Ethylene-induced Nasal Lesions in Rats: Understanding the Pathogenesis and the Potential Mode of Action

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Slides Prepared for the 2014 Symposium on Understanding the Health Risks of Lower Olefins

Overview

- **Background**
  - Rationale for conducting a 90-day study
- **90-Day Studies**
  - TERC study results and new findings
  - Hamner reproducibility study
- **Mode of Action Studies**
  - Time- and concentration-dependent effects on nasal morphology and regional gene expression
  - Differentiating immune-mediated from irritant-induced effects
- **Summary and Conclusions**
  - Current hypothesis and next steps

Background: Previous Studies

- Subchronic Inhalation (Rhudy *et al.,* 1978 [unpublished report])
  - SD rats
  - 0, 300, 1000, 3000, or 10,000 ppm ethylene
  - 6 h/day, 5 days/wk for 14 wks
  - no exposure-related mortality & no evidence of systemic toxicity or histopathologic effects
- Chronic Inhalation/Carcinogenicity (Hamm *et al*., 1984)
  - Fischer 344 rats
  - 0, 300, 1000, or 3000 ppm ethylene
  - 6 h/day, 5 days/wk for 106 wks
  - histopathologic analysis of tissues (including nasal airways) revealed no chronic toxicologic or carcinogenic effects
  - based on a chronic NOAEL of 3000 ppm, and PK/metabolism data, the ACGIH (2001) set a TLV-TWA (8 h) of 200 ppm

Background: Previous Studies

- Biomarker of effect (genotoxicity)
  - Micronucleus (MN) Assay (Vergnes *et al.*, 1994)
    - Rats and mice
      - Up to 3000 ppm ethylene; 6 h/day, 5 days/wk for 4 wks
    - *In vivo* bone marrow MN tests were negative
- Biomarker of exposure
  - Hemoglobin (Hb) Adducts (Walker *et al*., 2000)
    - Rats and mice
      - 0, 40, 1000, 3000 ppm ethylene; 6 h/day, 5 days/wk for 4 wks
    - Endogenous N7-(2-hydroxyethyl)valine (HEVal) Hb adduct levels (0 ppm controls) were 50 & 100 pmol/g Hb in rats and mice, respectively
    - Exposure-dependent increase in HEVal levels, but not dose-proportionate
    - HEVal levels in 1000 & 3000 ppm-exposed animals were similar
      - Indicates near saturated ethylene metabolism at 1000 ppm

Background: 90-day Study Rationale

- Confirm results of the previous 90-day ethylene inhalation study (Rhudy *et al.,* 1978)
- Extend the range of high dose 90-day data for:
  - Biomarker of exposure – hemoglobin adducts (HEVal)
  - Biomarker of effect – micronucleus (MN) induction
    - Assess exposure-dependent effects on thermoregulation (Potential confounding factor in MN assay)
- Provide supporting evidence of lack of genotoxicity for high level, repeated exposures to ethylene
- Provide guideline- and GLP-compliant, subchronic inhalation toxicity data for inhaled ethylene in rats

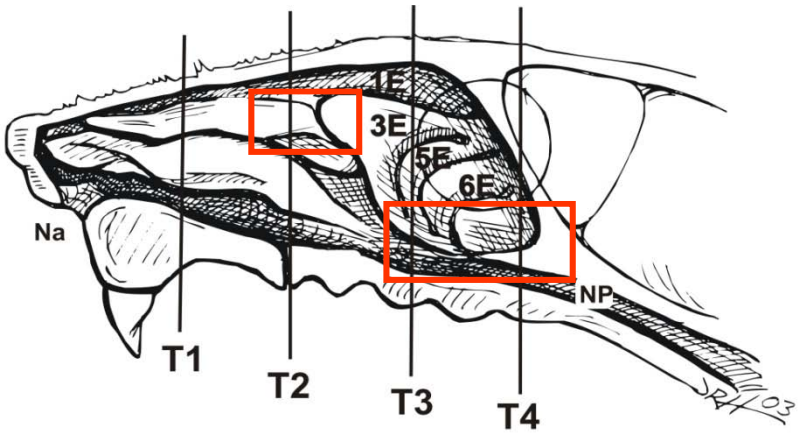
TERC 90-day Study: Experimental Design

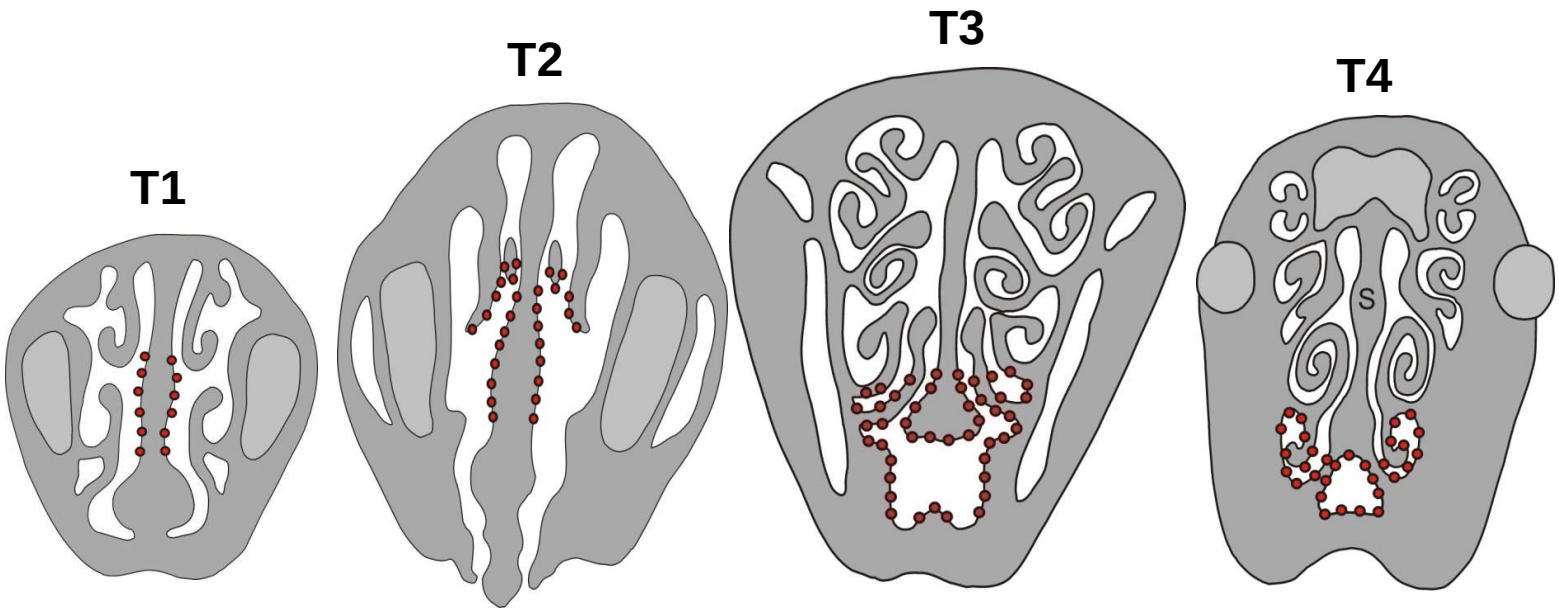
- F344 rat, male & female, 10/sex/concentration
- Whole-body vapor inhalation exposures
- 0, 300, 1000, 3000, & 10,000 ppm ethylene
  - 6 h/day, 5 d/wk for 13 wks (65 exposure days),
- Body temperature monitored pre- & post-exposure
- Rats sacrificed 1 day following last exposure
  - Standard subchronic inhalation study endpoints
  - MN induction in peripheral blood reticulocytes evaluated by flow cytometry after 5 and 65 exposure days
  - MN induction in bone marrow after 65 exposure days
  - HEVal Hb adduct levels after 65 exposure days

TERC 90-day Study: Results

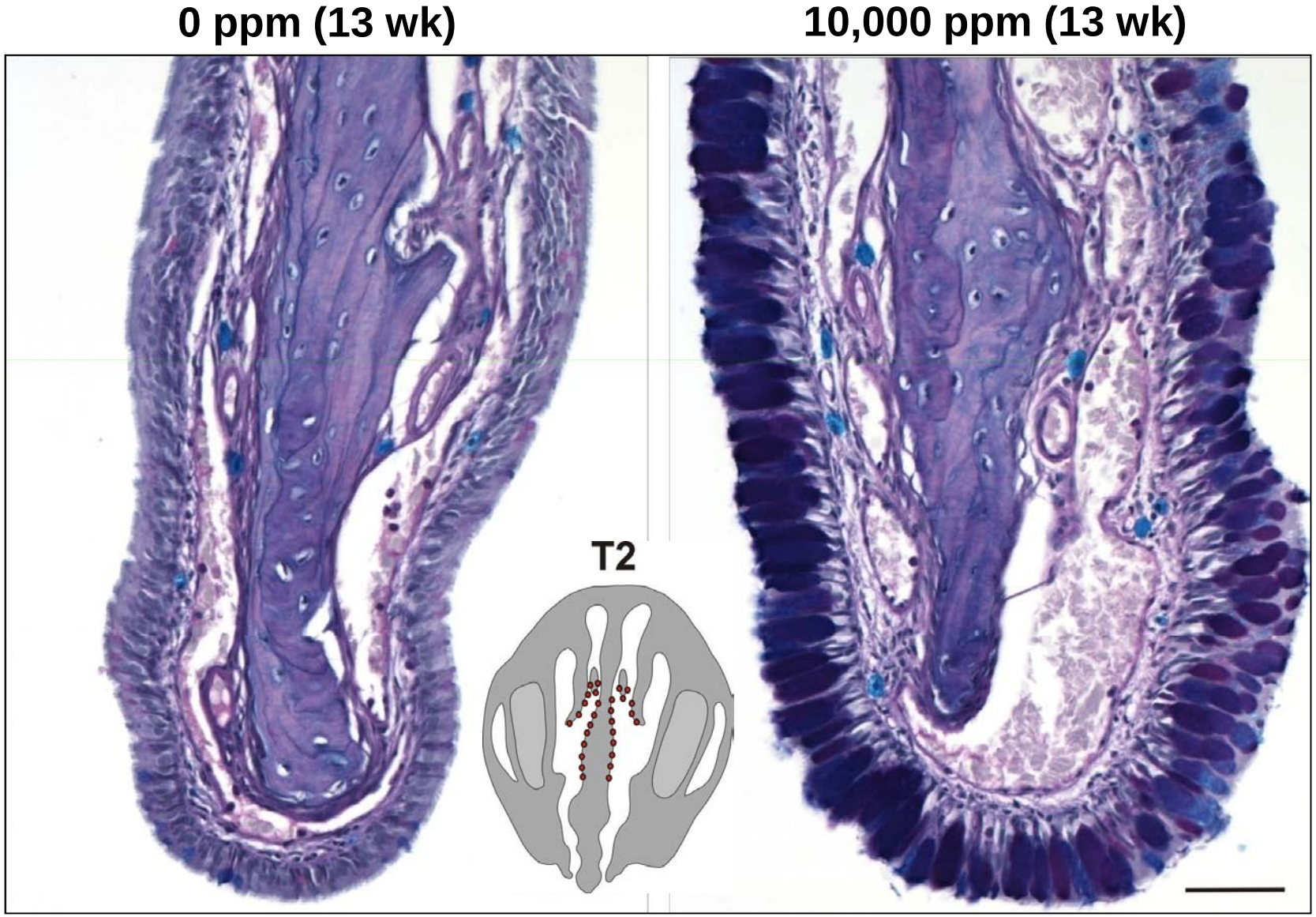
- No mortality or treatment-related clinical observations
- No systemic effects identified: **NOEL = 10,000 ppm**
- No neurotoxicity: **NOEL = 10,000 ppm**
- No cytogenetic damage: **NOEL = 10,000 ppm**
  - No ↑ in micronucleated peripheral blood reticulocytes (1 or 13 wk) or MN polychromatic reticulocytes in bone marrow (13 wk)
- Exposure-dependent increase in HEVal Hb adducts
  - Not dose-proportional
  - Evidence of saturation at 3000 ppm
- Histopathology identified lesions in nasal respiratory epithelium at all exposure levels: **LOEL = 300 ppm**
  - Mucous cell hyperplasia/metaplasia
  - Hyalinosis
  - Eosinophilic inflammation (mucosal eosinophils)

Ethylene-induced Nasal Lesions



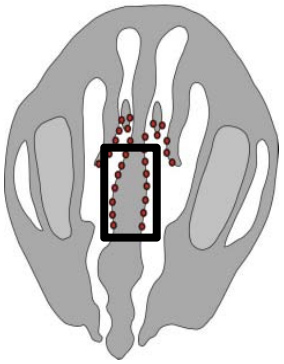


Mucous Cell Hyperplasia/Metaplasia

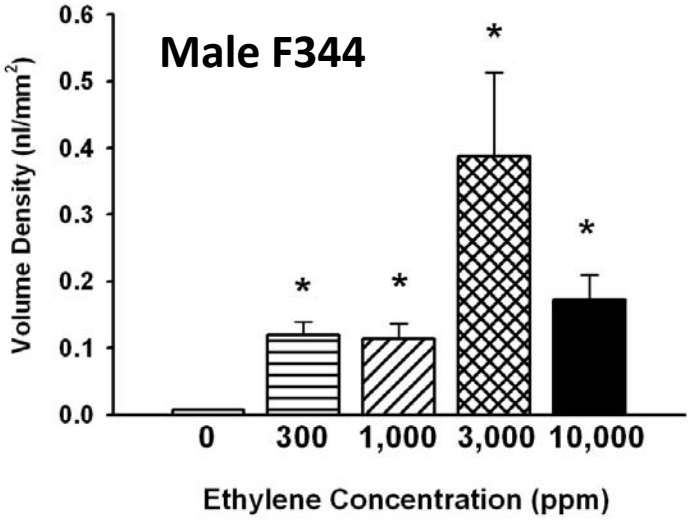


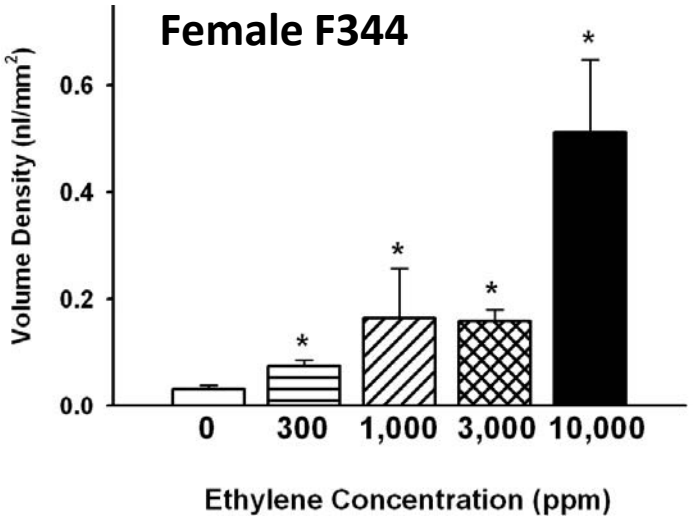
Mucous Cell Hyperplasia/Metaplasia

T2



Mid-Septum





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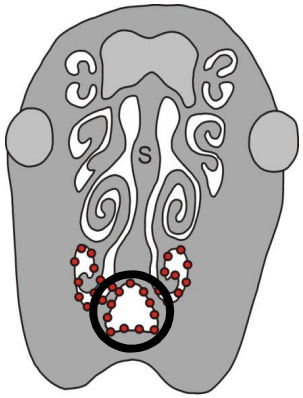
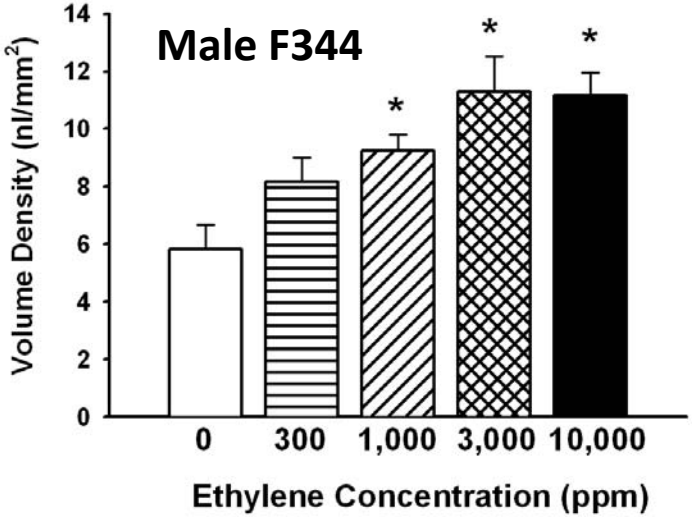
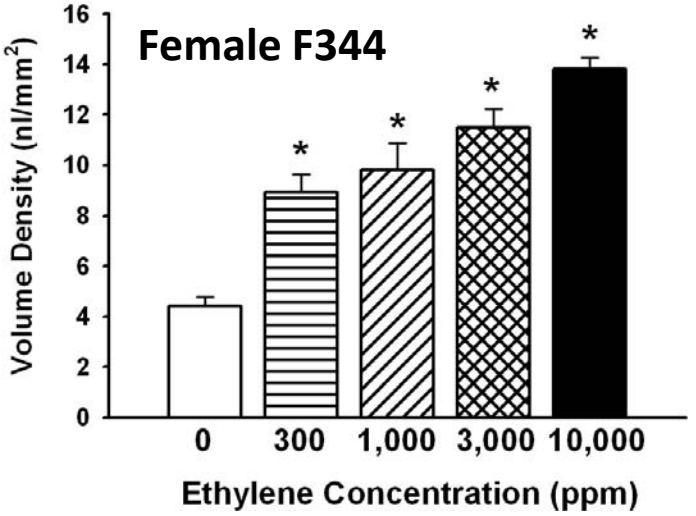
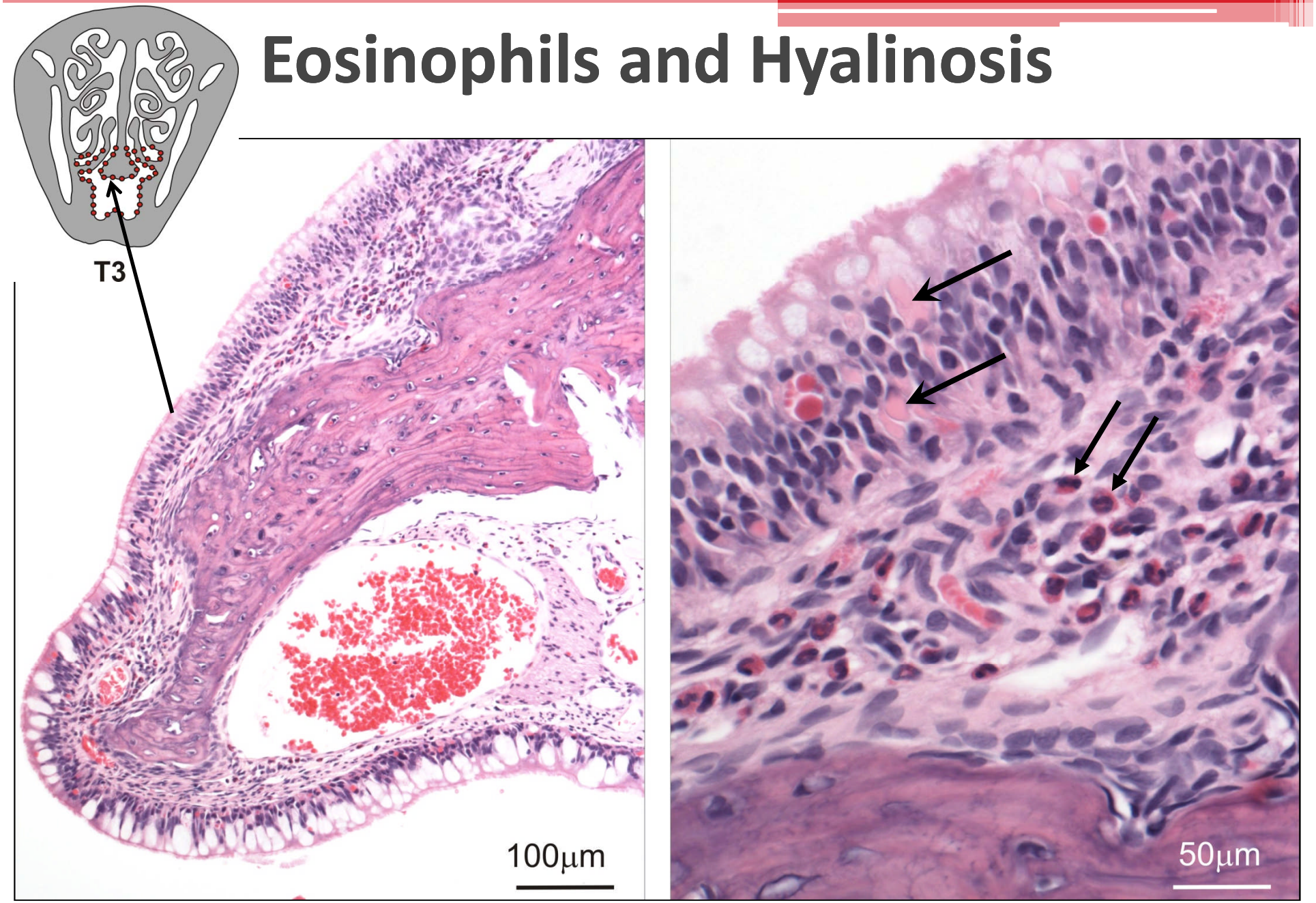


Nasopharynx





Eosinophils and Hyalinosis



Eosinophilic Inflammation

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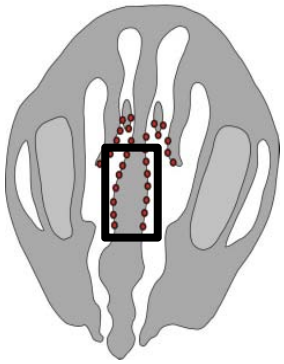
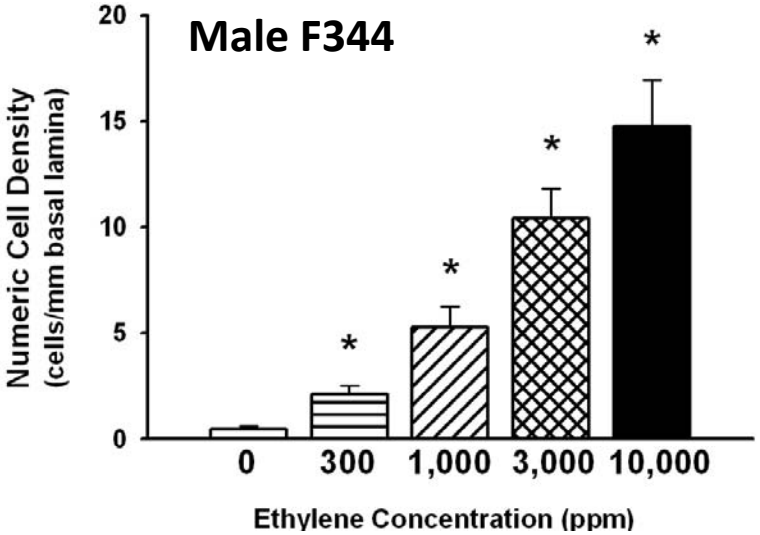
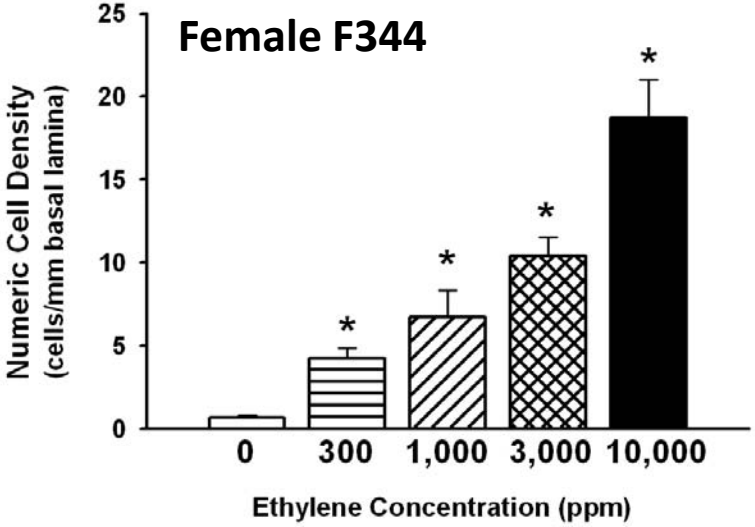


Mid-Septum





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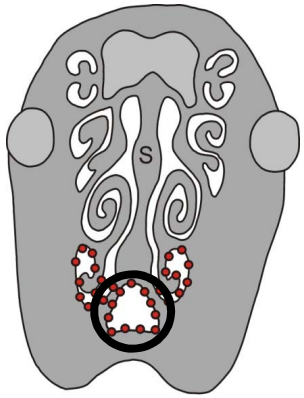
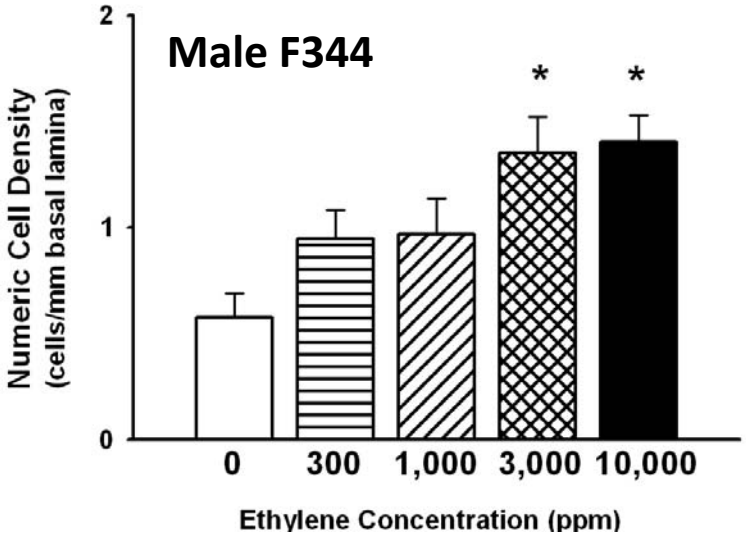
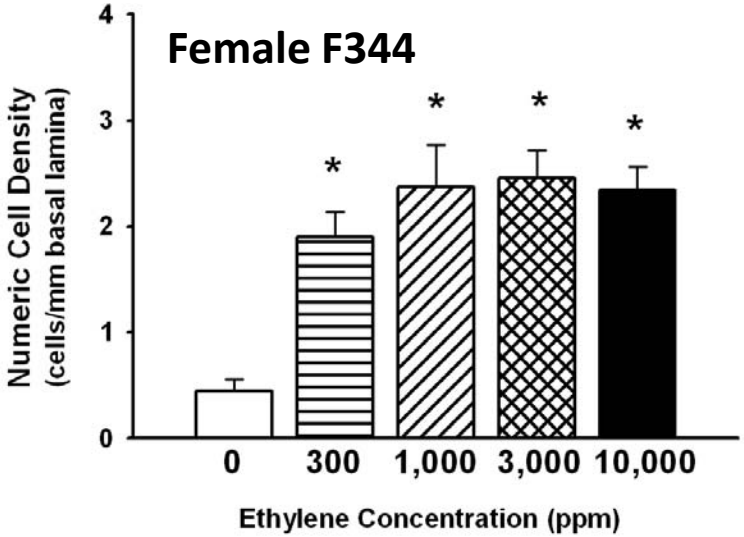
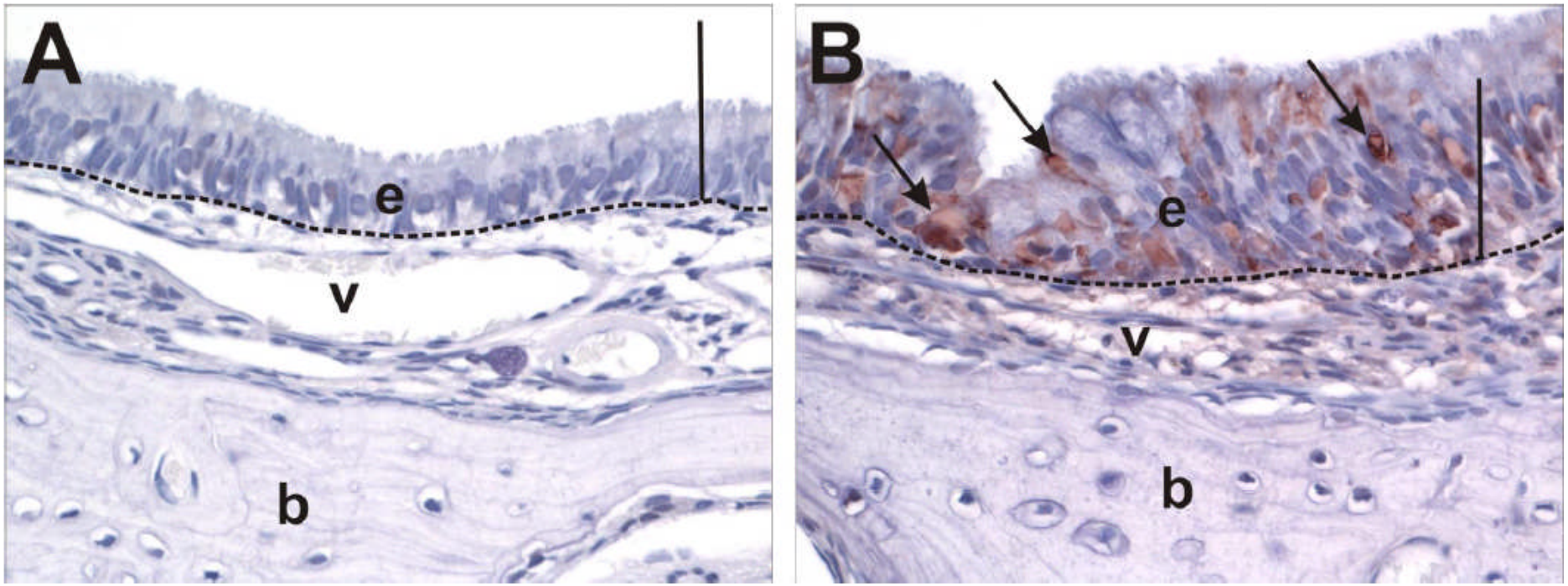


Nasopharynx





Epithelial Hyalinosis: Ym 1/2 Chitinase



- Mammalian chitinase Ym1/2 protein present in hyaline droplets of ethylene-exposed F344 rats
- Increased human chitinase-expression in lung and serum of asthmatics and with Th2-mediated inflammation

Hamner Reproducibility Study

- Test Lab
  - The Hamner Institutes for Health Sciences
- Species/Strain
  - F344 and Wistar rats
    - Different source of test animals
- Exposure levels
  - 0 ppm and 10,000 ppm
    - Different test lot of ethylene
- Exposure duration
  - 4 wks - F344 only
    - Time-dependency of lesion development
  - 13 wks - F344 and Wistar
    - Strain comparison
- Histopathologic examination (limited to likely target tissues)
  - Nose, larynx, trachea, lung, draining lymph nodes (nasal)

Hamner Reproducibility Study

- Location and character of ethylene-induced nasal lesions similar to TERC 90-day study
- Lesions present in F344 rats after 4 wks exposure but increased in severity after 13 wks exposure
- Similar, but markedly less severe, lesions in Wistar rats as compared to F344 rats (13 wks)
- No exposure-related changes in other tissues, except for slight increase in eosinophils in medullary sinuses of draining lymph nodes in ethylene-exposed F344 rats after 13 wks exposure

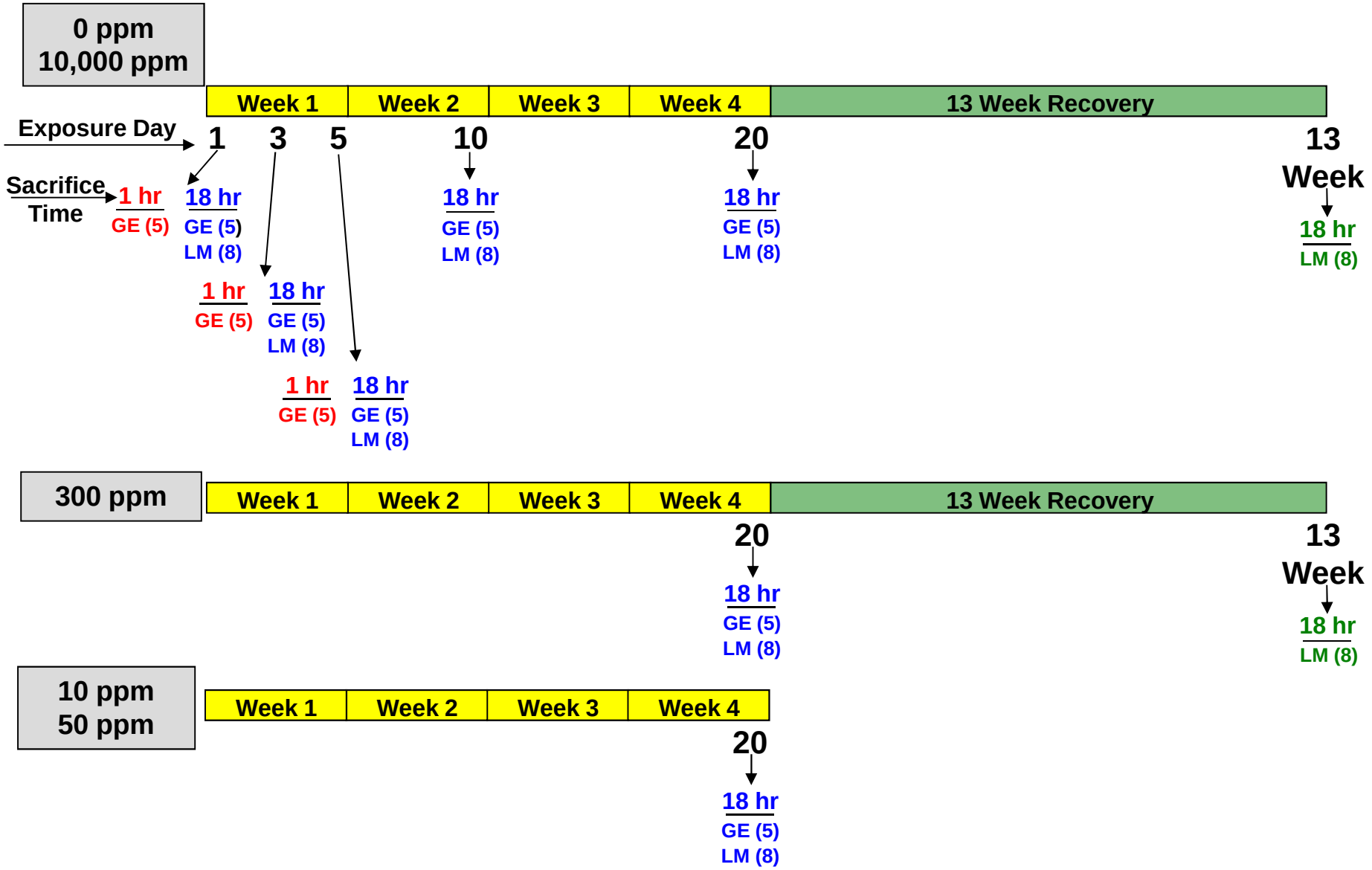
Interim Summary 1: What did we learn?

- Subchronic inhalation of ethylene ≥ 300 ppm induces site-specific morphologic changes in the nasal respiratory epithelium of F344 rats
  - Eosinophilic inflammation
  - Mucous cell hyperplasia/metaplasia
  - Hyalinosis
    - Ym1/2 expression
- Ethylene-induced nasal lesion is present after 4 wk exposure to 10,000 ppm
- Strain-related differences in the magnitude of response
  - F344 rats > Wistar rats

Identification of Key Cellular and Molecular Events Involved in the Pathogenesis of Ethylene-Induced Nasal Epithelial Lesions in F344/DuCrl Rats

- **Hypothesis 1**
  - Ethylene exposure induces acute cellular inflammation, nasal epithelial DNA synthesis, & increased Th2 cytokine, chitinase, & mucin mRNA expression that precede the development of morphologically identifiable nasal epithelial lesions
- **Hypothesis 2**
  - Nasal epithelial lesions induced by repeated inhalation exposure of rats to ethylene are both time- and concentration-dependent and reversible
- **Study Endpoints**
  - Cell proliferation
  - Mucous cell hyperplasia/metaplasia (MCM)
  - Eosinophilic inflammation
  - Hyalinosis (YM 1/2)
  - Serum IgE and IgG1/IgG2a ratio
  - Regional gene expression

4-Wk MOA Study: Experimental Design



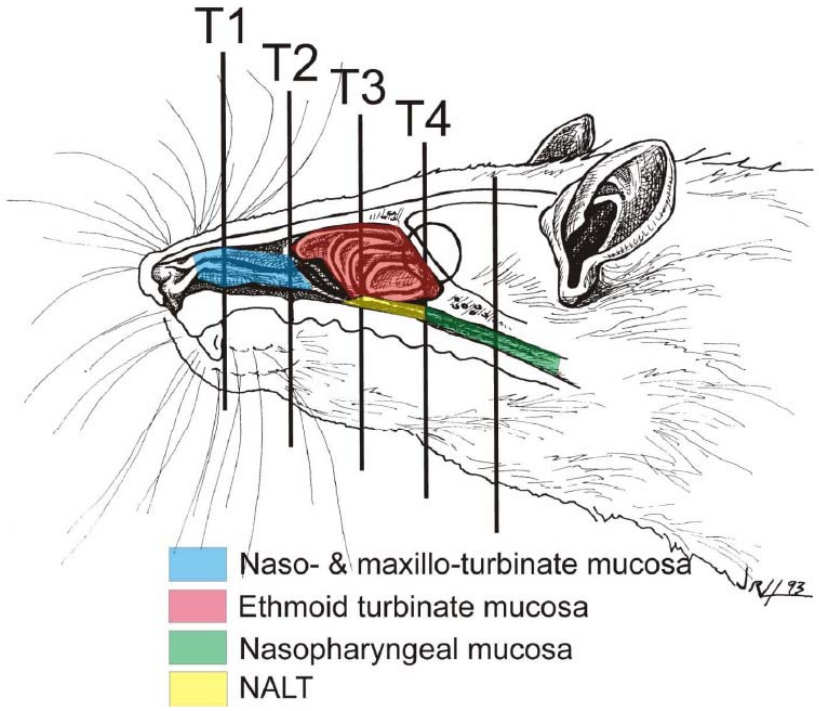
Summary: Concentration-Response

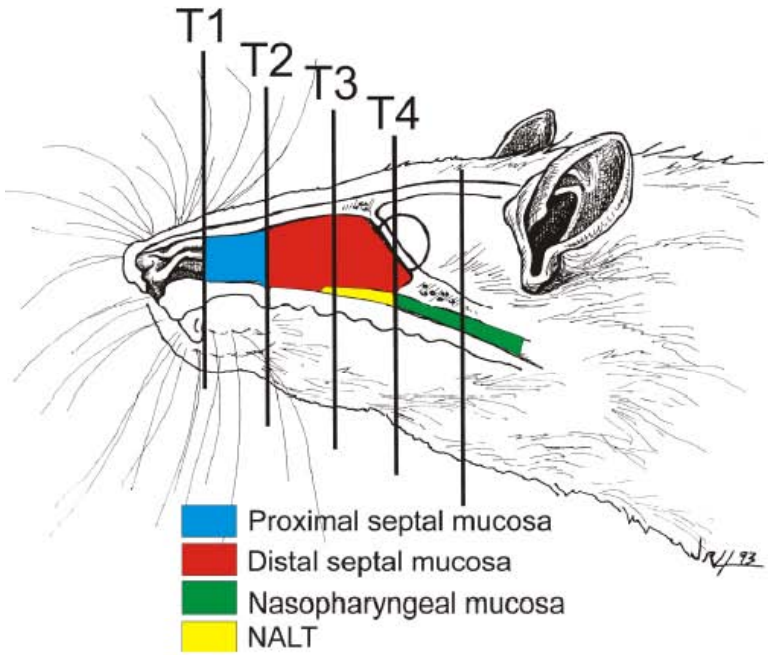
20 days of exposure	10 ppm	50 ppm	300 ppm	10,000 ppm
Eosinophils T2	+	+	+	+
Eosinophils T4	–	–	–	+
MCM T2	–	–	–	+
MCM T4	+	+	+	+
Hyalinosis (Ym1/2)	–	–	–	–
BrdU Increase	–	–	–	–

Summary: Time-Response

10,000 ppm	Day 1	D 3	D 5	D 10	D 20
Eosinophils T2	–	–	–	+	+
Eosinophils T4	–	–	–	–	+
MCM T2	–	–	–	–	+
MCM T4	–	–	+	+	+
Hyalinosis (Ym1/2)	–	–	–	–	–
BrdU Increase	–	–	–	–	–

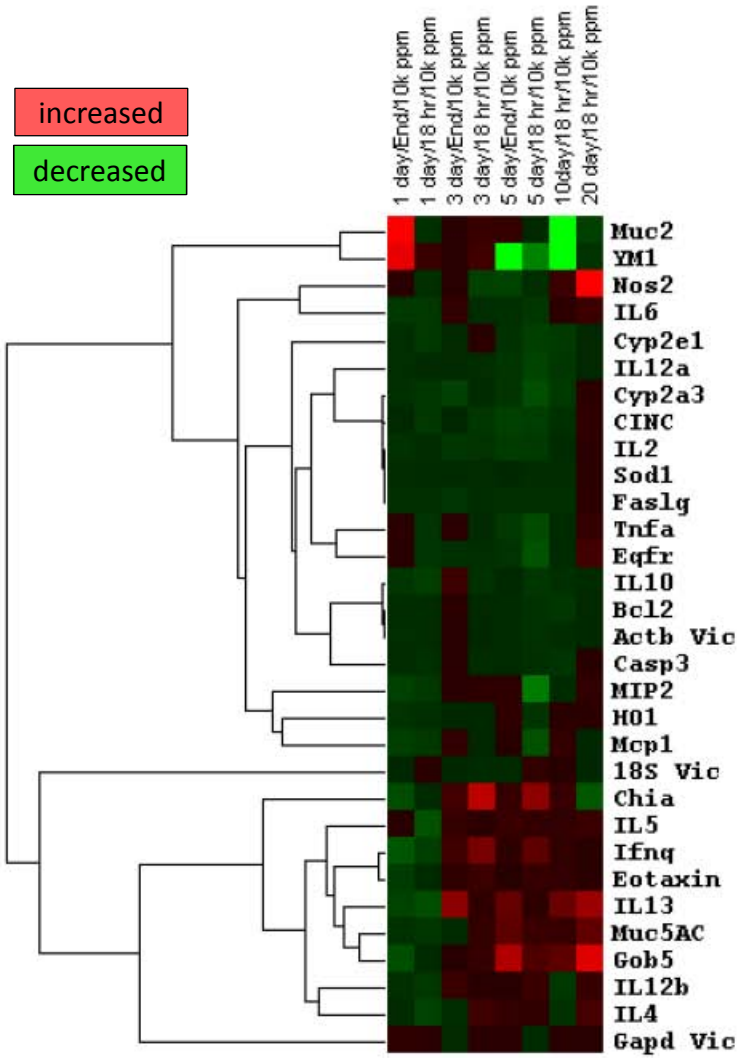
Sample Sites: Gene Expression Assays

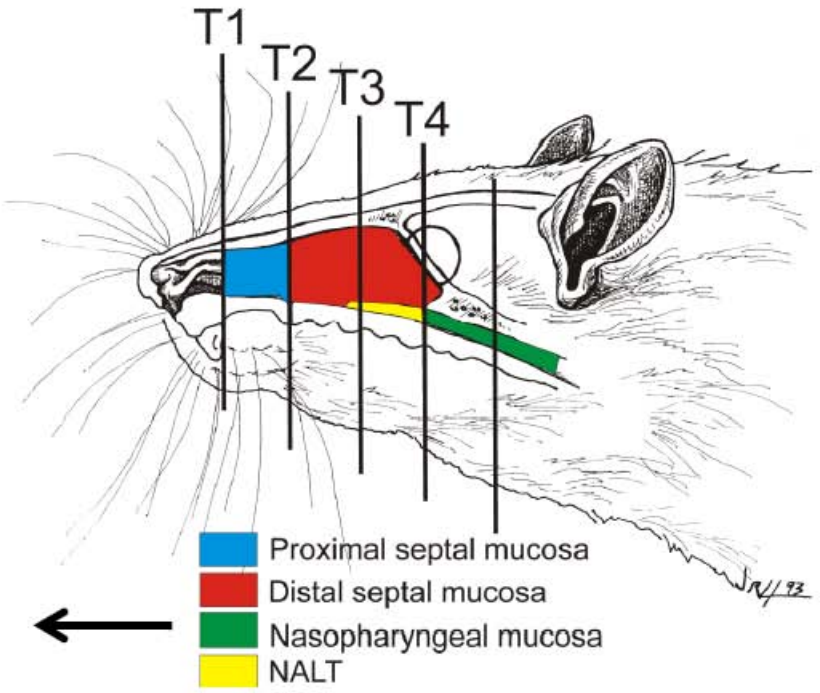




- Nasal airways flushed and stored in RNAlater®
- Nasal mucosal tissues microdissected from airways
- RNA extracted from site-selected nasal mucosal tissues
- Conducted PCR-arrays (pooled cDNA by group) and qRT-PCr assays (cDNA from individual rats)

Gene Expression: PCR-Array





10,000 ppm time-response data

Gene Expression: qRT-PCR

769 DS 18S	Concentration-Response					Time-Response				
						Day 1	Day 3	Day 5	Day 10	Day 20
	0 ppm	10 ppm	50 ppm	300 ppm	10k ppm	10k ppm	10k ppm	10k ppm	10k ppm	10k ppm
Vic	1.00+/-0.01	-1.01+/-0.01	1.06+/-0.04	-1.10+/-0.17	1.01+/-0.02	-1.02+/-0.02	-1.02+/-0.04	-1.03+/-0.02	-1.06+/-0.03	1.01+/-0.02
Chia	1.00+/-0.12	1.55+/-0.30	1.53+/-0.21	1.42+/-0.34	1.90+/-0.22	-1.15+/-0.12	1.43+/-0.14 *	-1.25+/-0.14	1.52+/-0.32	**1.90+/-0.22 ***
YM1	1.00+/-0.04	1.24+/-0.16	1.19+/-0.10	1.42+/-0.32	**2.41+/-0.46 ***	-1.01+/-0.02	1.06+/-0.08	1.01+/-0.07	1.41+/-0.21	2.41+/-0.46
Gob5	1.00+/-0.20	32.92+/-28.22	22.52+/-10.35	**43.32+/-22.01 ***	**165.62+/-71.99 ***	3.40+/-2.55	-1.06+/-0.29	**5.47+/-1.29 ***	**17.66+/-10.34 ***	**165.62+/-71.99 ***
EGFR	1.00+/-0.02	-1.10+/-0.03	-1.15+/-0.04	1.14+/-0.24	-1.10+/-0.04	-1.05+/-0.03	1.18+/-0.03	-1.17+/-0.04	-1.05+/-0.02	-1.10+/-0.04
IFNg	1.00+/-0.22	1.17+/-0.24	1.50+/-0.36	1.08+/-0.14	-1.52+/-0.35	-1.11+/-0.10	1.33+/-0.34	-1.86+/-0.39	-1.28+/-0.20	-1.52+/-0.35
IL4	1.00+/-0.12	-1.21+/-0.10	-1.08+/-0.18	1.15+/-0.17	-1.30+/-0.18	-1.28+/-0.19	-1.38+/-0.17	-1.06+/-0.22	-1.38+/-0.04 *	-1.30+/-0.18
IL5	1.00+/-0.11	2.26+/-0.70	**2.42+/-0.26 ***	**3.18+/-0.96 ***	**6.17+/-1.31 ***	-1.17+/-0.09	**3.00+/-0.46 ***	1.33+/-0.18	**2.96+/-0.38 ***	**6.17+/-1.31 ***
IL13	1.00+/-0.17	**4.12+/-1.32 ***	**4.76+/-0.88 ***	**5.79+/-2.17 ***	**15.51+/-4.49 ***	-1.88+/-0.41	2.43+/-0.67	1.29+/-0.27	**6.75+/-1.64 ***	**15.51+/-4.49 ***
Muc5 AC	1.00+/-0.63	8.96+/-4.75	5.17+/-1.12	**8.04+/-2.78 ***	**13.84+/-4.97 ***	-12.85+/-5.72	-6.30+/-1.99	5.07+/-4.17	1.78+/-1.07	**13.84+/-4.97 ***
NOS2	1.00+/-0.11	1.74+/-0.46	1.47+/-0.31	1.94+/-0.59	1.14+/-0.07	1.17+/-0.24	-1.11+/-0.35	-1.86+/-0.28	-1.01+/-0.12	1.14+/-0.07
TNFa	1.00+/-0.03	1.04+/-0.10	1.01+/-0.02	1.33+/-0.17	1.19+/-0.08	1.03+/-0.10	1.19+/-0.04	-1.59+/-0.14	1.20+/-0.05	1.19+/-0.08
ACTB	1.00+/-0.01	1.01+/-0.02	-1.04+/-0.03	1.13+/-0.14	1.01+/-0.02	-1.01+/-0.02	1.01+/-0.02	1.03+/-0.01	1.05+/-0.02	1.01+/-0.02
GAPD	1.00+/-0.01	1.00+/-0.01	-1.02+/-0.01	1.11+/-0.15	-1.02+/-0.02	1.03+/-0.01	-1.01+/-0.03	-1.01+/-0.01	1.00+/-0.02	-1.02+/-0.02

- qRT-PCR analysis of selected gene expression (increased decreased)
- Concentration- and Time-dependent effects on site-specific gene expression
- Data represent fold-difference compared to 0 ppm (control) mucosal samples from the same site and experimental group (distal septum data)

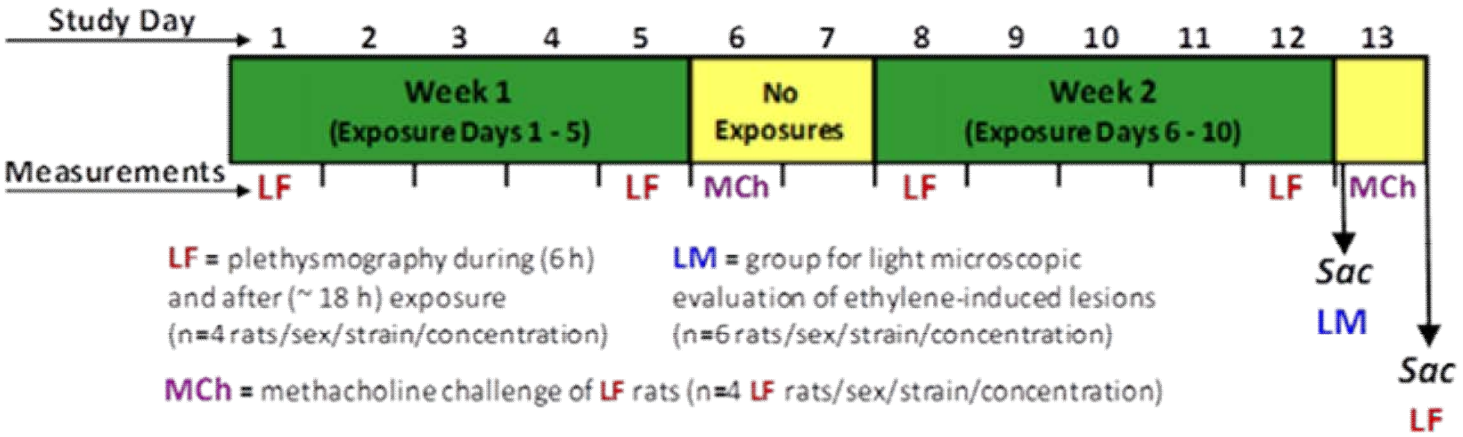
Interim Summary 2: What did we learn?

- An increase in eosinophils (mid-septum) and in stored mucosubstances (nasopharynx) was detected in all exposure groups after 4 wks exposure to 10,000 ppm ethylene
  - Eosinophils significantly increased after 2 wks exposure
  - Mucosubstances significantly increased after 1 wk exposure
- Cell death and epithelial regeneration not part of the pathogenesis of ethylene-induced nasal lesions
- No increase in serum IgE or IgG isotypes - does not support immune-mediated mechanism
- Nasal lesions in rats exposed 4 wks to 300 or 10,000 ppm were not present in 13-wk recovery groups
- Enhanced gene expression of Th2 cytokines (IL-5, IL-13), Ym1/2, and airway mucin genes (Muc5AC) in ethylene-exposed rats

ACC Expert Panel Summary

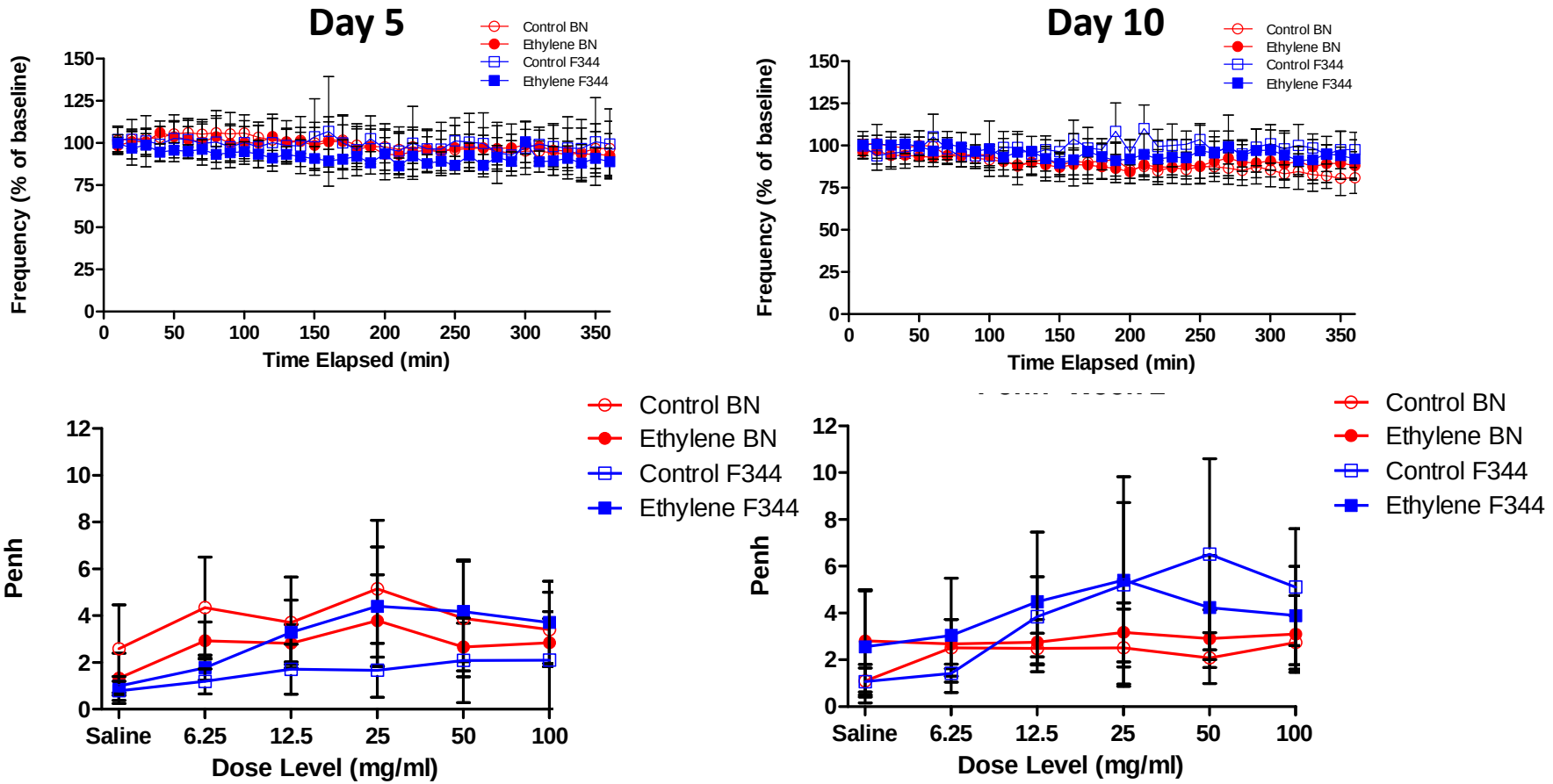
- All previous exposures have been based on standard toxicologic investigation inhalation exposure paradigm
- Animal models of respiratory sensitization use an **episodic exposure paradigm to induce sensitization** with **a separate single challenge (elicitation) exposure** to assess responses in naïve and "sensitized" groups
- Animal models of respiratory sensitization use mice or rats predisposed to allergic (Th2) responses
- Recommended **comparison of Brown-Norway (BN) rat responses** (histopathology, morphometry, gene expression, pulmonary function) to **ethylene**, and to a **known respiratory sensitizer**, and a **known irritant**

BN and F344 Rat Strain Comparison



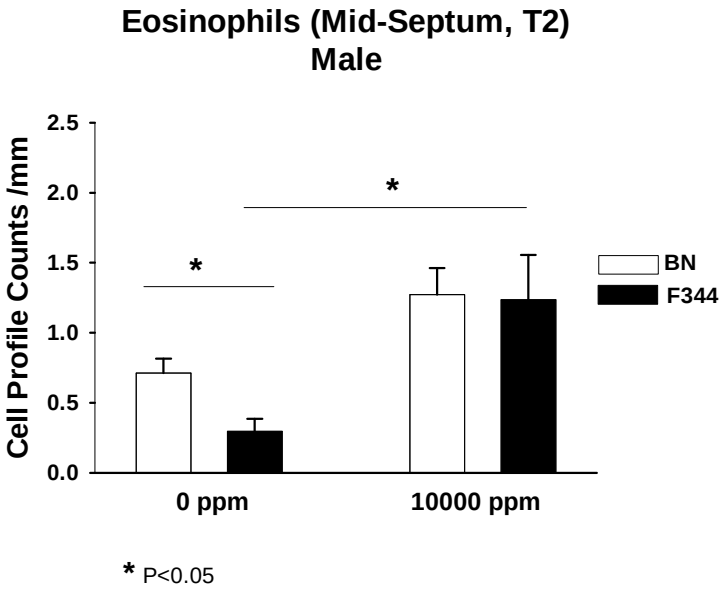
- BN and F344 rats exposed 6 h/day, 5 days/week for up to 2 wks to 0 or 10,000 ppm ethylene
  - n=10 rats/sex/strain/exposure concentration
- Lung Function (LF) Group – Hotchkiss/TERC
  - n=4/sex/strain/concentration
- Light Microscopy (LM) Group – Harkema/MSU
  - n=6/sex/strain/concentration

Functional Pulmonary Measurements

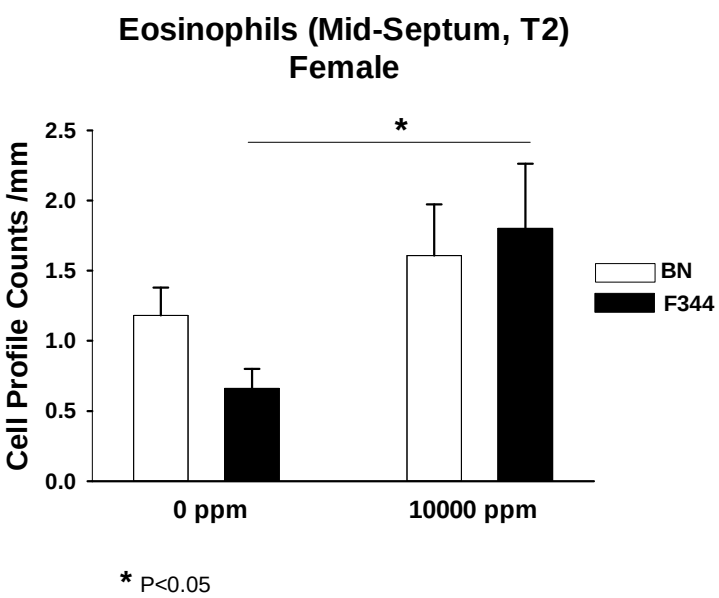


- No effect during or after exposure (f, MVb, TVb, Penh)
- Slight increase in airway reactivity in F344 rats after 1 week of exposure

Eosinophils: Mid-Septum



* P<0.05



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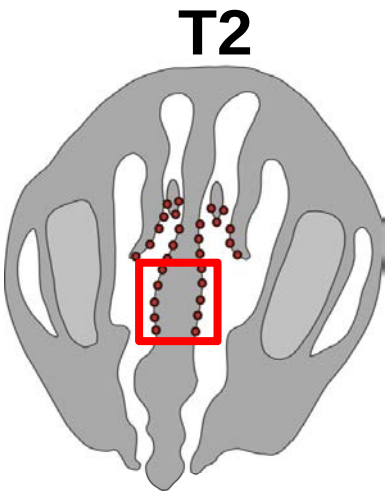
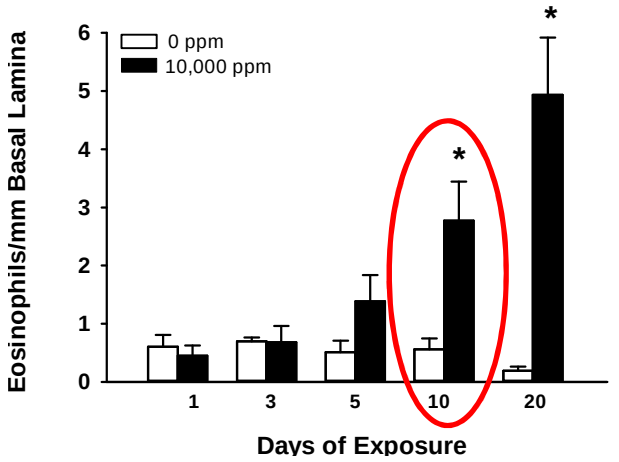
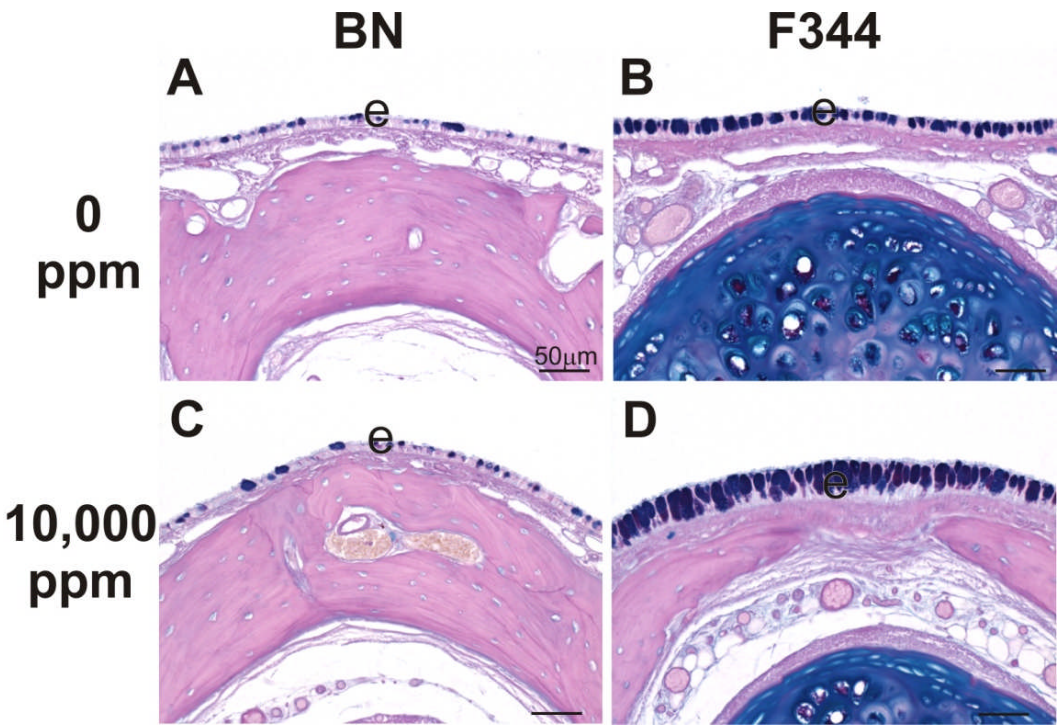


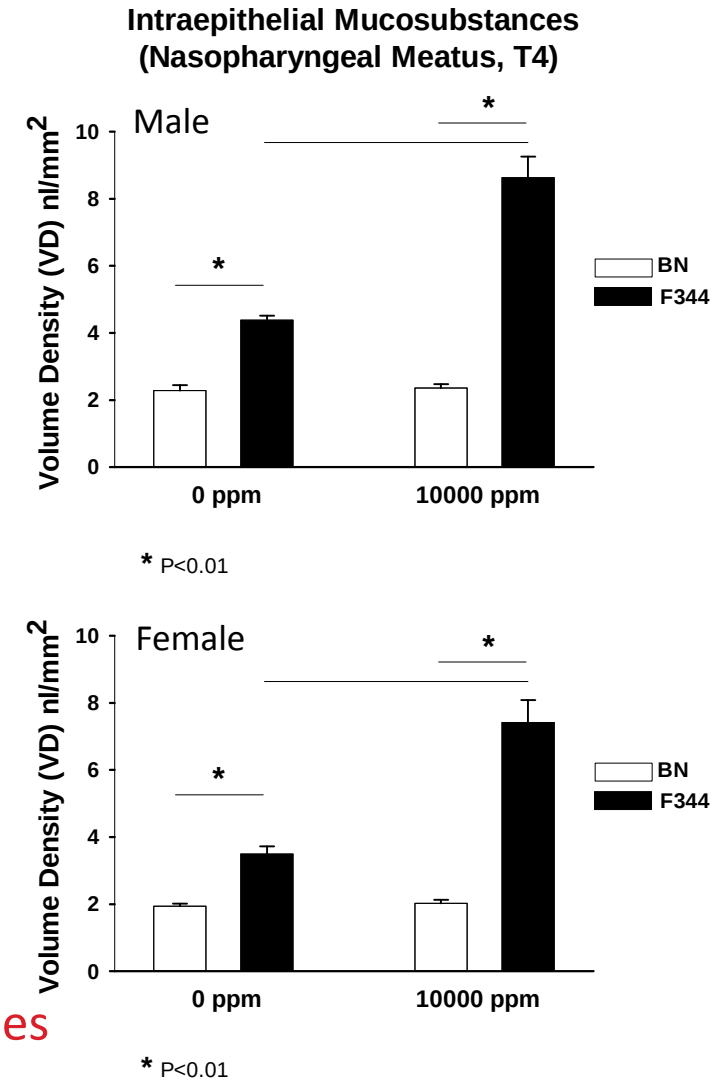
- 2-wk exposure to 10,000 ppm ethylene induced eosinophil influx in F344 but not BN rats
  - Control BN rats have slightly higher numbers of mucosal eosinophils than F344 control rats
  - Similar magnitude of eosinophilic inflammation in nose-only and whole-body exposed F344 rats



MOA Study: T2 Eosinophils
Whole-body Exposure

Ethylene-Induced Mucous Cell Metaplasia (MCM)





- 2-wk exposure to 10,000 ppm ethylene induced MCM only in F344 rats
  - F344 control rats have more stored mucosubstances in nasopharynx epithelium than BN control rats

Interim Summary 3: What did we learn?

- Exposure mode (nose-only vs whole-body) does not affect ethylene-induced nasal epithelial remodeling or eosinophilic inflammation in F344 rats
- The BN rat is not a suitable animal model for future MOA studies on these endpoints
  - No ethylene-induced eosinophilic inflammation or mucus cell hyperplasia/metaplasia
- There are pronounced strain-related differences in nasal epithelial responses to ethylene exposure
  - F344 rats >> Wistar rats >>> Brown Norway rats

Summary

- Repeated inhalation of ethylene causes eosinophilic rhinitis with epithelial remodeling (MCM, Hyalinosis) and concurrent Th2 cytokine expression in the nasal mucosa of F344 rats
- Nasal responses in F344 rats but not in BN rats
- Ethylene-induced histopathologic effects limited to respiratory epithelium lining the nasal airways
  - No effects on larynx, trachea, or lung
- The potential mechanisms by which ethylene specifically induces these morphologic and molecular responses has yet to be determined

Acknowledgements

- **American Chemistry Councils's Olefins Panel**
  - Dr. Marcy Banton, E/P Workgroup Chair
- **The Dow Chemical Company - Toxicology and Environmental Research and Consulting (TERC)**
  - Drs. Hotchkiss, Pottenger and Greenwood, Shannon Krieger MS and TERC respiratory toxicology lab
- **Michigan State University**
  - Drs. Harkema and Brandenberger

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