LABORATORY ANIMAL MODELS AND RESEARCH STRATEGIES FOR ASSESSING THE RELATIVE HEALTH HAZARDS OF COMPLEX COMBUSTION/PYROLYSIS PRODUCTS

Joe Mauderly et many al.

- **Only LRRI studies**
- **Only research published, in press, or submitted**
- **Excludes:**
  - **Immunosuppression by nicotine**
  - **Retarded particle clearance**

NATIONAL ENVIRONMENTAL RESPIRATORY CENTER

Because You Never Breathe Only One Pollutant !

Lovelace Respiratory Research Institute

Albuquerque, NM

TOPICS

1. **Experience with chronic and subchronic bioassays of inhaled cigarette smoke**
   - Lung cancer in rats and mice
   - Tumorigenesis in A/J mice
   - COPD in rats and mice

3. **Assays of immunological effects**
   - Effect of fetal exposure on postnatal allergic airway sensitization
   - Effect of fetal exposure on resistance to respiratory infection

4. **Health assays in use to assess hazards of other inhaled complex source emissions**
   - Bio-directed fractionation
   - Identifying putative causal agents by multivariate analysis

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INHALATION CARCINOGENESIS BIOASSAY IN COMMON STRAINS OF RATS AND MICE

Animals:

Male and female F344 rats, 6 wk old, n = 81-178/gender/group

Female B6C3F1 mice, 8 wk old, n = 330/group

Exposure:

Mainstream smoke from unfiltered cigarettes puffed 2/min at 70 ml

Rats : 100 (LS) or 250 mg (HS) TPM/m^3 (1R3)

Mice : 250 mg TPM/m^3 (2R1)

HEPA-filtered ambient air as negative control

Maintained and exposed in whole-body exposure chambers

Exposed 6 hours/day, 5 days/week, for 30 months

Abbreviations:

CS = cigarette smoke
MS = mainstream CS
SS = sidestream CS
FA = filtered air control

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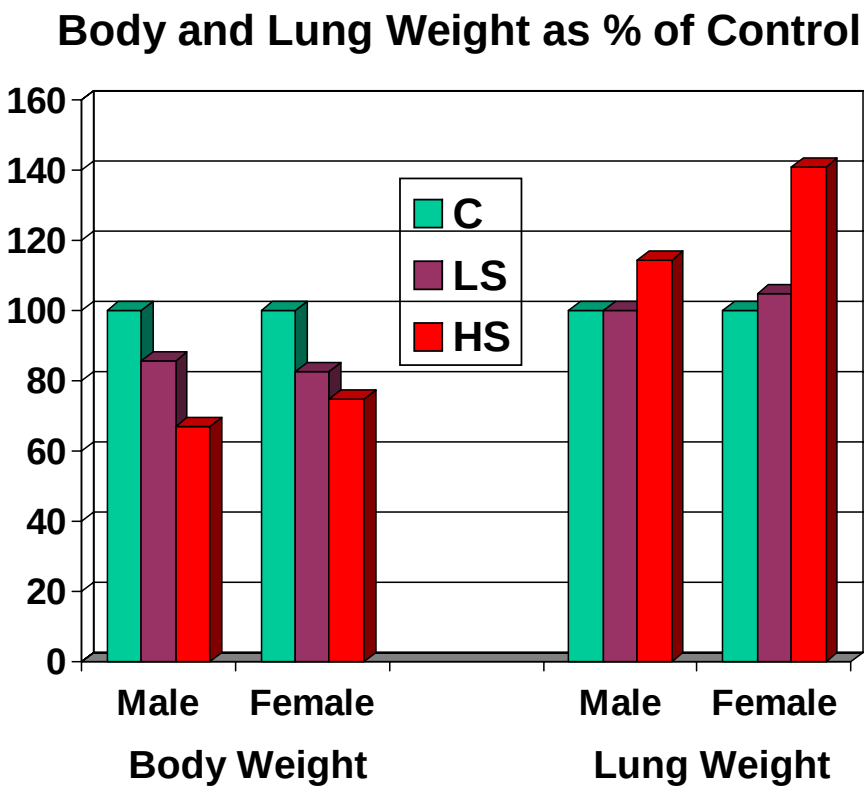
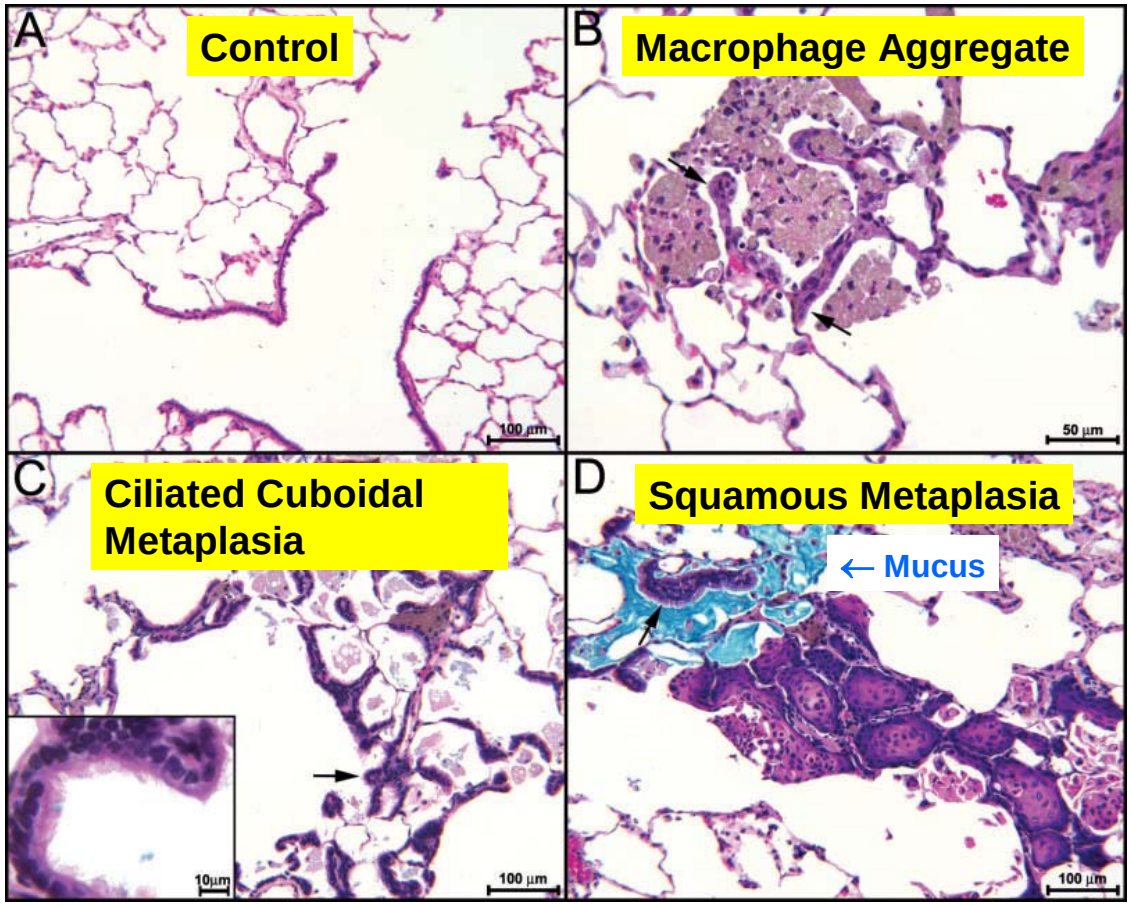
CHRONIC WHOLE-BODY EXPOSURE INDUCED NON-CANCER CHRONIC LUNG DISEASE

Alterations in Rats at End of Exposure

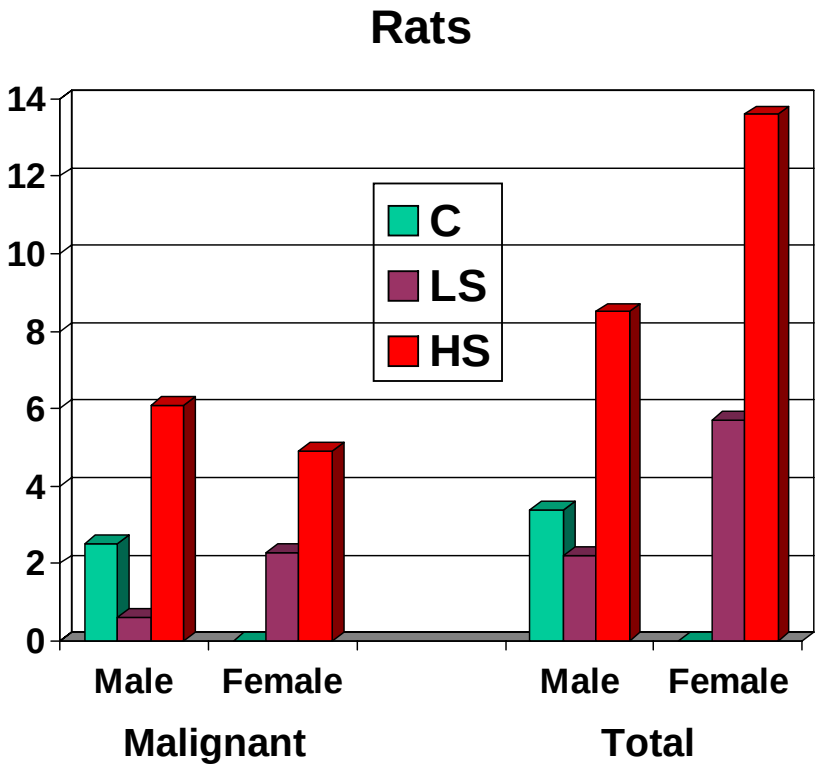


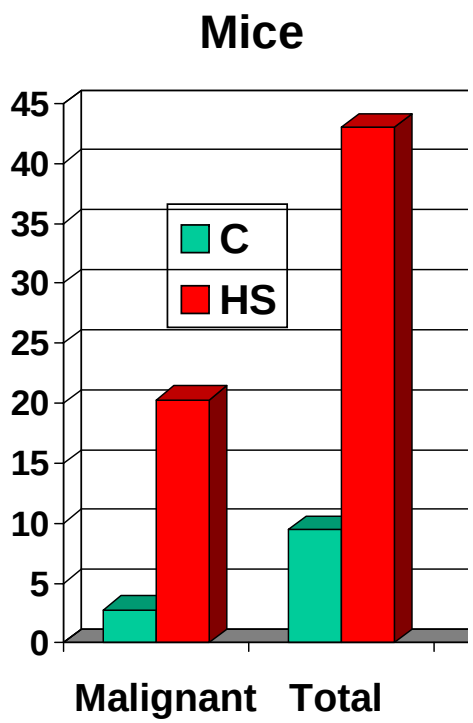


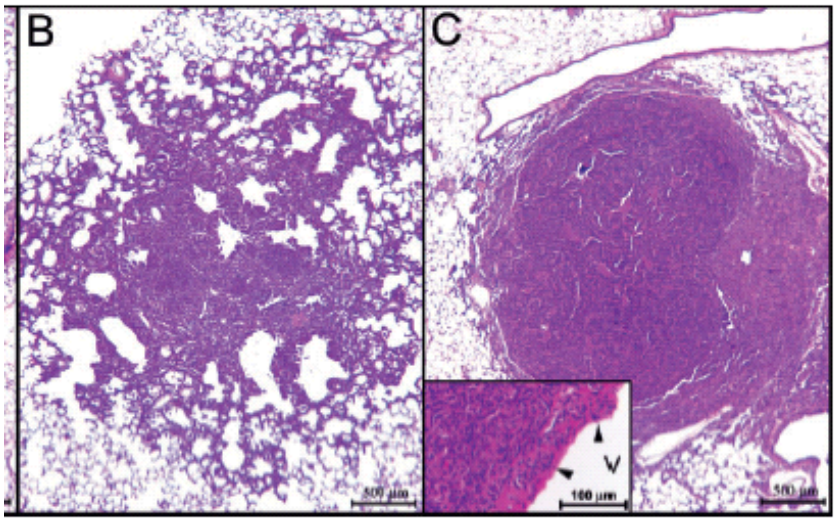
[Mauderly et al., *Toxicol. Sci.* 81: 280-292, 2004]

LUNG TUMOR INCIDENCE WAS INCREASED

% Incidence of Lung Neoplasia







B.A Adenoma B.A. Carcinoma

- Whole-body exposure produces tumors
- Mice had greater response
- Bronchoalveolar carcinoma is now most predominant tumor type in human smokers

[Mauderly et al., *Toxicol. Sci.* 81: 280-292, 2004]
[Hutt et al., *Carcinogenesis*, in press, 2005]

THERE ARE SIMILARITIES OF MOLECULAR LESIONS IN LUNG TUMORS IN RODENTS AND HUMANS

Genetic Lesions e.g., Mutational spectra of codon 12 K-ras

Humans: 181 tumors from smokers
B6C3F1 mice: 45 tumors from exposed

- Mutations in both species were predominantly transversions
- Overlap of mutational spectra:

	TGT	GTT	GAT	CGT	AGT
Mouse	-0-	31%	20%	9%	4%
Human	35%	13%	24%	4%	15%

Epigenetic Lesions e.g., Hypermethylation of DAP-K and RAR-β

- Involved in cell differentiation and apoptosis

  DAP-K = death-associated protein - kinase
  RAR-β = retinoic acid receptor - beta

- In tumors from both humans and mice:

  These genes often inactivated by promoter region hypermethylation

  Affected similarly in tumors from smokers and non-smokers

[Pulling et al., *Cancer Res.* 63: 4842-4848, 2003]
[Vuillemenot et al., *Carcinbogenesis* 25: 623-629, 2004]
[Hutt et al., *Carcinogenesis*, in press, 2005]

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EXPERIENCE WITH A/J TUMORIGENESIS ASSAY

The Assay (e.g., Witschi et al. *Toxicol. Sci.* 68: 332-330, 2002):

1. Expose A/J mice subchronically
2. Hold mice without exposure for a few months
3. Evaluate lung adenoma incidence and multiplicity

Our Experience:

1. **Finch et al. Cancer Lett. 99: 161-167, 1996**
   - Female A/J exposed 6 hr x 5 d/wk x 26 wks and held for 5 wks
   - MS at 250 mg TPM/m^3 (1R3)
   - One-half of controls and exposed also treated with NNK

   Results:

   CS did not increase adenomas

   Increase caused by NNK was not further increased by cig. smoke

2. **Reed et al. Inhal. Toxicol. 16: 177-193, 2004** (diesel only)
   - Male and female A/J exposed 6 hr x 7 d/wk x 6 mo and held for 6 mo
   - Diesel emissions or hardwood smoke at 1000, 300, 100, or 30 µg PM/m^3

   Results:

   Neither exposure increased adenomas

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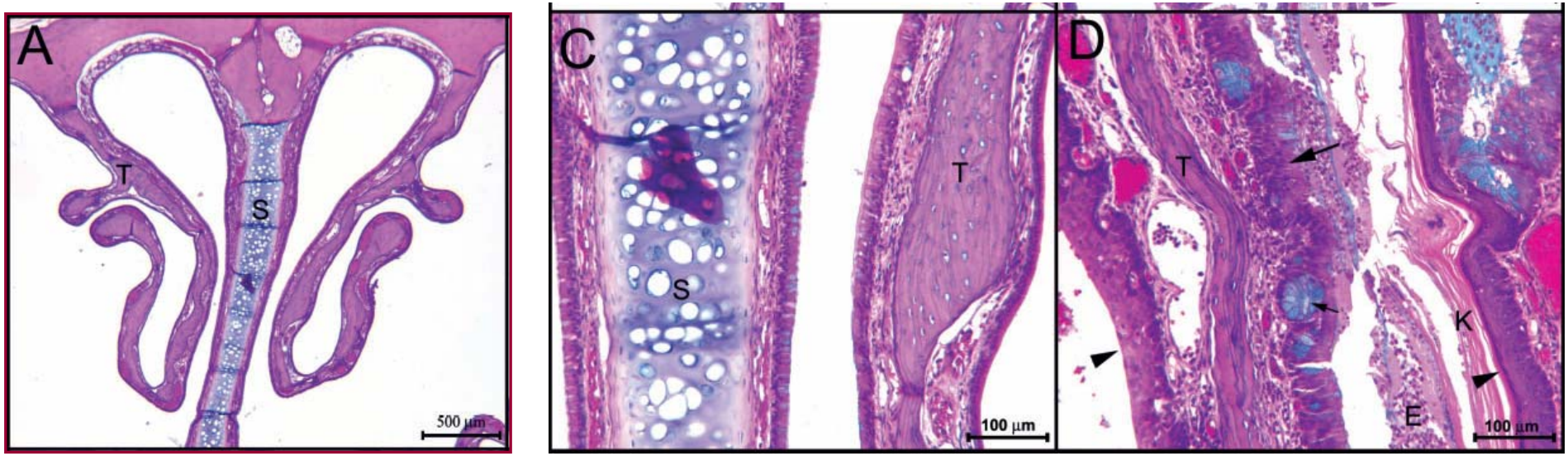
CS EXPOSURE CAUSES CHRONIC, PROGRESSIVE NASAL INFLAMMATION, METAPLASIA, AND NEOPLASIA

Non-Neoplastic Nasal Alterations in Rats at End of Chronic Exposure



Low Power View **Control** **Exposed**

Neutrophilic inflammmation with exudates **(E)**
Epithelial hyperplasia **(large arrows)**
Mucous metaplasia **(small arrows)**
Squamous metaplasia **(arrowheads)**
Keratinization **(K)**

- **Nasal epithelium at this location mimics large airway epithelium in humans**
- **Best rat/mouse model of increased mucus production**

[Mauderly et al., *Toxicol. Sci.* 81: 280-292, 2004]

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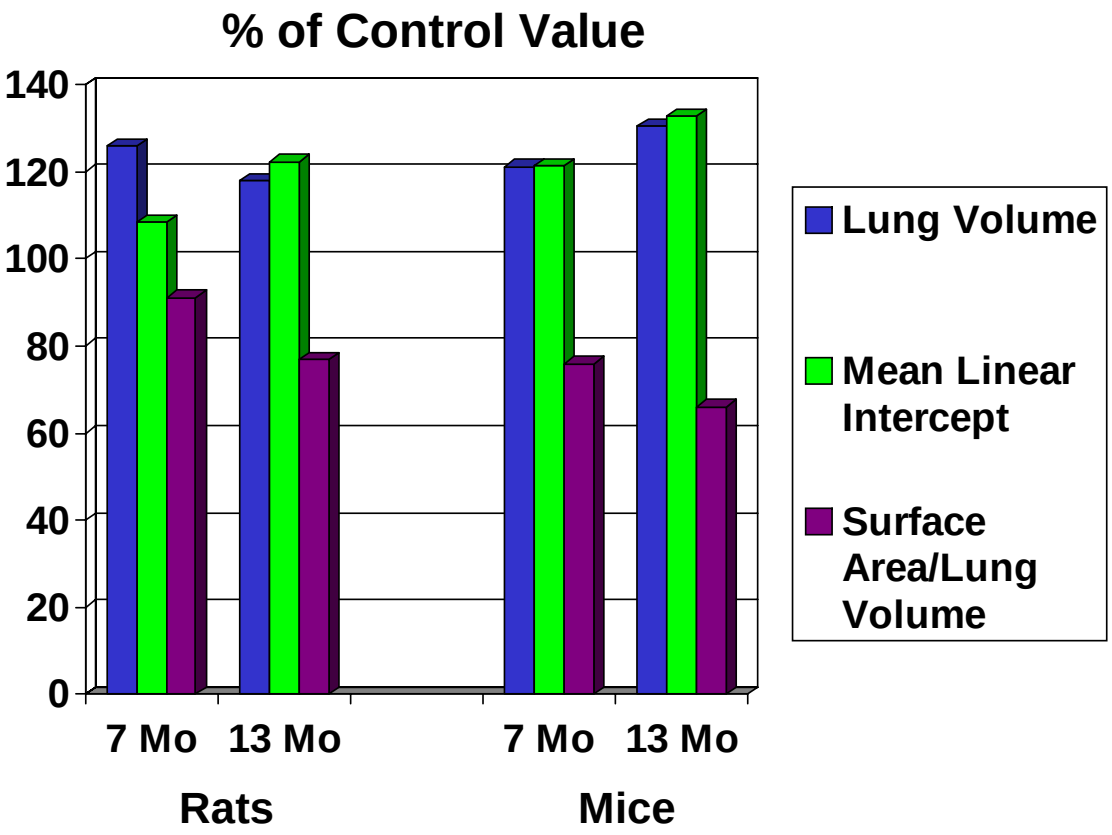
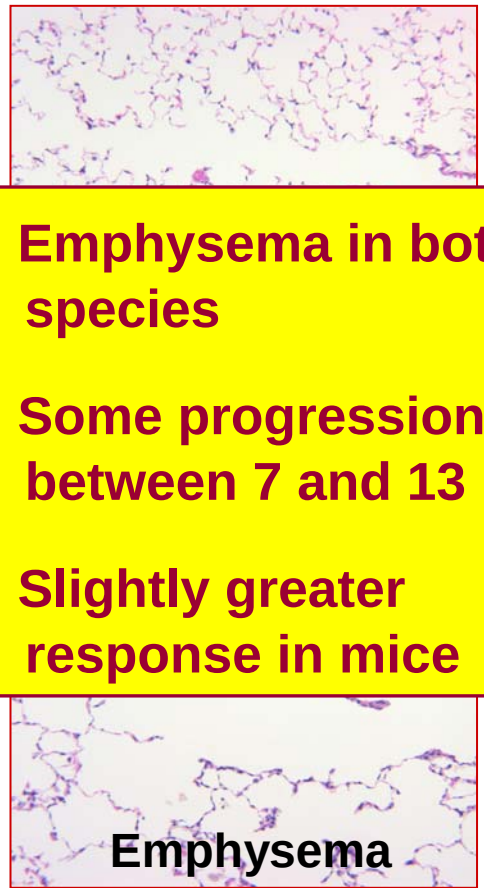
RODENT MODELS OF COPD FROM SUBCHRONIC EXPOSURE TO MAINSTREAM CIGARETTE SMOKE

Effect of Species and Exposure Time on Emphysema

- Female F344 rats and B6C3F1 mice
- Exposed 6 hr x 5 d/wk for 7 or 13 months to MS at 250 mg TPM/m^3 (2R1)
- Emphysema by histopathology and morphometry





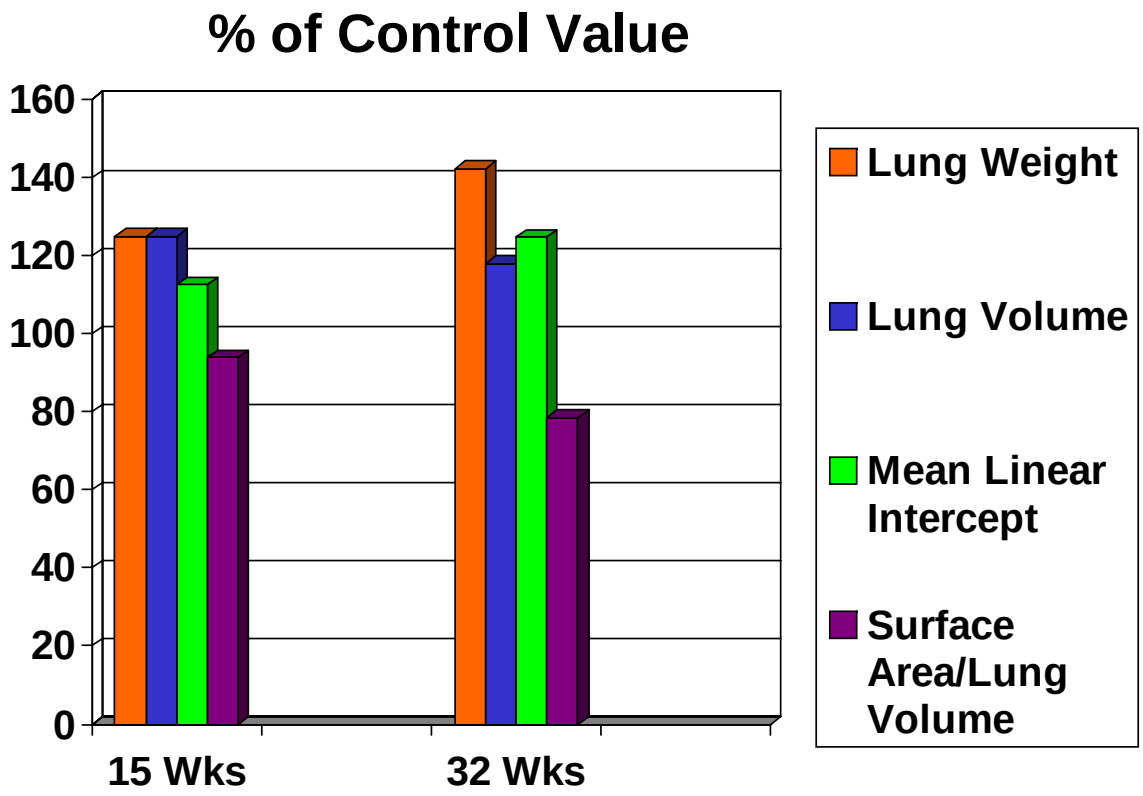
- Emphysema in both species
- Some progression between 7 and 13 mo
- Slightly greater response in mice

[March et al., *Toxicol. Sci.* 51: 289-299, 1999]

RODENT MODELS OF COPD

Effect of Exposure Time on Emphysema in Mice

- Female B6C3F1 mice
- Exposed 6 hr x 5 d/wk for 15 or 32 weeks to MS at 250 mg TPM/m^3 (2R1)
- Emphysema by histopathology and morphometry



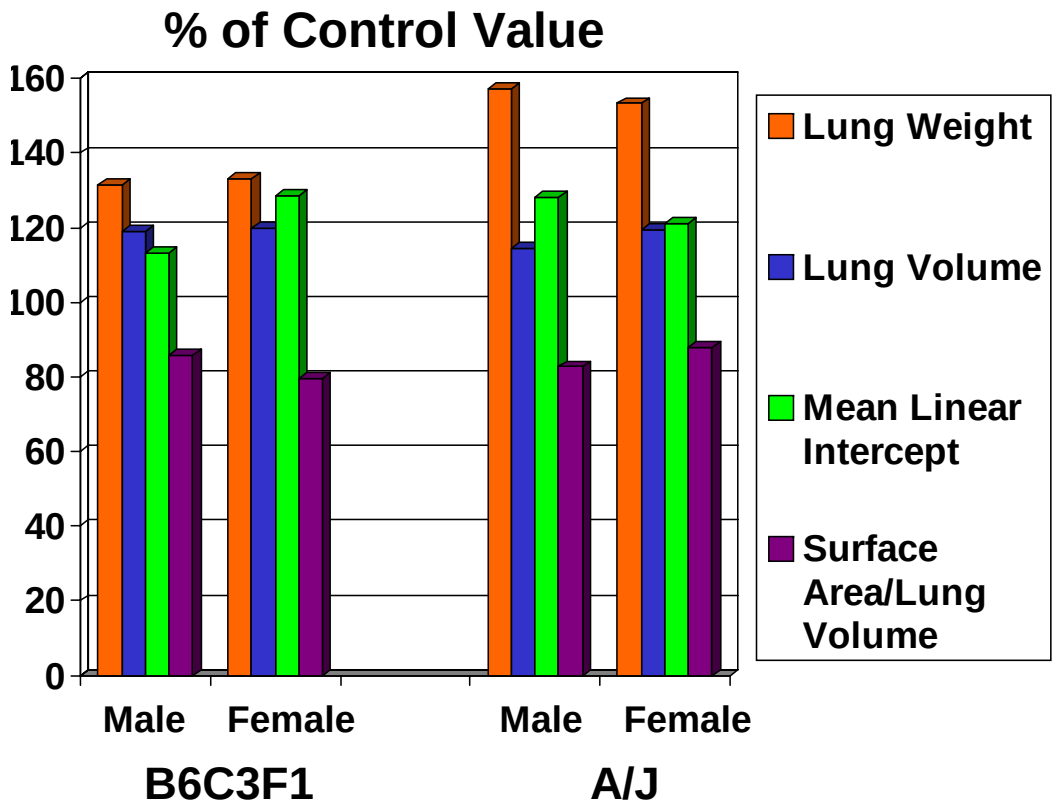
- 15 weeks produces evidence of emphysema
- Modest progression of all changes except lung volume

[March et al., *Inhal. Toxicol.* 14: 1187-1213, 2002]

RODENT MODELS OF COPD

Susceptibility of Mouse Strains and Genders

- Male and Female **B6C3F1** and **A/J** mice
- Exposed 6 hr x 5 d/wk for **15 weeks** to MS at 250 mg TPM/m^3 (2R1)
- Emphysema by histopathology and morphometry



- Overall, neither strain nor gender made a large difference
- A/J had greater change in lung weight
- Authors cited A/J as more responsive on basis of Lm/BW, which was largely a function of BW

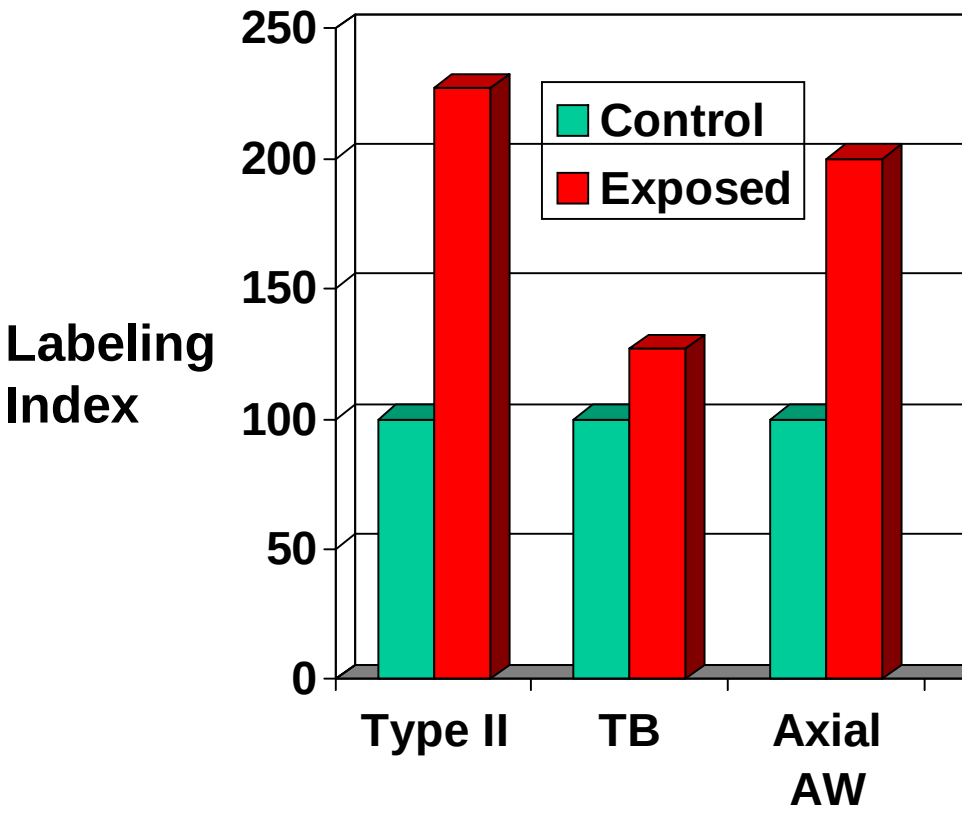
In this study, all-trans-retinoic acid had no effect on emphysema

[March et al., *J. COPD.* In press, 2005]

RODENT MODELS OF COPD

Epithelial Proliferation and Airway Mucosubstances

- Male and Female F344/N rats
- Exposed 6 hr x 5 d/wk for 2 weeks to MS at 250 mg TPM/m^3 (1R3)
- Cell proliferation by BrdU labeling
- Differential stain and stereology for mucosubstance type and amount



- 2 weeks exposure caused epithelial cell proliferation
- 2 weeks recovery reduced labeling index
- Mucosubstance was not increased in axial airway, but shifted to more acid type

[March et al., *Toxicol. Appl Pharmacol.* 161: 171-179, 1999]

RODENT MODELS OF IMMUNOLOGICAL EFFECTS

Effect of Fetal and Postnatal Exposure on Allergic Airway Hyperresponsiveness in Genetically Predisposed Mice

1. F BALB/c bred to M DO11.10 OVA T cell receptor hemizygous (+/–)
2. Exposed 6hr x 7 d/wk during gestation to MS at 100 mg TPM/m^3 (2R1)
3. Exposed young for 4 wks to FA or SS 6 hr x 5 d/wk at 5 mg TPM/m^3
4. Exposed young for 6 wks to FA, Ovalbumin (aerosol 6 hr x 5 d/wk at 5 mg/m^3), SS, or OVA+SS
5. Measured airway Hyperresponsiveness (AHR) to methacholine, OVA-specific IgG & IgE, and BAL cells

Results in predisposed (+/–) and non-predisposed (–/–):

SS ↑ AHR in both –/– and +/–

SS + OVA ↑ AHR in both –/– and +/–

OVA ↑ AHR only in +/–

IgE and IgG in +/– was lower in SS+OVA than in OVA

- Postnatal SS increased airway reactivity in mice with and without genetic predisposition
- Effect not dependent on eosinophilia or allergic antibody

[Barrett et al., *Am. J. Respir. Crit. Care Med.* 165: 1410-1418, 2002]

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RODENT MODELS OF IMMUNOLOGICAL EFFECTS

Effect of Fetal Exposure to on Allergic Airway Hyperresponsiveness

1. **BALB/c mice exposed to FA or MS 6 hr x 7 d/wk at 103 mg TPM/m^3 (2R1) for 2 wk, then during breeding and gestation**
2. **Young exposed to FA or SS 6 hr x 7 d/wk at 5 mg TPM/m^3**
3. **Young instilled with Aspergillus extract at 15 wks of age, then AHR measured with methacholine & Penh at 48 hrs (+ other measures)**

Results:

AHR ↑ by fetal or fetal+postnatal exposure

Postnatal exposure alone did not ↑ AHR

Exposure did not affect AHR in adults

- **Fetal exposure increased airway reactivity by modulating lung cAMP through changes in phosphodiesterase-4D activity**
- **Effect was independent of inflammatory cell recruitment into the lung**

[Singh et al., *Am. J. Respir. Crit. Care Med.* 168: 342-347, 2003]

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RODENT MODELS OF IMMUNOLOGICAL EFFECTS

Resistance to Respiratory Viral Infection

- Newborn naïve (no in utero exposure) BALB/c mice
- Exposed 6 hr x 7 d/wk days 1 - 35 to FA or SS at 1.5 mg TPM/m^3 (1R4F)
- One-half infected with RSV A2 on days 7 and 28
- Evaluated at 2, 4, or 7 d after 1st RSV, and 4 or 7 days after 2nd RSV
  - RSV gene expression by RT-PCR
  - Clara cell secretory protein (CCSP) and mucus by IHC staining
  - Histopathology, BAL, cytokines in lung homogenate

Effects of RSV in <u>normal</u> mice:

Few effects 4 days after primary (1st) infection (most virus is cleared)

Marked effects 4 days after challenge (2nd) infection:

- Obvious viral expression (not cleared by 4 days)
- Neutrophilic inflammation (BAL and peribronchiolar)
- ↑ mucus production (mucous cell metaplasia)
- ↓ CCSP (a protective cellular protein)

[Barrett et al., *Am. J. Physiol. Lung Cell, Molec. Physiol.*, submitted 2005]

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  - RSV gene expression by RT-PCR
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  - Histopathology, BAL, cytokines in lung homogenate

Results:

SS alone (no RSV):

- ↓ IL-12 and IFN-γ

SS vs FA after 2nd RSV:

- ↑ RSV expression
- ↑ BAL eosinophils
- ↓ neutrophilic inflammation
- ↓ mucus production
- ↓ CCSP

- Postnatal SS reduced resistance to repeated RSV infection
- Postnatal SS altered immunological status

[Barrett et al., *Am. J. Physiol. Lung Cell, Mol. Physiol.*, submitted 2005]

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STRATEGIS FOR STUDYING REDUCED-HARM PRODUCTS ARE SIMILAR TO THOSE FOR STUDYING COMPLEX ENVIRONMENTAL POLLUTANT MIXTURES

Study every component individually ?

- Too many
- Combinations are important

Predict effects using structure-function models ?

- Don’t have sufficient data

Study combinations of components using factorial designs ?

- Becomes intractable beyond 3-4 components

Study example mixtures ?

- “Menu” of real or synthetic mixtures
- Can dissect by bio-directed fractionation

Apply multivariate analyses to data from different mixtures studied using identical protocols ?

- OK – if you have such a database

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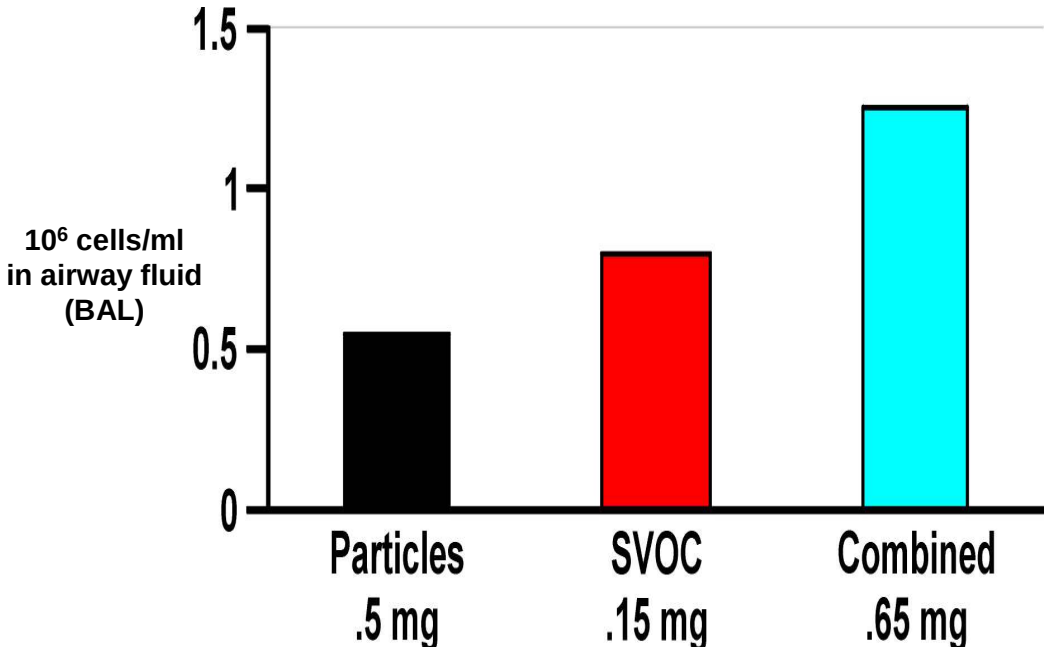
EXAMPLE OF FACTORIAL APPROACH USING IN VIVO BIODIRECTED FRACTIONATION

Importance of Vapor-Phase Semi-Volatile Organic Compounds in Traffic Tunnel Samples

1. Particles (filter mass) and vapor-phase SVOC (PUF-XAD) collected from traffic tunnel and instilled into rat lungs
2. Measured Inflammatory cells in airway fluid 24 hr later



- Vapor-phase SVOC caused majority of effect
- 4X more potent than PM per unit mass

[Seagrave et al., *Toxicologist* 60:192, 2001]

EXAMPLE OF A MULTIVARIATE APPROACH:

Toxic Components of Engine Emission Samples

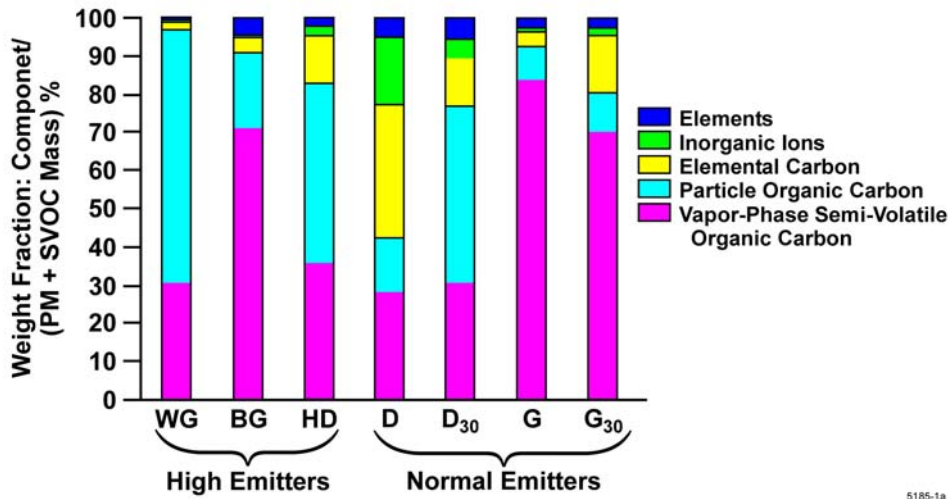
1. Collected emissions from in-use vehicles
2. PM and vapor-phase SVOCs collected during urban driving cycle
3. Analyzed composition in detail
4. Re-combined the 2 fractions in their original ratio
5. Measured:

   Ames mutagenicity

   Inflammation after instillation into rat lungs

Samples	
Gasoline (5)	G
Gasoline 30º	G_{30}
White smoker gas.	WG
Black smoker gas.	BG
Diesel (3)	D
Diesel 30º	D_{30}
High-emitter diesel	HD

Composition



[Seagrave et al. *Toxicol. Sci.* 70: 212-226, 2002]

[Zielinska et al., *J. Air Waste Man. Assoc.* 54: 1138-1150, 2004]

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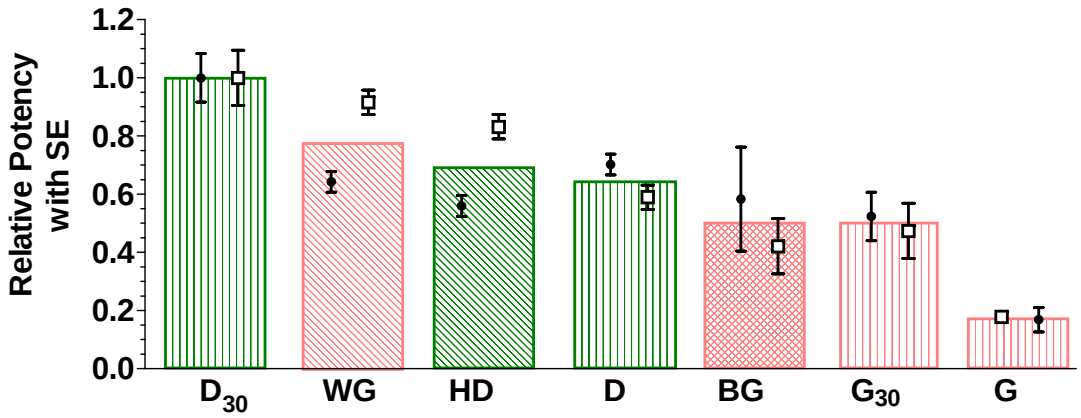
THERE WAS A 5-FOLD RANGE OF POTENCY AMONG THE SAMPLES AT EQUAL MASS DOSES

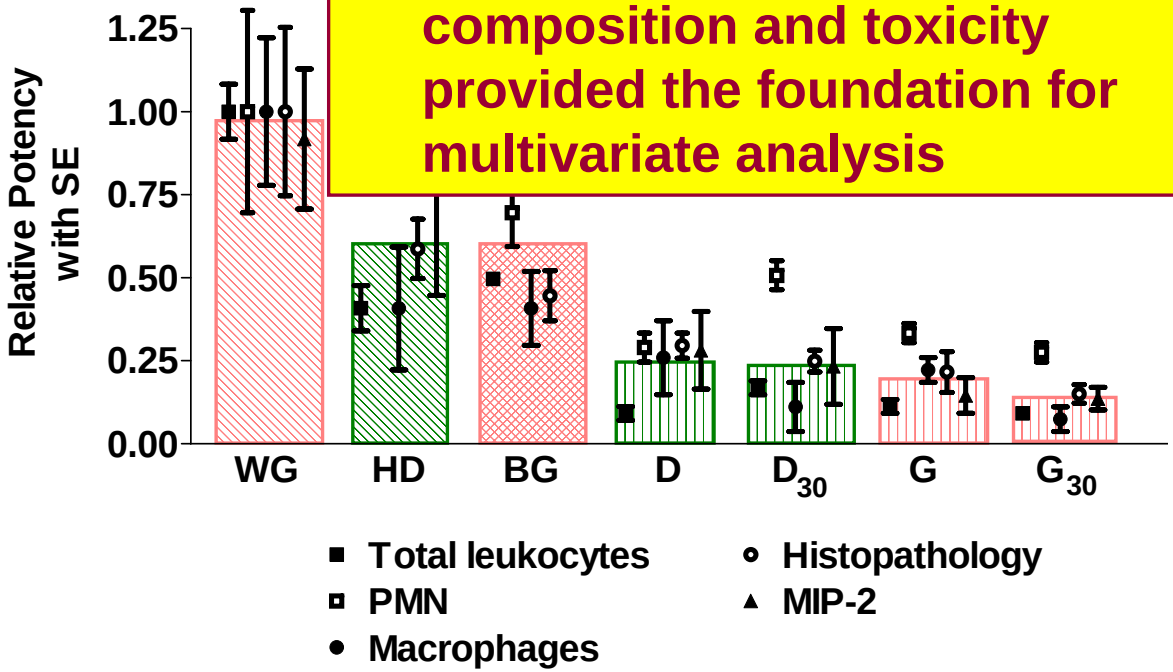
Ames Mutagenicity

(TA 100 ± S9)



Lung Inflammation

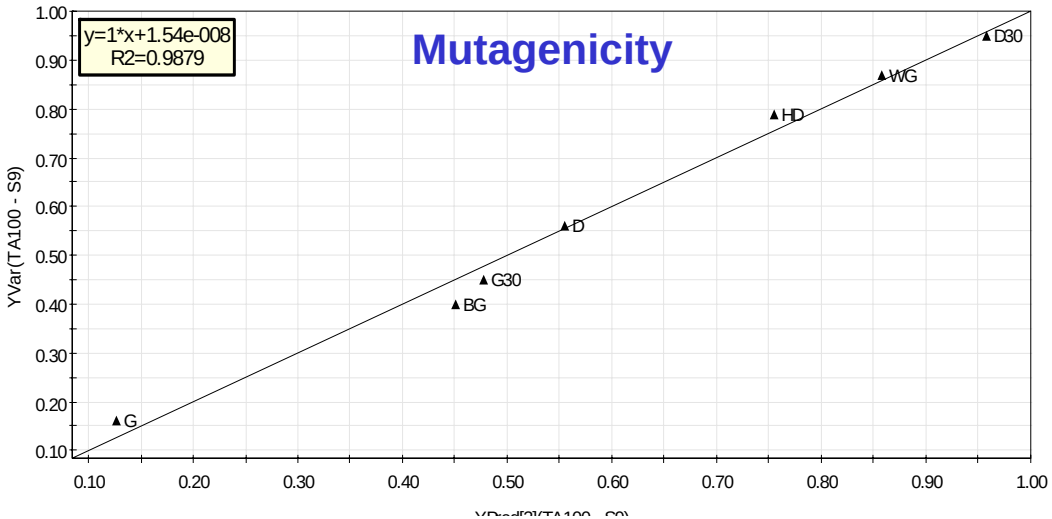
(5 parameters)

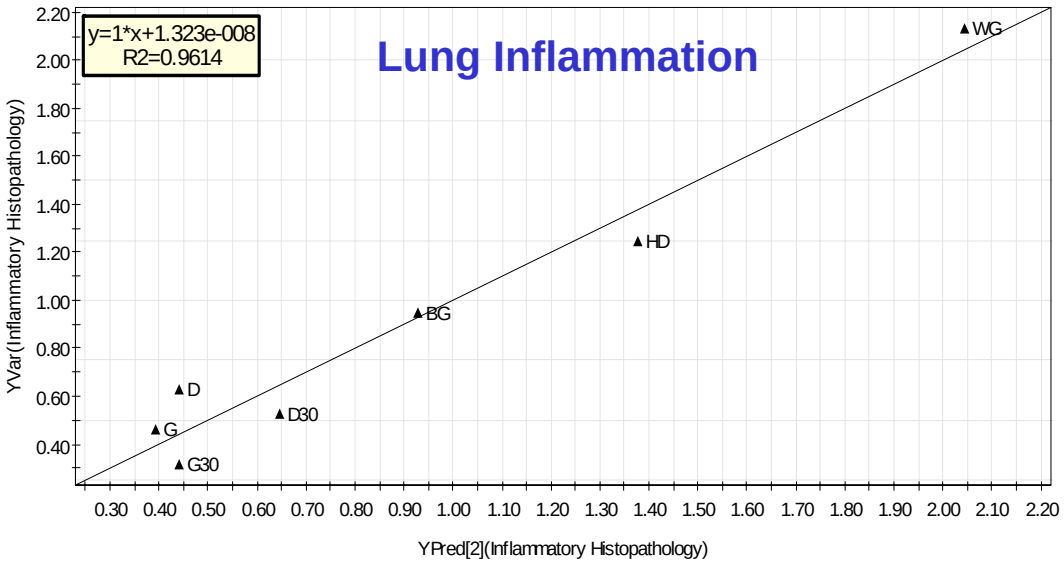


- **Substantial differences in <u>both</u> composition and toxicity provided the foundation for multivariate analysis**

[Seagrave et al. *Toxicol. Sci.* 70: 212-226, 2002]

PCA/PLS WAS USED TO IDENTIFY CHEMICAL SPECIES CO-VARYING MOST CLOSELY WITH EFFECTS





- **Obtained models that fit well to both responses**
- **Certain (but not total) nitro-aromatics co-varied most closely with mutagenicty** *(of course)*
- **Hopanes and Stearanes co-varied most closely with inflammation**

McDonald et al., *Environ. Health Perspect. 112: 1527-1538, 2004*]

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IDENTIFYING PUTATIVE CAUSAL COMPONENTS AMONG INHALED ENVIRONMENTAL MIXTURES

National Environmental Respiratory Center

Strategy:

- Create a composition-concentration-response data matrix by applying identical protocols to source-based complex atmospheres
- Use univariate and multivariate analyses to:
  - Detect significant adverse effects
  - Define exposure-response relationships
  - Compare effects of different exposures (sources)
  - Determine physical-chemical species co-varying most closely with different health responses
- Involve stakeholders in planning and support of research
- Base experimental design on expert consensus
- Vest approval authority in independent advisory body
- Make unique resources available to other investigators

[www.nercenter.org]

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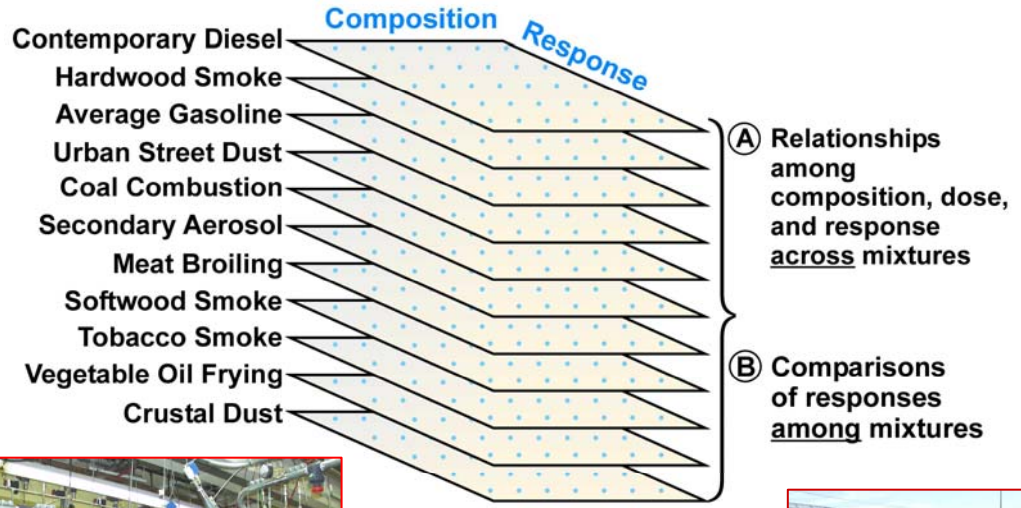
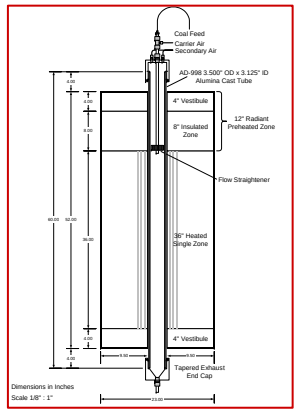
WE ARE BUILDING THE NERC COMPOSITION-CONCENTRATION-RESPONSE DATABASE





- Expose rodent models by whole-body inhalation
- Evaluate exposure-response relationships (4 treatment groups + control)
- Expose 6 hr x 7 d/wk for times ranging from a few days to 6 mo
- Characterize exposure at highest practical level of detail (>500 analytes)
- Measure health outcomes in 5 general categories (>200 parameters)

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NERC MEASURES OF BIOLOGICAL RESPONSES

General toxicity in F344/CrlBR rats and A/J mice

- Body & organ weights of rats and mice
- Hematology, clinical chemistry, clotting factors of rats
- Bronchoalveolar lavage of rats
- Histopathology of all major organs of rats
- Lung gene expression in rats by microarray

Pulmonary immune responses in BALB/C mice:

- Development of allergic responses (fetal exposure)
- Exacerbation of allergic responses in pre-sensitized

Resistance to respiratory infection in C57/BL6 mice

- Instilled *Pseudomonas aeruginosa*
- Instilled Respiratory Syncytial Virus

Cardiac effects in SHR/Crl rats & ApoE-deficient mice

- Heart rate and variability
- ECG Waveform abnormalities
- Heart and vessel histopathology

Carcinogenic potential in F344 rats and A/J mice:

- DNA Methylation (global and gene-specific methylation)
- Oxidative DNA damage (aldehydic lesions and 8-OhdG adducts)
- Micronuclei in circulating erythrocytes (mice only)

Ames mutagenicity of PM & vapor-phase SVOC

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THREE GENERAL CONCLUSIONS

There are lots of animal models that have been demonstrated to be responsive to different effects of tobacco smoke

- **I've only shown some that we have used – there are others**

There is no single model that is "best" – it depends on the effect of interest

- **There are multiple approaches even for each effect**
- **There is no single "accepted standard" for most effects**

There are "shortcuts" to screening potential reduced-harm products, but verification will be necessary

- **In vitro assays and "omics" can provide useful rapid and broad-based screening data – but are not confirmatory**
- **To be convincing, reduced harm will have to be demonstrated using in vivo models (expression of phenotype)**

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