20. doi: 10.1016/j.tox.2010.05.005; Stinn et al. (2013a) Toxicology. 2013, 305:49-64. doi: 10.1016/j.tox.2013.01.005; Stinn et al. (2013b) Toxicol Sci. 2013 Feb;131(2):596-611. doi: 10.1093/toxsci/kfs312

Study Design



*) In-life observations; body weight; food and water consumption; biomarkers of exposure; clinical chemistry; hematology; urinalysis etc.

Based on Stinn et al. (2013), there is no difference in cigarette smoke-induced lung tumor incidence and multiplicity in male and female A/J mice; female mice take up more smoke and are more sensitive to toxicity.

26.8 µg/L nicotine concentration in THS aerosol represents 56 Sticks/day (*FDA, 2005. Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. Food and Drug Administration, Washington, DC. http://www.fda.gov/cder/guidance.), Stinn et al. (2013) Toxicology. 2013, 305:49-64. doi: 10.1016/j.tox.2013.01.005

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Study Endpoints (1)

Parameter	OECD Toxicology	Lung inflammation, emphysema	Carcinogenicity
Body weight	√		
In-life observation	√		
Hematology	√		
Blood - clinical chemistry	√		
Organ weights	√		
Urinalysis	√		
Blood FACS	√		
Lung inflammation		√	
Lung function		√	
Lung morphometry		√	
Histopathology, respiratory tract	√	√	√
Histopathology, nonrespiratory organs	√		√

Study Endpoints (2)

Tissue	Methylome	Mutation profile	Transcriptome	Proteome
Lung parenchyma		√	√	√*
Tumor nodules		√	√	
Nasal epithelium			√	√
Larynx			√	
Blood	√		√	√
Liver			√	

* Month 1 only

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RESULTS

I. OECD Toxicology

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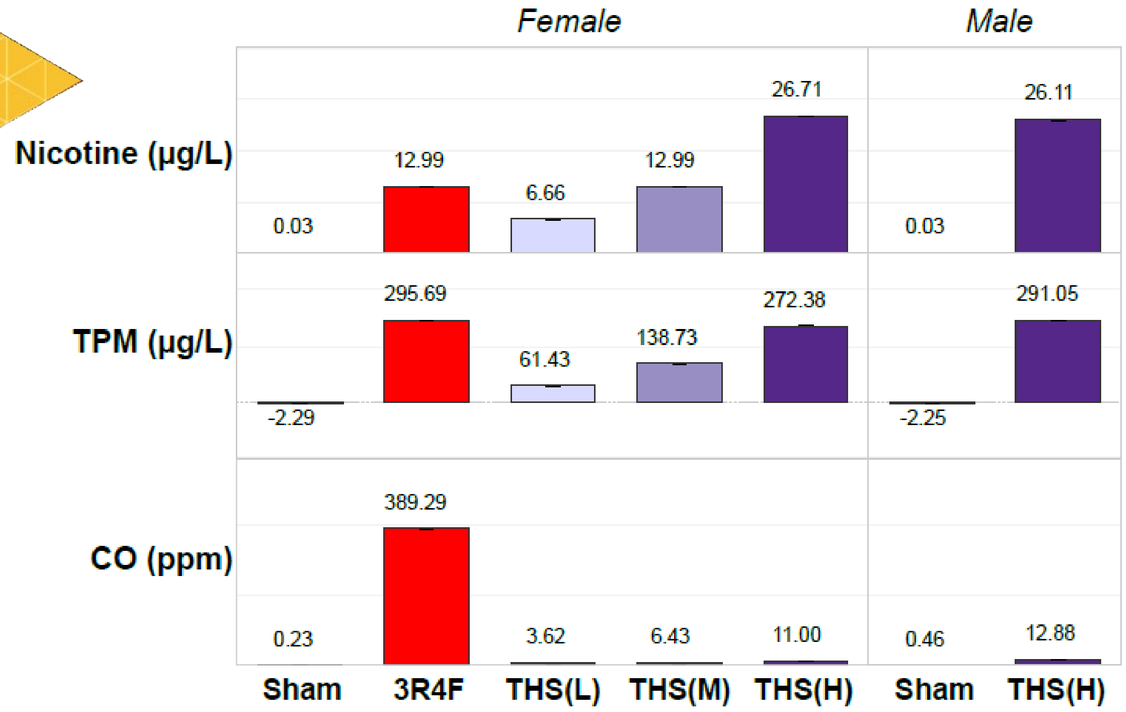
Study Endpoints

Parameter	OECD Toxicology	Lung inflammation, emphysema	Carcinogenicity
Body weight	√		
In-life Observation	√		
Hematology	√		
Blood - clinical chemistry	√		
Organ weights	√		
Urinalysis	√		
Blood FACS	√		
Lung inflammation		√	
Lung function		√	
Lung morphometry		√	
Histopathology, respiratory tract	√	√	√
Histopathology, nonrespiratory organs	√		√

Test Atmosphere Characterization



Average study data from between 332 and 397 daily means except for Sham (fresh air)

- Aerosol delivery was highly reproducible
- Nicotine levels in the test atmosphere were within +/- 10% of target concentrations throughout the study
- CO and carbonyl levels (not shown) in the test atmosphere reflected chemical composition of the two aerosols

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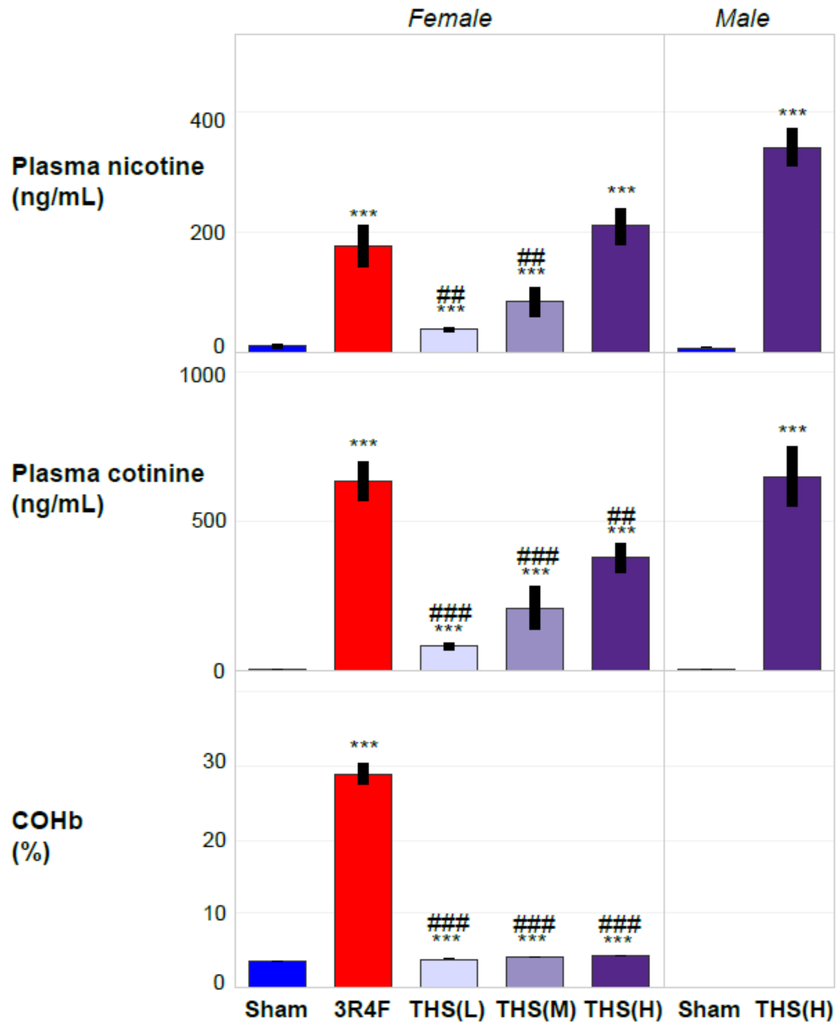
Test Atmosphere Uptake

1. Biomarkers of Exposure in Blood



- Plasma nicotine and cotinine levels confirmed aerosol uptake commensurate with aerosol nicotine concentrations
- Carboxyhemoglobin (COHb) levels also reflected the exposure group

Data from Months 12 (male mice; no COHb at this time point; N=8-10) and 16 (female mice; N=9-10) are presented as mean ±SEM; ***: $p<0.001$ versus Sham (fresh air); ##: $p<0.01$; ###: $p<0.001$ versus 3R4F

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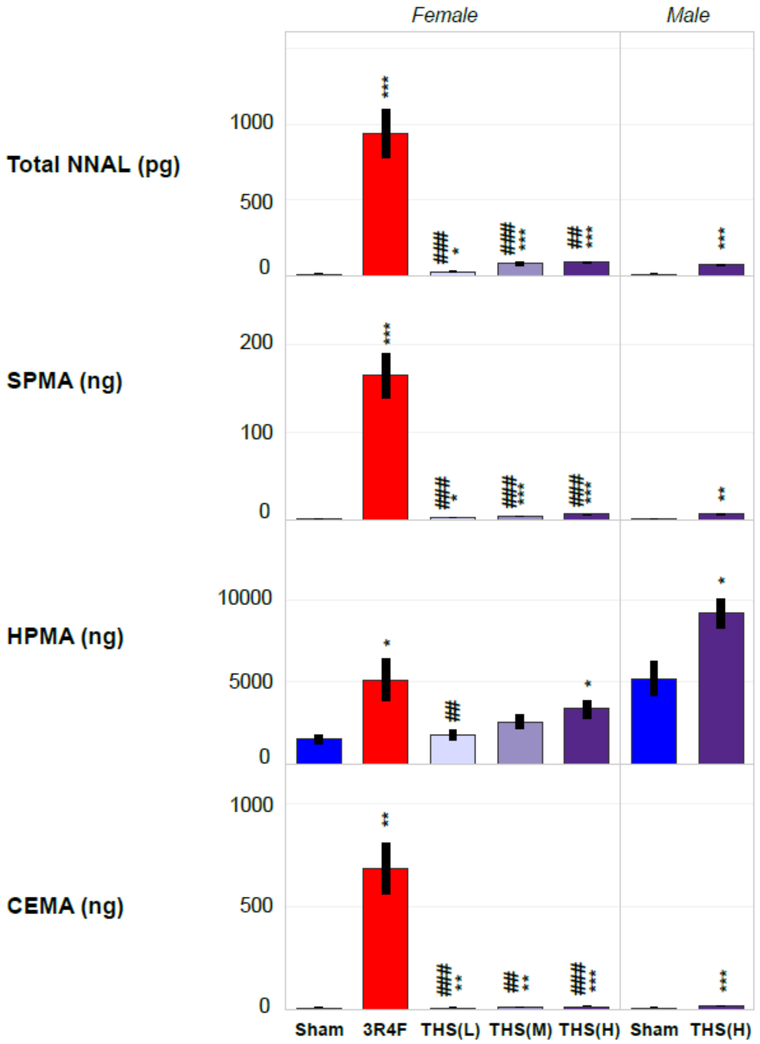
Test Atmosphere Uptake

2. Biomarkers of Exposure in Urine



Data from Months 14 (male mice; N=7-11) and 16 (female mice; N=8-14); *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ versus Sham (fresh air); ##: $p<0.01$; ###: $p<0.001$ versus 3R4F

- Total NNAL, SPMA, HPMA, and CEMA were recovered at a higher quantity in the urine of mice exposed to 3R4F cigarette smoke than to those receiving the THS 2.2 aerosol
- This is consistent with the exposure groups and the chemical composition of the aerosol

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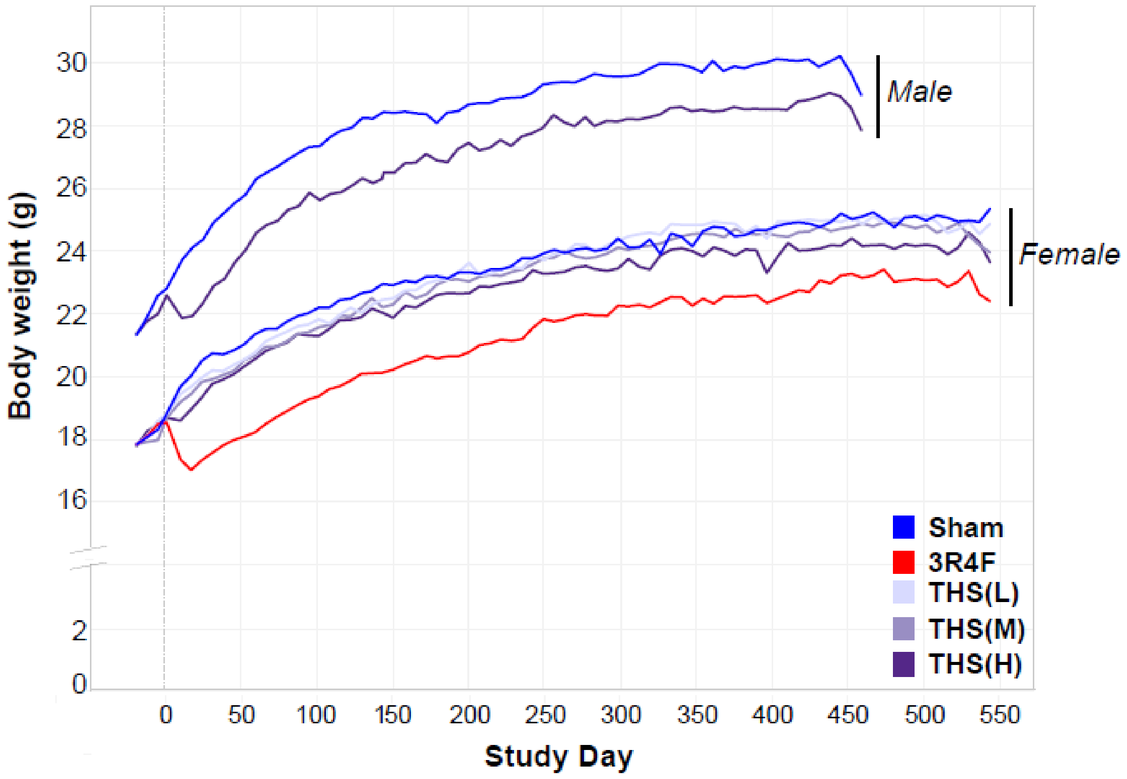
Systemic Toxicity

1. Body Weight



The body weight progression over time for female and male exposure groups (average body weight per exposure group per time point) is presented. SEM were removed for clarity.

- After an initial drop in body weight during the first 2–3 weeks of the exposure adaptation phase, all animals gained weight progressively throughout the study

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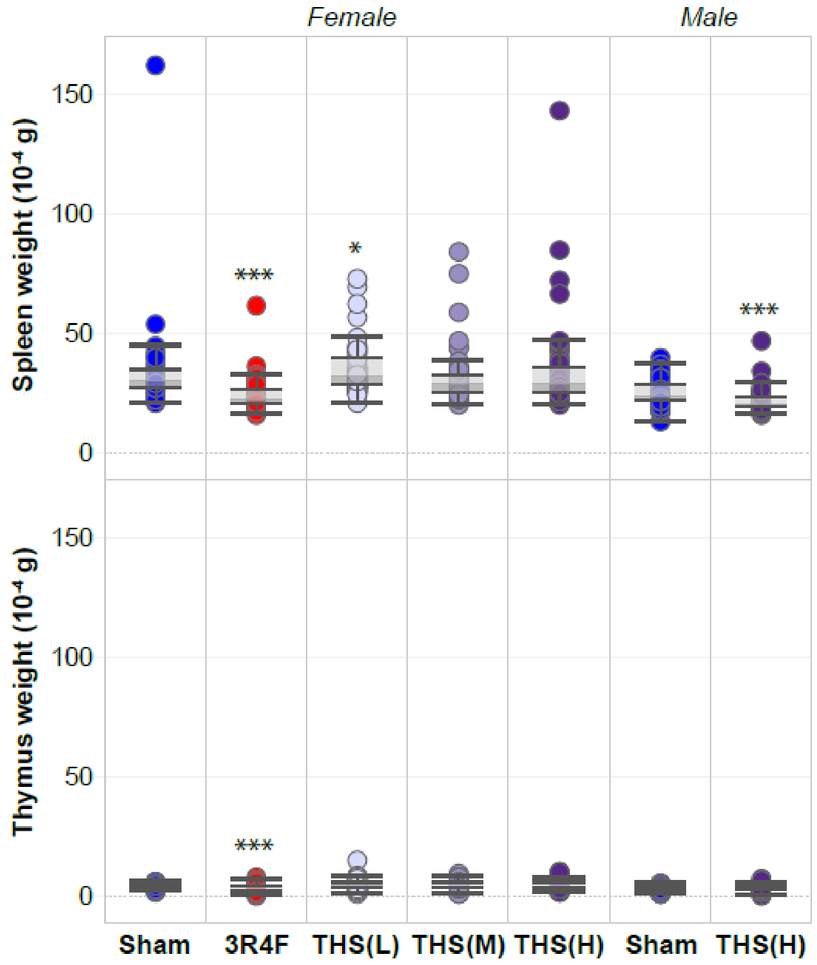
Systemic Toxicity
2. Organ Weights



Data for organ weights relative to body weight after exsanguination from Month 15 (male mice, N=61-99) or Month 18 (female mice, N=55-63) are presented as means ± SEM; *: p<0,05; **: p<0.01; ***: p<0.001 versus Sham (fresh air); #: p<0.05; ##: p<0.01 versus 3R4F

- Absolute and relative spleen weights were lower in 3R4F cigarette smoke-exposed female and THS 2.2 aerosol-exposed male mice, but higher in THS 2.2 aerosol-exposed female mice, relative to Sham
- No histopathological correlates; most likely related to nicotine exposure
- Absolute and relative thymus weights were lower in 3R4F cigarette smoke-exposed compared to THS 2.2 and Sham groups

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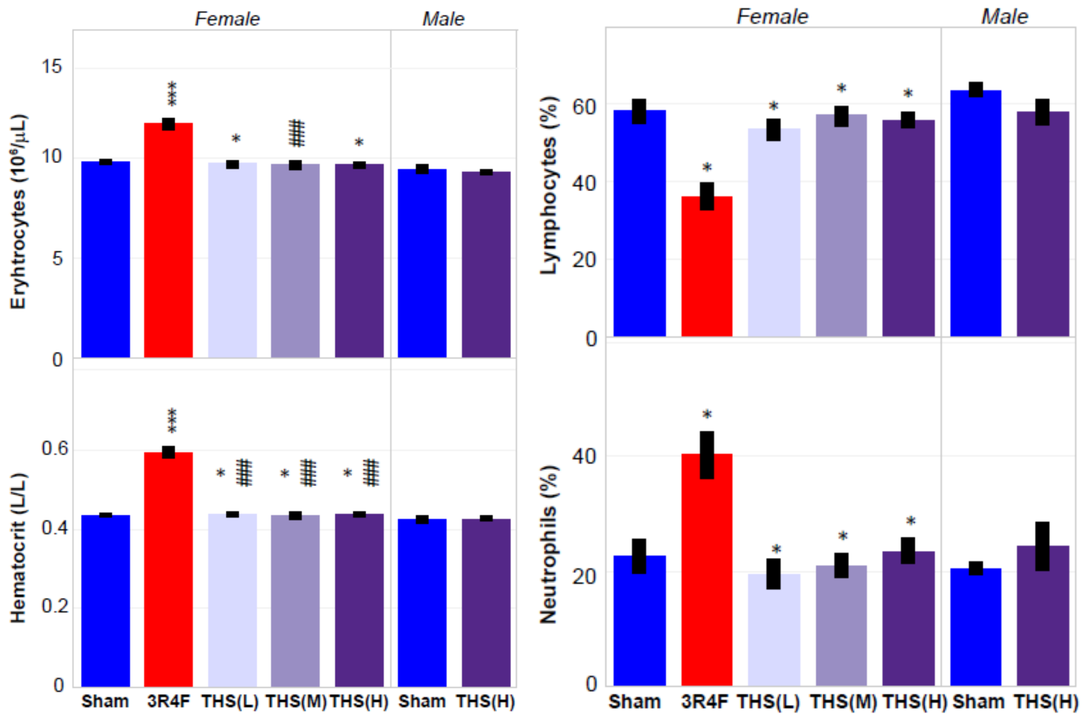
Systemic Toxicity

3. Hematology



- Higher erythrocyte counts and increased hemoglobin-related parameters in 3R4F cigarette-exposed female mice
- Lower absolute and relative neutrophil and lymphocyte counts in 3R4F cigarette and THS 2.2 aerosol-exposed female mice

Data from Months 14 (male mice; N=7-11) and 16 (female mice; N=8-14) are presented as mean ± SEM; *: $p<0.05$; ***: $p<0.001$ versus Sham (fresh air); ###: $p<0.001$ versus 3R4F

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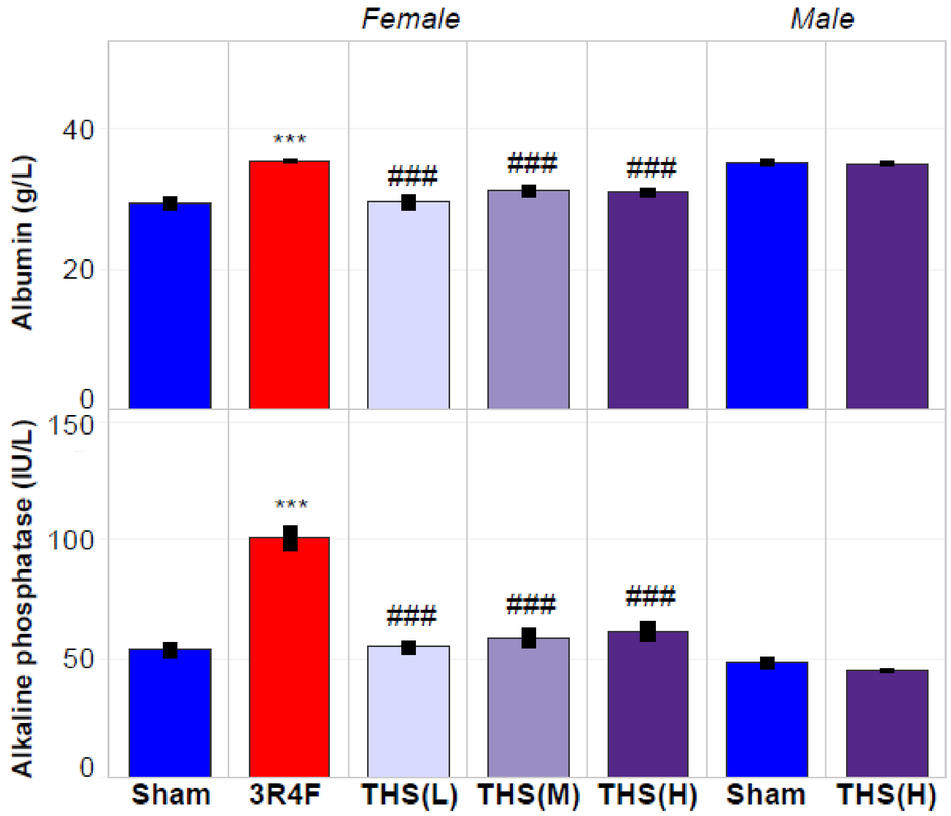
Systemic Toxicity
4. Clinical Chemistry



- Higher serum levels of liver-derived proteins and alkaline phosphatase activity in 3R4F cigarette smoke-exposed mice compared to Sham and THS 2.2 groups

Data from Month 15 (male mice, N=15-18) or Month 18 (female mice, N=11-13) are presented as means ± SEM; ***: p<0.001 versus Sham (fresh air); ###: p<0.001 versus 3R4F

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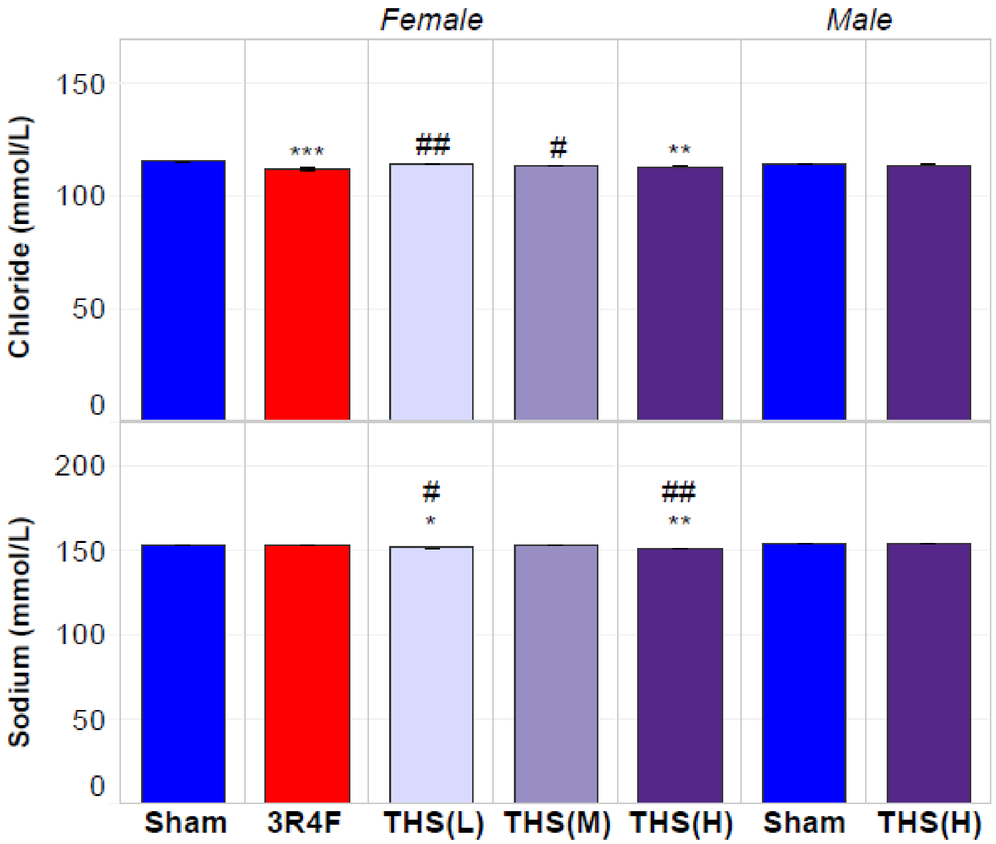
Systemic Toxicity
4. Clinical Chemistry



Data from Month 15 (male mice, N=18-20) or Month 18 (female mice, N=11-14) are presented as means ± SEM; *: p<0,05; **: p<0.01; ***: p<0.001 versus Sham (fresh air); #: p<0.05; ##: p<0.01 versus 3R4F

- Lower serum chloride concentrations in 3R4F cigarette smoke-exposed mice and mice exposed to THS 2.2 aerosol at the highest concentration compared to Sham
- Lower sodium concentrations in serum of mice exposed to THS 2.2 aerosol at the lowest and highest concentrations
- Effects are subtle; changes are within physiological range for these parameters in A/J mice

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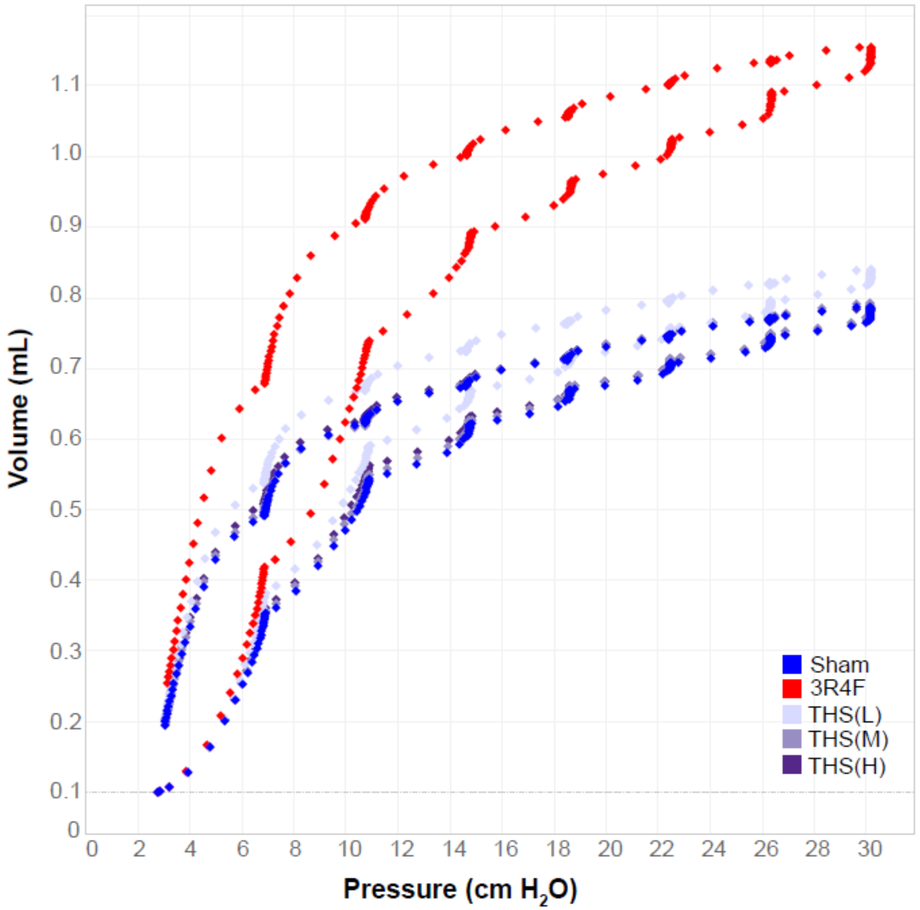
Respiratory Tract Pathology
1. Lung Function



Average data from Month 5 (female mice, N=8-10) are presented; error bars removed for clarity

- No changes in lung function of THS 2.2 aerosol-exposed A/J mice
- No changes in compliance and airway resistance in THS 2.2 aerosol-exposed A/J mice
- Upward and leftward shift of the pressure-volume (P-V) loops for both the inflation and deflation phases and higher lung volumes at specified pressure in mice exposed to 3R4F cigarette smoke compared to the Sham group

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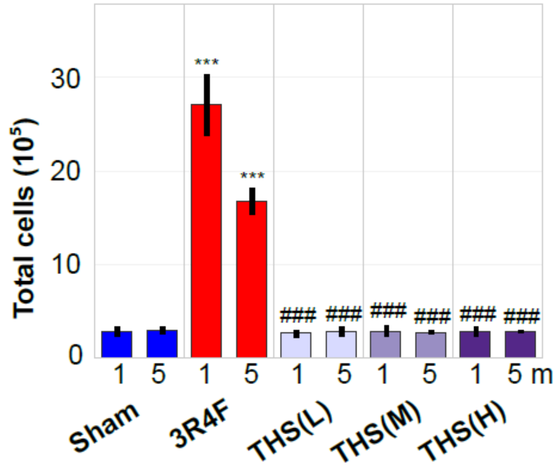
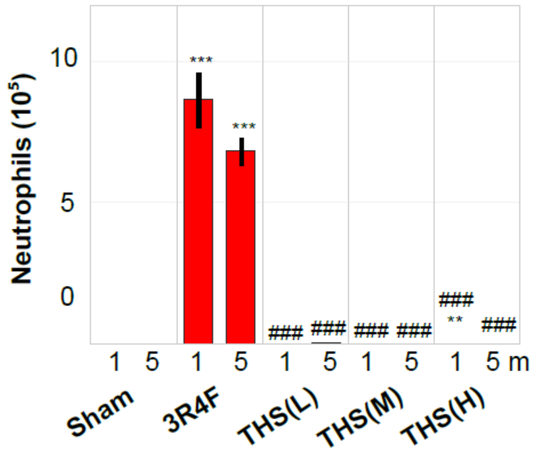
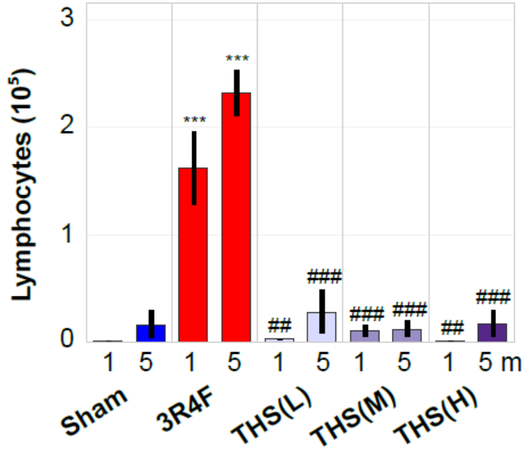
Respiratory Tract Pathology
2. Lung Inflammation







- No lung inflammation seen in THS 2.2 aerosol-exposed mice
- 3R4F cigarette smoke exposure results in higher total cell count and higher neutrophil and lymphocyte counts in the bronchoalveolar lavage fluid (BALF)

Data from female mice (N=10); **: $p<0.01$; ***: $p<0.001$ versus Sham (fresh air); #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$ versus 3R4F

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Respiratory Tract Pathology

2. Lung Inflammation (cont'd)

	Month 1				Month 5			
	♀3R4F	♀THS(L)	♀THS(M)	♀THS(H)	♀3R4F	♀THS(L)	♀THS(M)	♀THS(H)
vWF	4.75	0.83	0.79	0.80	5.33	1.20	1.23	1.02
VEGF-A	8.83	1.05	0.97	1.19	4.00	1.12	1.04	0.99
VCAM-1	11.1	1.31	1.45	1.02	13.4	1.15	1.15	1.17
TNF-alpha	7.37	1.38	1.00	1.00	7.97	1.14	1.32	1.00
TIMP-1 Mouse	10.7	1.12	1.14	1.09	6.74	1.13	1.22	1.05
Thrombopoietin	5.01	1.00	1.00	1.00	5.70	1.23	1.42	1.41
SCF	7.42	1.09	0.85	0.90	6.83	1.02	0.91	0.74
SAP	1.36	1.00	1.00	1.00	1.93	1.00	1.18	1.00
Resistin	1.30	0.94	0.97	1.17	1.04	0.85	0.89	0.83
PAI-1	5.00	1.03	0.99	1.01	4.37	1.01	1.05	1.07
Oncostatin-M	6.38	1.00	1.00	1.00	6.25	1.00	1.00	1.00
Myoglobin	0.84	1.61	1.12	1.38	4.32	2.40	2.79	7.64
MCP-5	70.6	1.00	1.00	1.00	29.2	1.00	1.00	1.00
MCP-3	350	1.00	1.51	1.00	200	1.07	1.19	0.79
MCP-1	1498	1.00	1.78	1.00	417	1.12	0.88	0.76
MMP-9	181	0.51	0.89	0.47	51.8	1.22	0.70	0.62
MIP-3 beta	4.72	0.91	0.88	0.87	4.62	1.24	1.10	1.13
MIP-2	6.43	0.94	1.03	1.13	3.55	1.12	1.04	0.94
MIP-1 gamma	17.5	1.05	1.07	0.93	12.4	0.83	0.97	1.03
MIP-1 beta	84.4	1.04	0.98	1.07	101	0.80	1.13	0.75
MIP-1 alpha	7.82	1.00	1.00	1.00	14.5	1.00	1.00	1.00
MDC	20.1	1.01	1.02	0.92	9.54	0.93	0.96	0.92
M-CSF-1	7.27	1.08	1.07	1.11	6.20	1.10	0.97	1.01
LIF	5.97	1.02	0.87	0.86	4.08	0.98	0.86	0.96
Leptin	0.92	0.76	0.87	0.89	0.70	0.63	0.89	0.73
IL-18	7.47	1.00	1.00	1.00	3.55	0.99	1.20	1.02
IL-11	2.32	1.00	1.00	1.00	2.24	1.00	1.00	1.00
IL-7	2.96	1.10	1.00	1.00	3.08	1.00	1.00	1.00
IL-6	8.97	1.27	1.22	1.00	6.32	1.00	1.14	1.00
IL-4	1.87	1.21	1.11	1.10	1.12	1.00	1.11	1.00
IL-1 beta	3.01	1.00	1.00	1.00	2.95	1.00	1.00	1.00
IL-1 alpha	13.7	1.00	1.00	1.00	18.6	1.00	1.00	1.00
IP-10	30.4	1.00	1.00	1.11	6.00	1.00	1.00	1.11
Insulin	1.08	1.27	0.78	1.17	0.68	0.71	0.96	0.87
IgA	24.8	0.81	5.35	0.66	146	1.01	1.08	0.93
Haptoglobin	1.00	0.97	0.97	0.98	1.02	1.00	1.00	1.00
KC/GRO	68.3	1.19	1.00	1.00	20.2	1.13	1.00	1.00
GM-CSF	6.28	1.00	1.00	1.00	2.59	1.00	1.00	1.00
GCP-2 Mouse	2.54	0.78	0.69	0.76	2.86	1.17	0.82	0.82
FGF-basic	1.40	1.00	1.00	1.00	1.87	0.90	1.44	1.00
Fibrinogen	5.23	0.86	0.90	0.85	4.32	1.11	2.02	1.31
EGF Mouse	4.40	1.00	1.00	1.00	9.52	0.63	0.89	0.95
Eotaxin	5.14	0.88	0.93	0.75	4.78	0.93	1.05	0.89
CRP Mouse	1.95	1.00	1.00	1.00	1.64	1.00	1.17	1.00
Apo A-I	1.56	0.81	0.51	0.59				

Significance and fold-change vs. respective Sham

▼ p<0.001 ▼ p<0.01 ▼ p<0.05

▲ p<0.001 ▲ p<0.01 ▲ p<0.05

n.s.

Data from female mice (N=10)

- Very few changes in secretion of inflammatory mediators into the BALF of THS 2.2 aerosol-exposed A/J mice
- Significant increases in levels of the majority of investigated inflammatory mediators in the BALF of A/J mice exposed to 3R4F cigarette smoke

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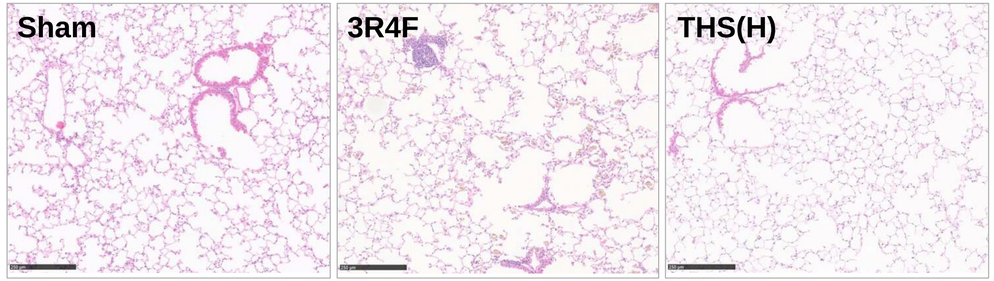
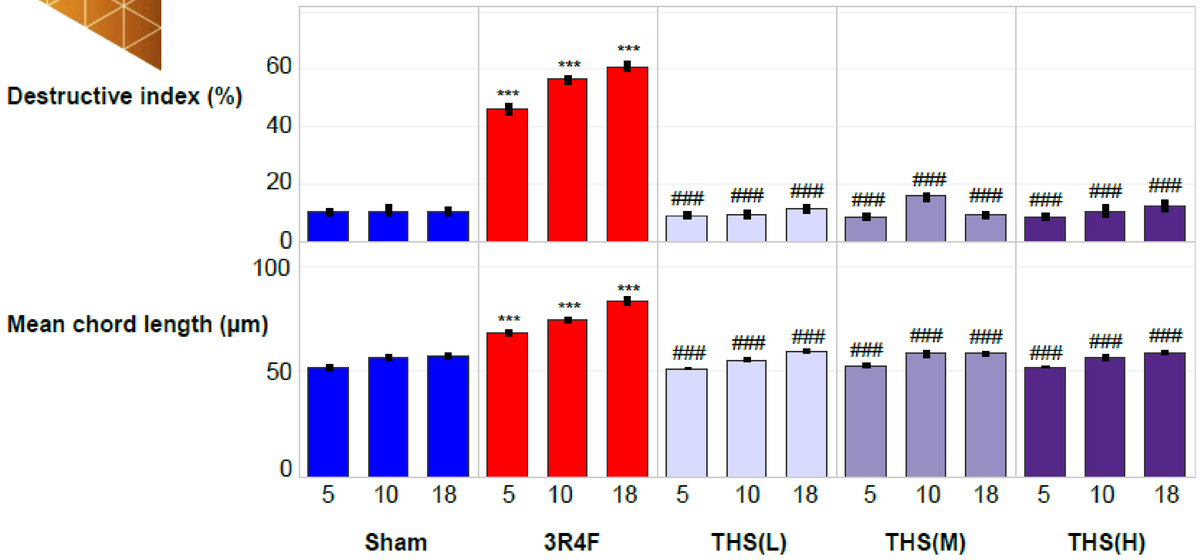
Respiratory Tract Pathology
1. Emphysema



10-month time point, scale bars: 250 µm



Data from female mice (N=8-10) are presented as mean ± SEM; ***: $p<0.001$ versus Sham (fresh air); ###: $p<0.001$ versus 3R4F

- Lung histopathology indicates moderate emphysema in the lungs of 3R4F cigarette smoke-, but not THS 2.2. aerosol-exposed A/J mice
- Changes in morphometric parameters such as destructive index and mean chord length confirm the presence of emphysematous changes in the lungs of 3R4F cigarette smoke-exposed A/J mice
- Based on morphometric analysis, only age-related emphysematous changes were observed in THS 2.2 aerosol-exposed A/J mice

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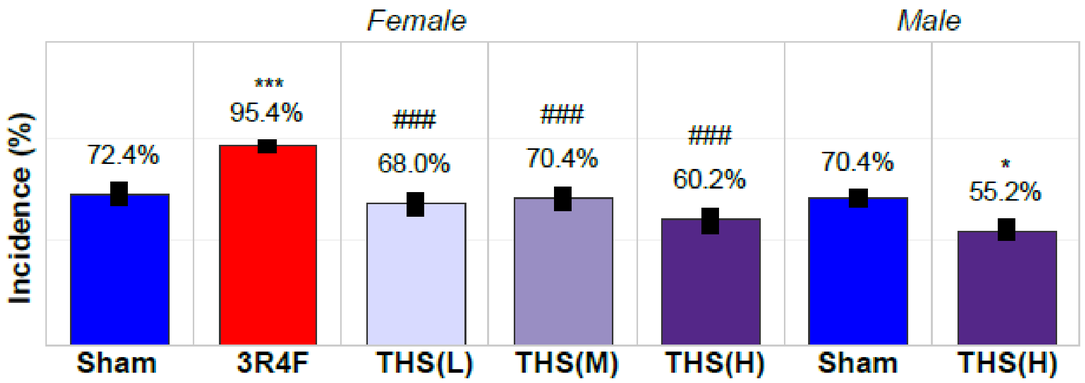
Carcinogenicity
1. Lung Tumor Incidence



Combined adenoma and/or adenocarcinoma incidence. Data are from terminal dissection and early death animals (study days 74-537), adjusted for survival by poly-k test at k=3; *: $p<0.05$; ***: $p<0.001$ versus Sham (fresh air); ###: $p<0.001$ versus 3R4F

- Lung adenoma/carcinoma incidence higher in 3R4F compared to Sham group
- Lung adenoma/carcinoma incidences lower in THS 2.2 aerosol-exposed mice compared to Sham animals
- No obvious dose-response relationship between lung tumor incidence and THS 2.2 aerosol concentration

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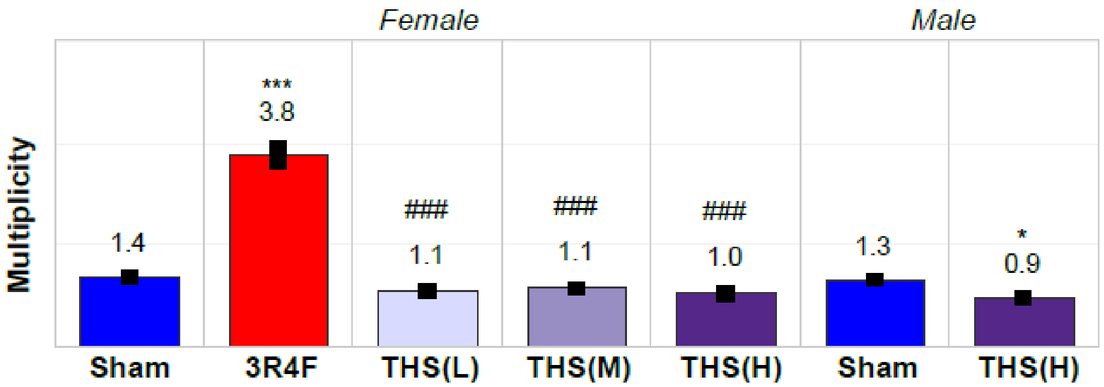
Carcinogenicity
2. Lung Tumor Multiplicity



Combined adenoma and/or adenocarcinoma multiplicity. Data are from terminal dissection and early death animals (study days 74-537), adjusted for survival with threshold of 400 days for female and 240 days for male animals; *: $p<0.05$; ***: $p<0.001$ versus Sham (fresh air); ###: $p<0.001$ versus 3R4F

- Lung adenoma/carcinoma multiplicities were higher in 3R4F compared to Sham and THS 2.2 groups
- Lung adenoma/carcinoma multiplicity lower in THS 2.2 aerosol-exposed than Sham mice
- No obvious dose-response relationship between lung tumor multiplicity and THS 2.2 aerosol concentration

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Summary

- Reproducible exposure was achieved; target concentrations were met.
- Signs of systemic toxicity reflect that stress-related effects and nicotine effects are less pronounced or absent in THS 2.2 aerosol-exposed mice, even at twice the concentration of nicotine in the aerosol.
- No lung inflammation and emphysematous changes were observed in THS 2.2 aerosol-exposed mice, even at twice the concentration of nicotine in the aerosol; clear inflammatory and emphysematous changes were observed upon 3R4F cigarette smoke exposure.
- No increased incidence and multiplicity in pre-neoplastic and neoplastic changes were observed in the lungs of THS 2.2 aerosol-exposed mice, even at twice the concentration of nicotine in the aerosol; clear effects were observed upon 3R4F smoke exposure.

RESULTS

II. Systems Toxicological Endpoints

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Study Endpoints (2)

Tissue	Methylome	Mutation profile	Transcriptome	Proteome
Lung parenchyma		√	√	√*
Tumor nodules		√	√	
Nasal epithelium			√	√
Larynx			√	
Blood	√		√	√
Liver			√	

* Month 1 only

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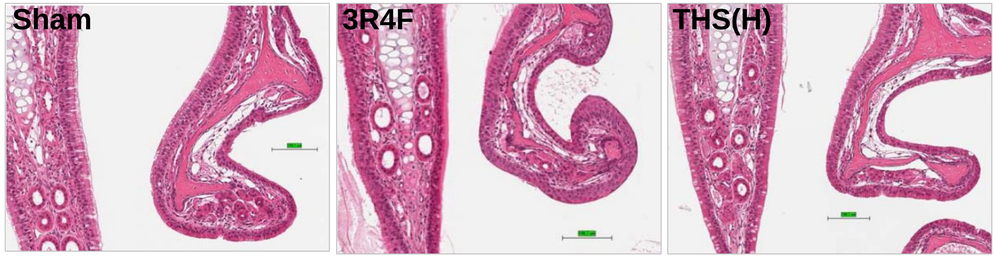
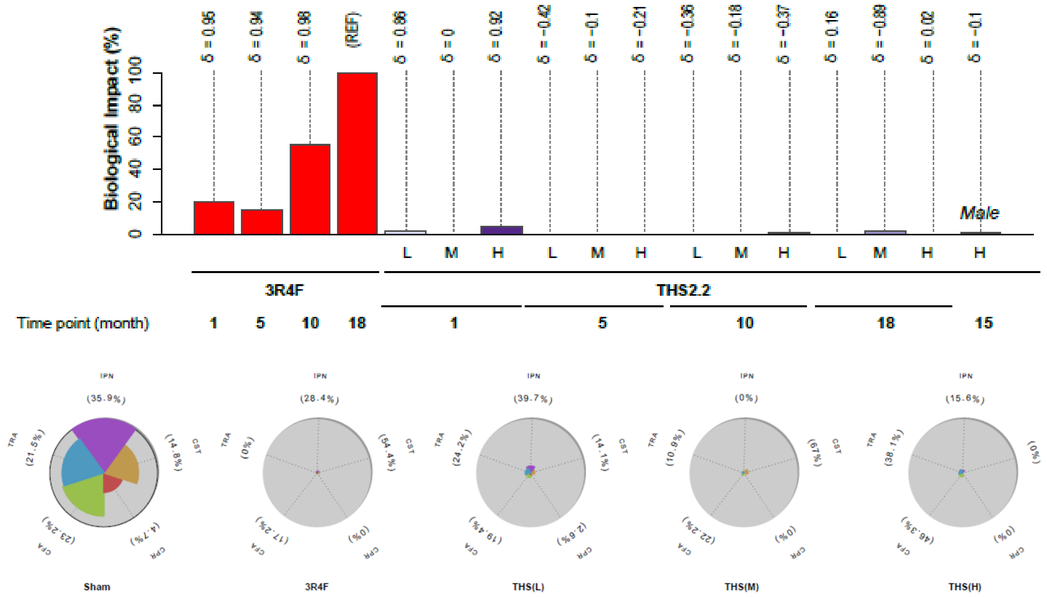
Exposure Effects on the Nose
1. Gene Expression



10-month time point, scale bars: 250 µm



- Histopathology indicates adaptive changes (e.g., hyperplasia, metaplasia, cornification) of the nasal epithelia of 3R4F cigarette smoke-, but not THS 2.2. aerosol-exposed A/J mice
- Highest biological impact seen following exposure to 3R4F cigarette smoke for 18 months; minimal impact of THS 2.2 aerosol exposure
- Processes affected by THS 2.2 aerosol exposure are limited to cellular stress responses (e.g., inflammation, oxidative stress) and tissue repair

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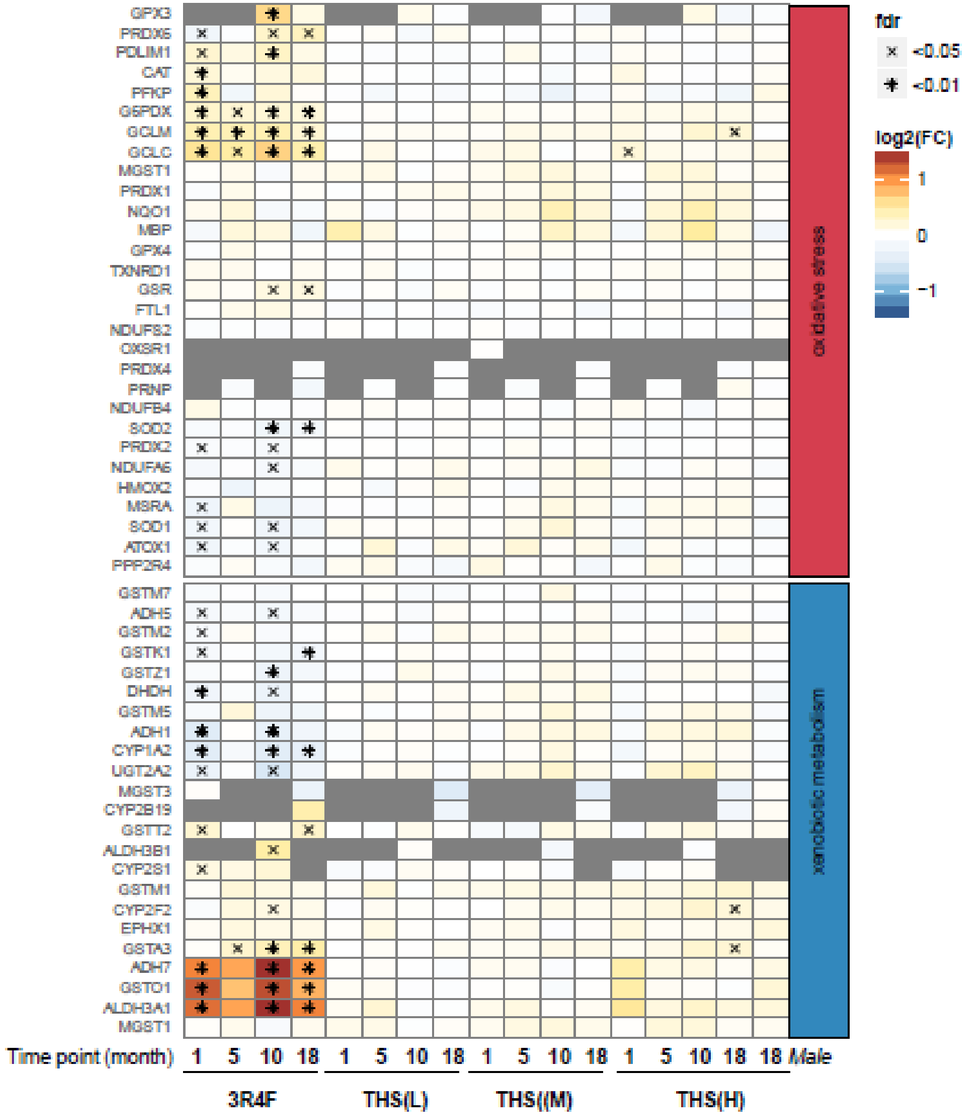
Exposure Effects on the Nose
2. Protein Expression



- Exposure to THS 2.2 aerosol exposure affects only few molecules involved in cellular stress responses (e.g., xenobiotic metabolism, oxidative stress) and tissue repair

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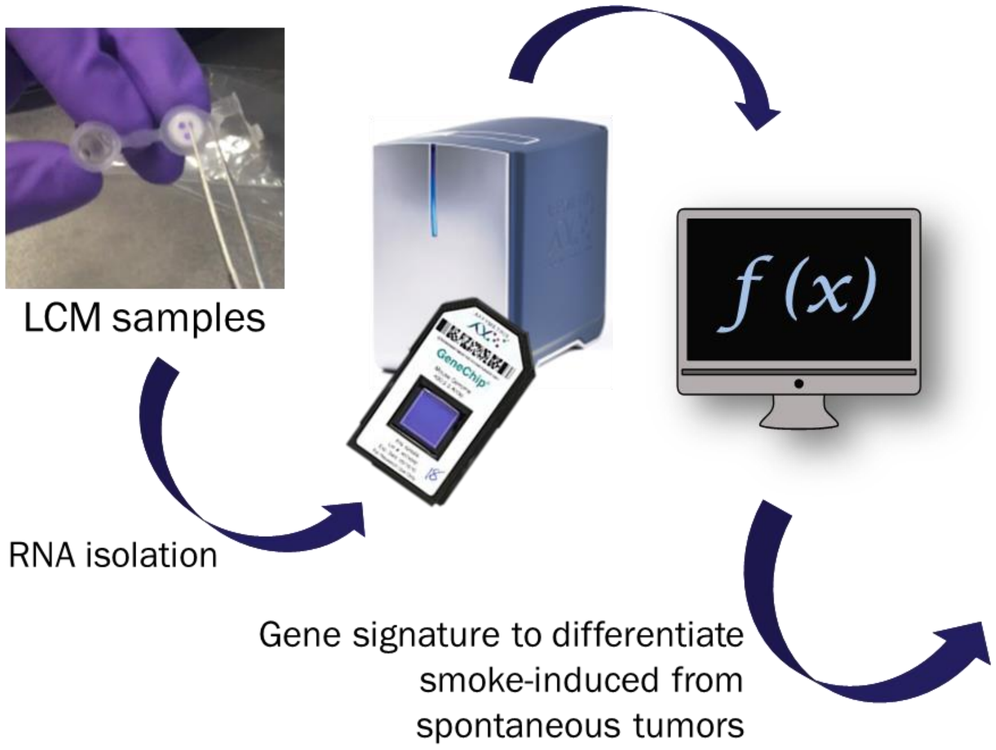
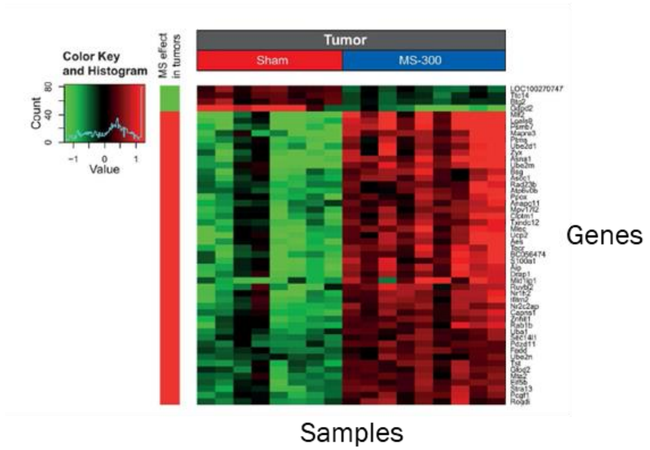
Lung Tumor Signatures
1. Gene Expression Signature





Gene expression signature. Heat map of a gene expression signature that could discriminate between spontaneous tumors in Sham controls and tumors from A/J mice exposed to 300 mg TPM/L from 3R4F cigarettes for 6 hours/day, 5 days/week (MS-300). (Luettich et al., 2014)

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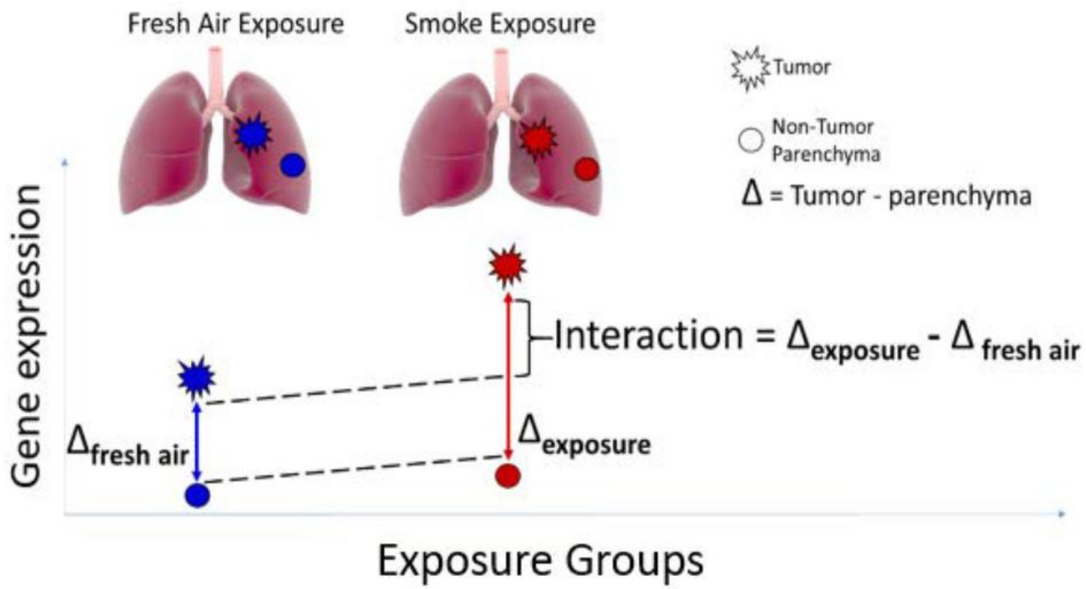
Luettich et al. (2014) Interdiscp Toxicol 7(2): 73–84, doi: 10.2478/intox-2014-0010

Lung Tumor Signatures
1. Gene Expression Signature



- Δ: difference in gene expression between excised tumor and parenchyma tissue
- Interaction defined as Δexposure – Δfresh air
- Interaction term estimates how differently genes behave in tumors of spontaneous vs. smoke-exposed mice
- 13 genes with the greatest interaction values were used for the gene signature (Luettich et al., 2014)

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Lung Tumor Signatures
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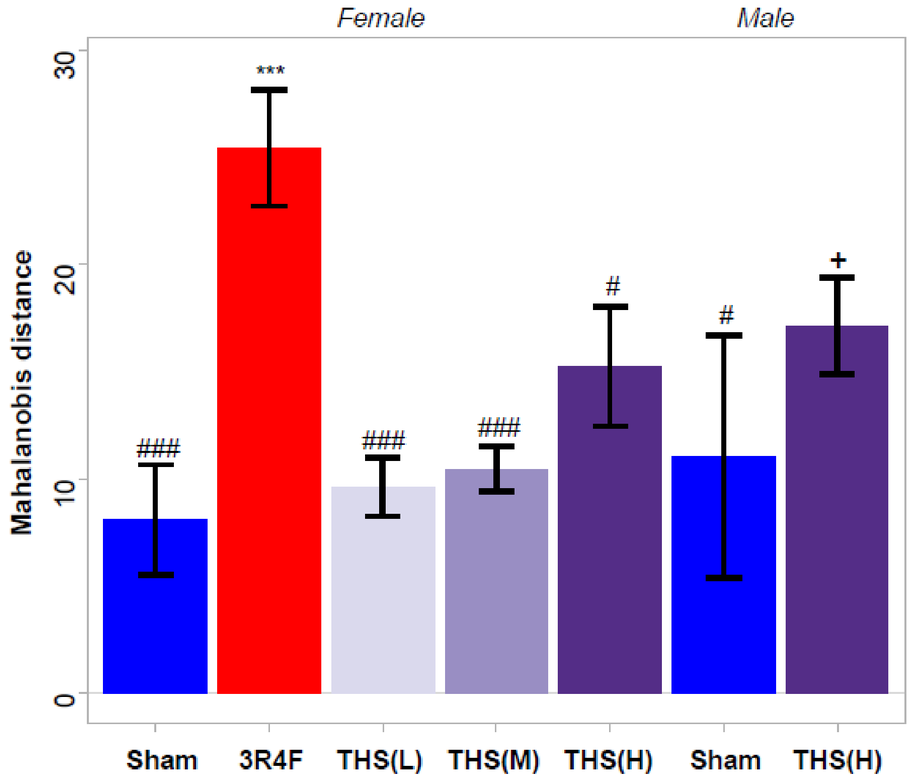

Data are presented as mean ± SEM (N=2-15); ***: *p*<0.001 versus Sham (fresh air); #: p<0.05; ###: p<0.001 versus 3R4F; +: Only 2 tumor samples

- Gene expression signature clearly distinguishes spontaneous tumors from cigarette smoke exposure tumors (*p*<0.001)
- Tumors from THS 2.2 aerosol-exposed mice were more similar to spontaneous tumors than cigarette smoke exposure tumors

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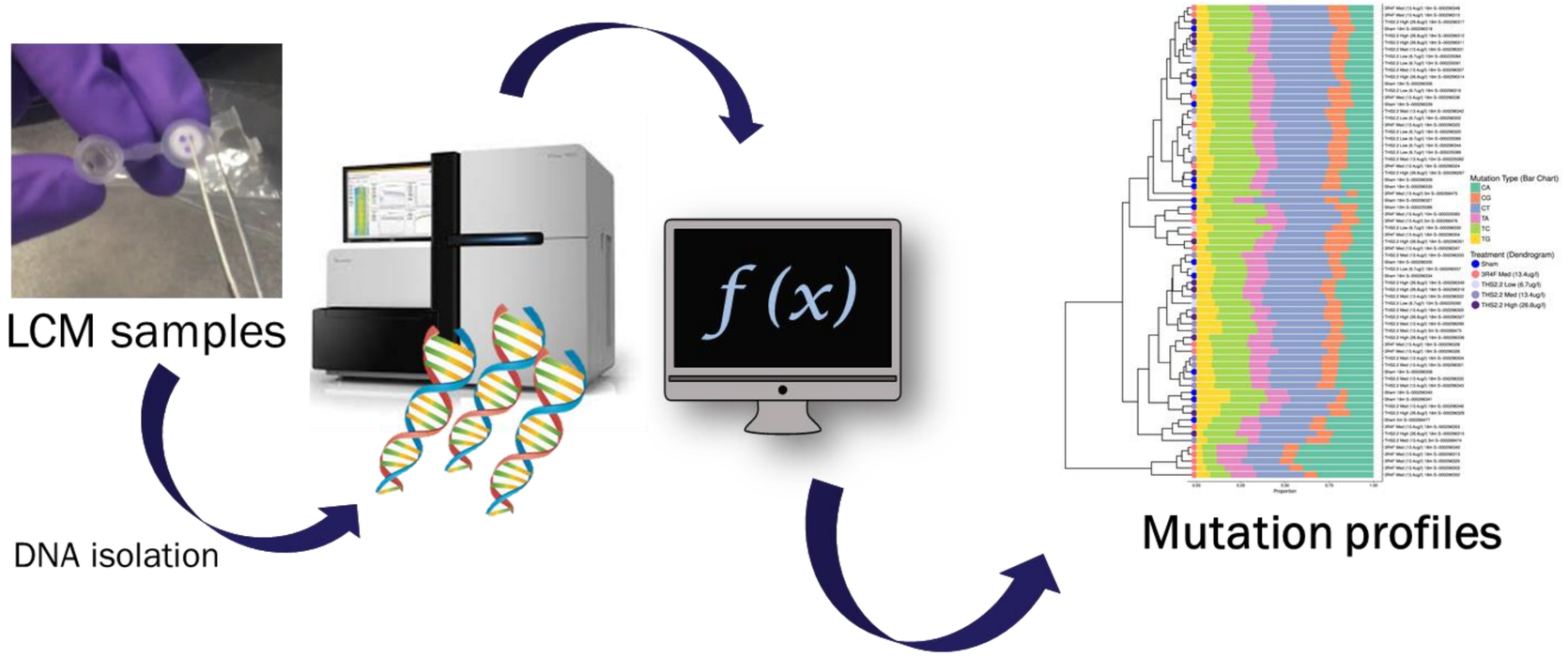
Lung Tumor Signatures
2. Mutation Signature



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Lung Tumor Signatures
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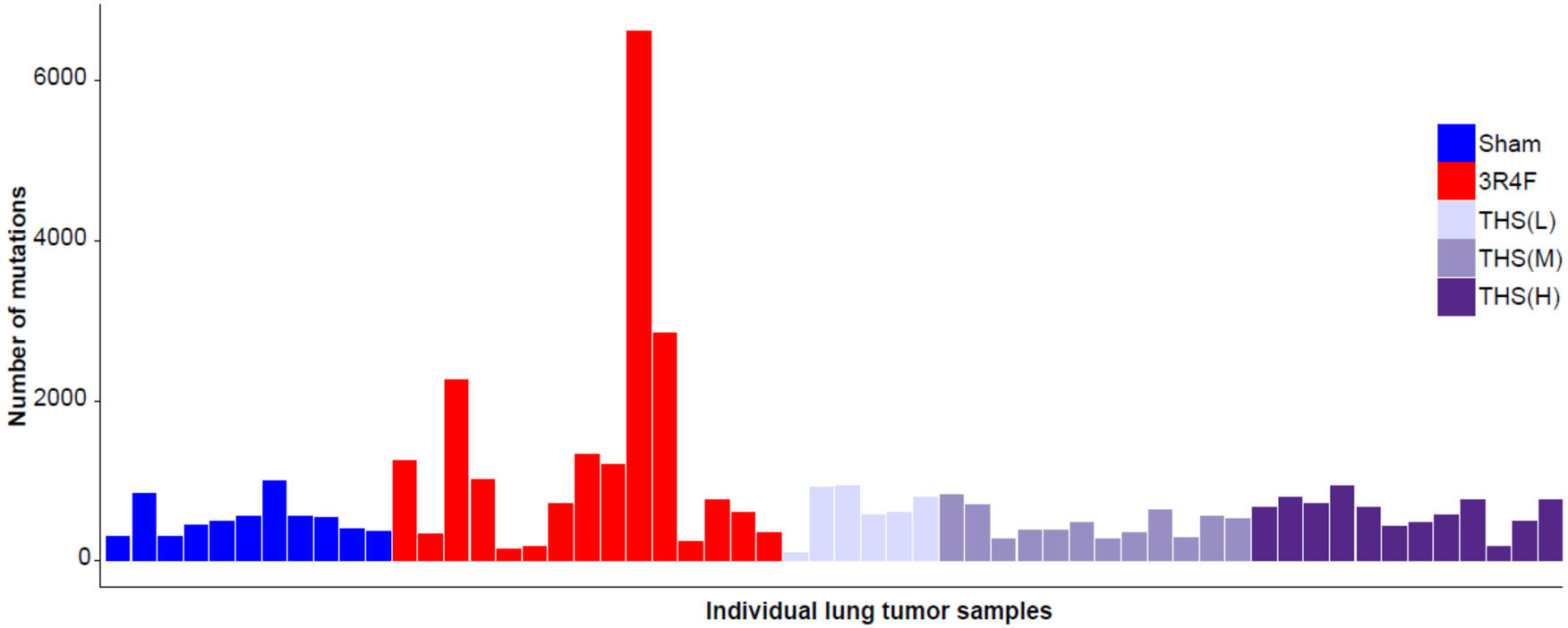

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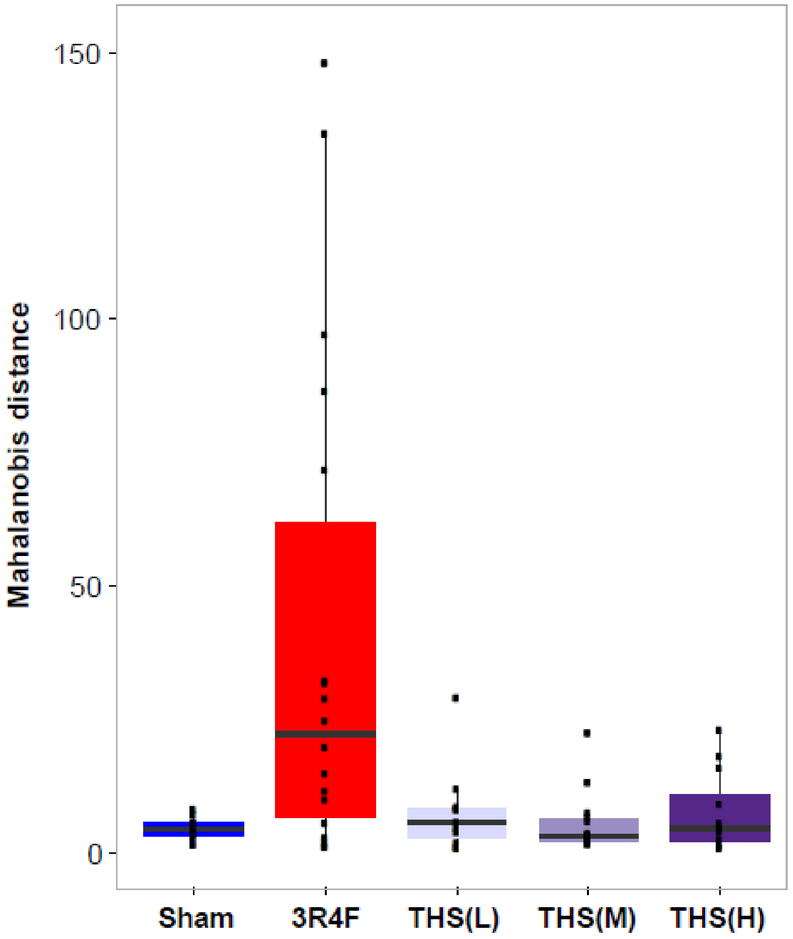
Lung Tumor Signatures
2. Mutation Signature



- Mutation profile signature clearly distinguishes spontaneous tumors from cigarette smoke exposure tumors
- Mutation profiles of lung tumors from THS 2.2 aerosol-exposed mice were more similar to those in spontaneous tumors than in those from cigarette smoke-exposed mice

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Summary

- Gene and protein expression analysis confirm minor effects of THS 2.2 aerosol exposure on the nasal epithelium; extensive exposure effects are seen with 3R4F cigarette smoke.
- A previously developed gene signature distinguishes lung tumors developing spontaneously from those arising in 3R4F cigarette smoke-exposed mice.
- The same gene signature also distinguishes the lung tumors from THS 2.2 aerosol-exposed mice from 3R4F cigarette smoke-exposed mice.
- Similarity analysis based on tumor mutation profiles confirms the molecular differences between the effects of 3R4F cigarette smoke and THS 2.2 aerosol exposures on lung tumors in A/J mice.

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sbv IMPROVER
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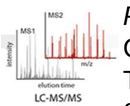
S Ansari (Biobanking)



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Thank you!