

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

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/m³ (1R3)

Mice : 250 mg TPM/m³ (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

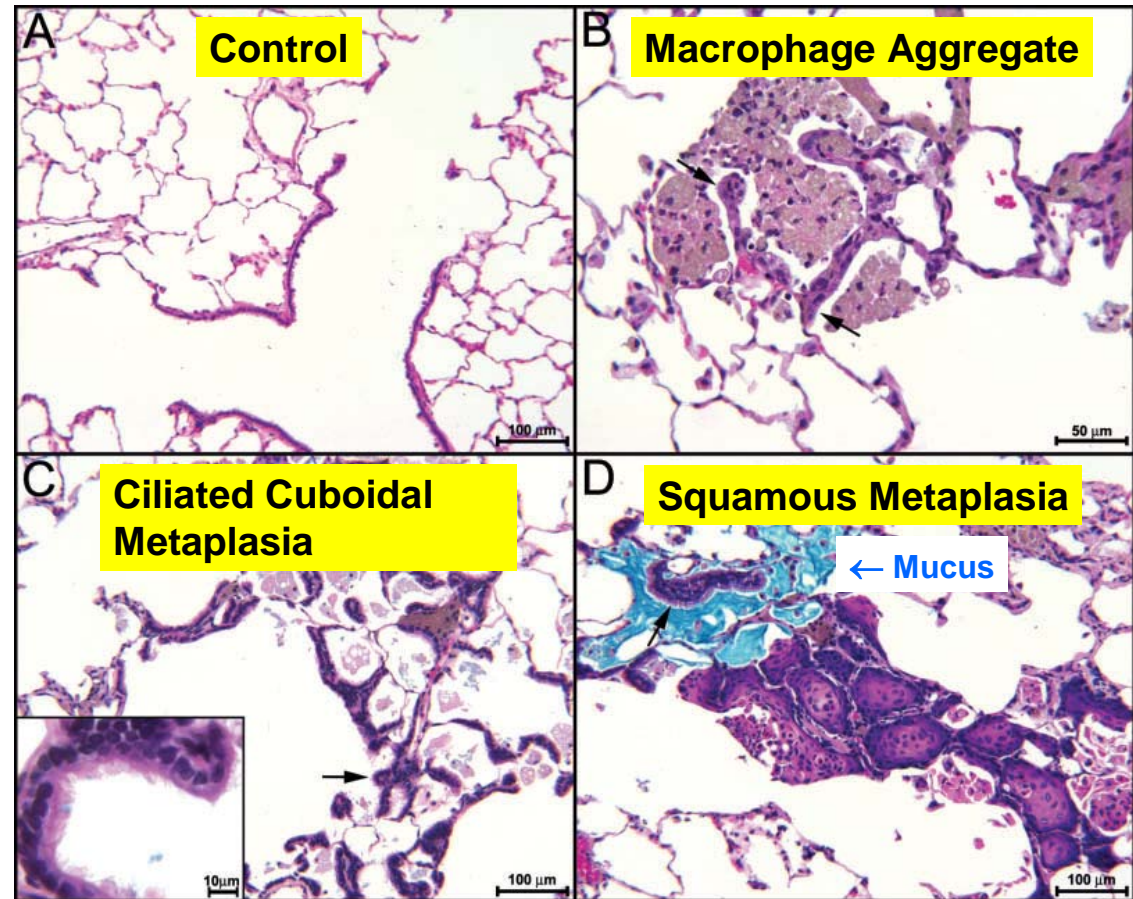
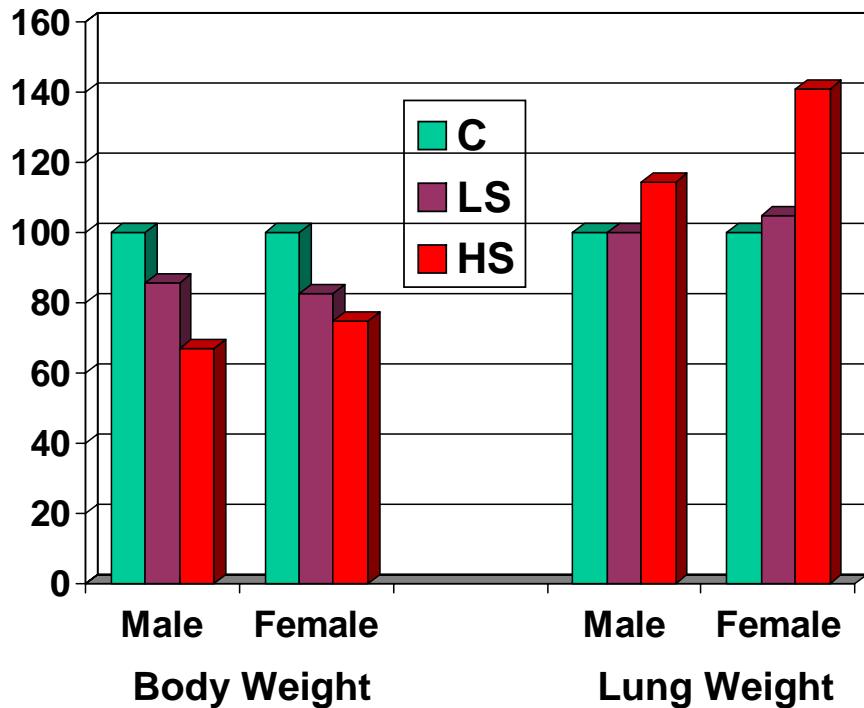
SS = sidestream CS

FA = filtered air control

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

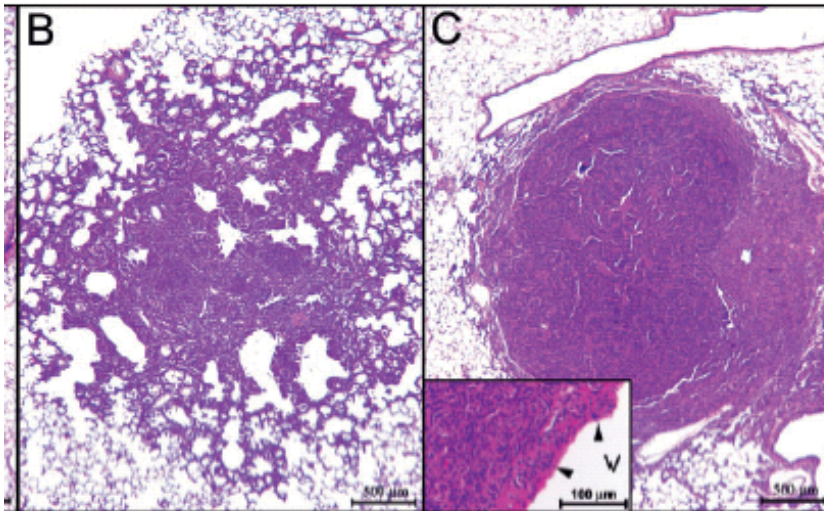
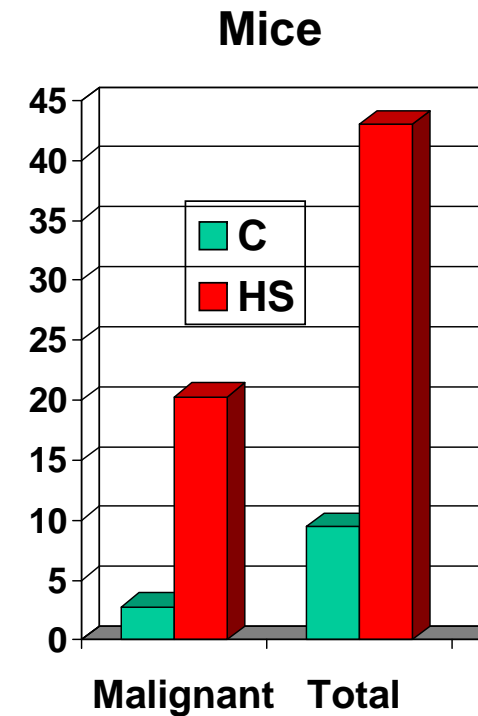
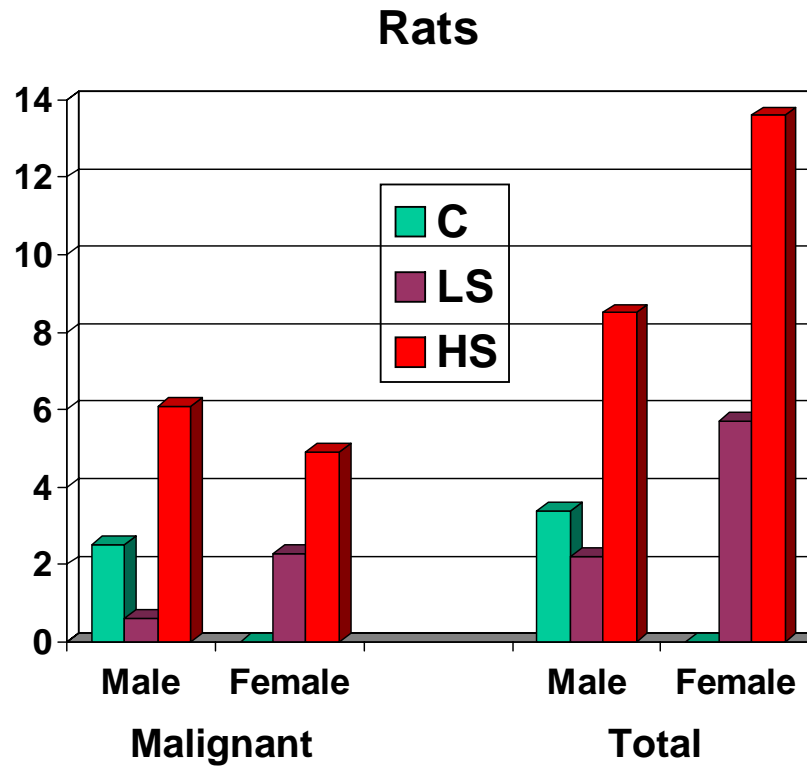
Alterations in Rats at End of Exposure

Body and Lung Weight as % of Control



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:

	<u>TGT</u>	<u>GTT</u>	<u>GAT</u>	<u>CGT</u>	<u>AGT</u>
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]

[Vuilleminot et al., *Carcinogenesis* 25: 623-629, 2004]

[Hutt et al., *Carcinogenesis*, in press, 2005]

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/m³ (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)

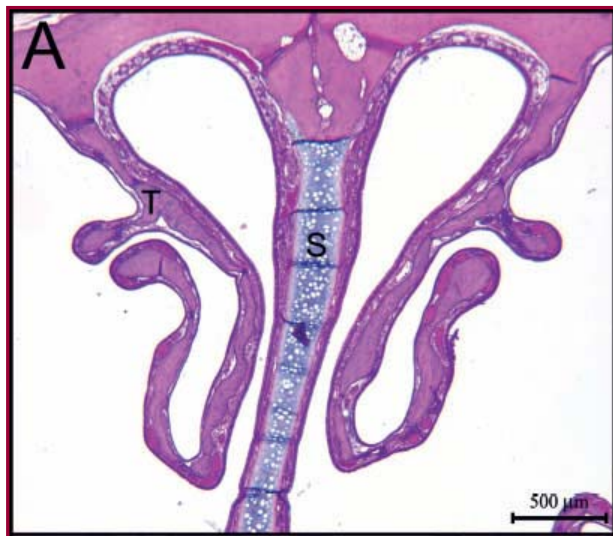
- Male and female A/J exposed 6 hr x 7 d/wk x 6 mo and held for 6 mo
- Diesel emissions or hardwood smoke at 1000, 300, 100, or 30 µg PM/m³

Results:

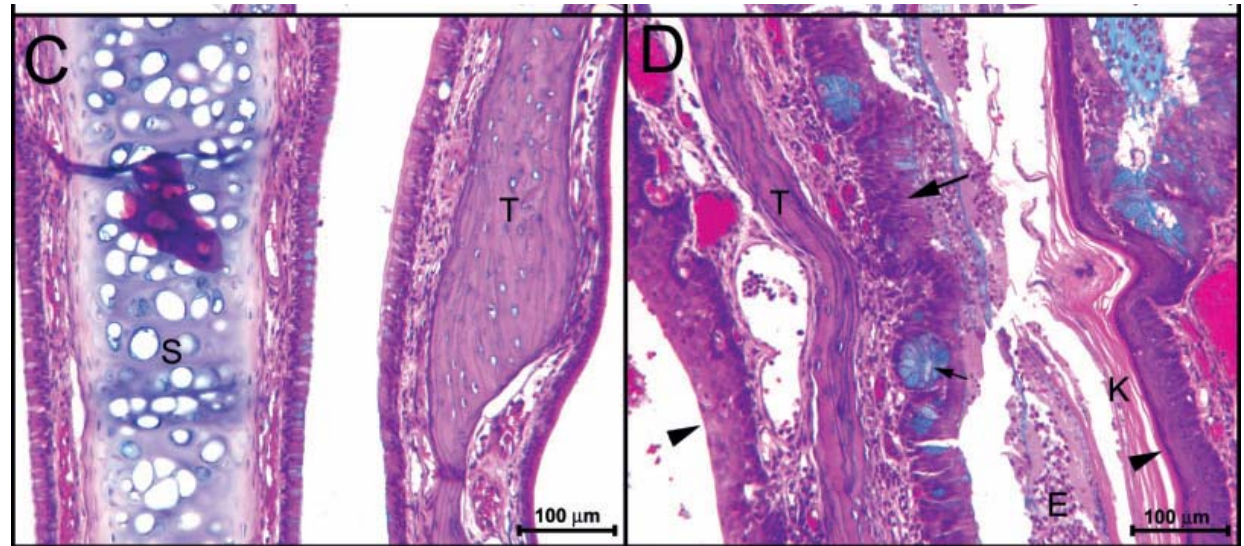
Neither exposure increased adenomas

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 inflammation with exudates (E)

Epithelial hyperplasia (large arrows)

Mucous metaplasia (small arrows)

Squamous metaplasia (arrowheads)

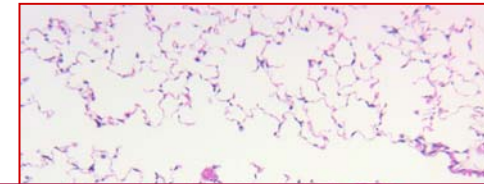
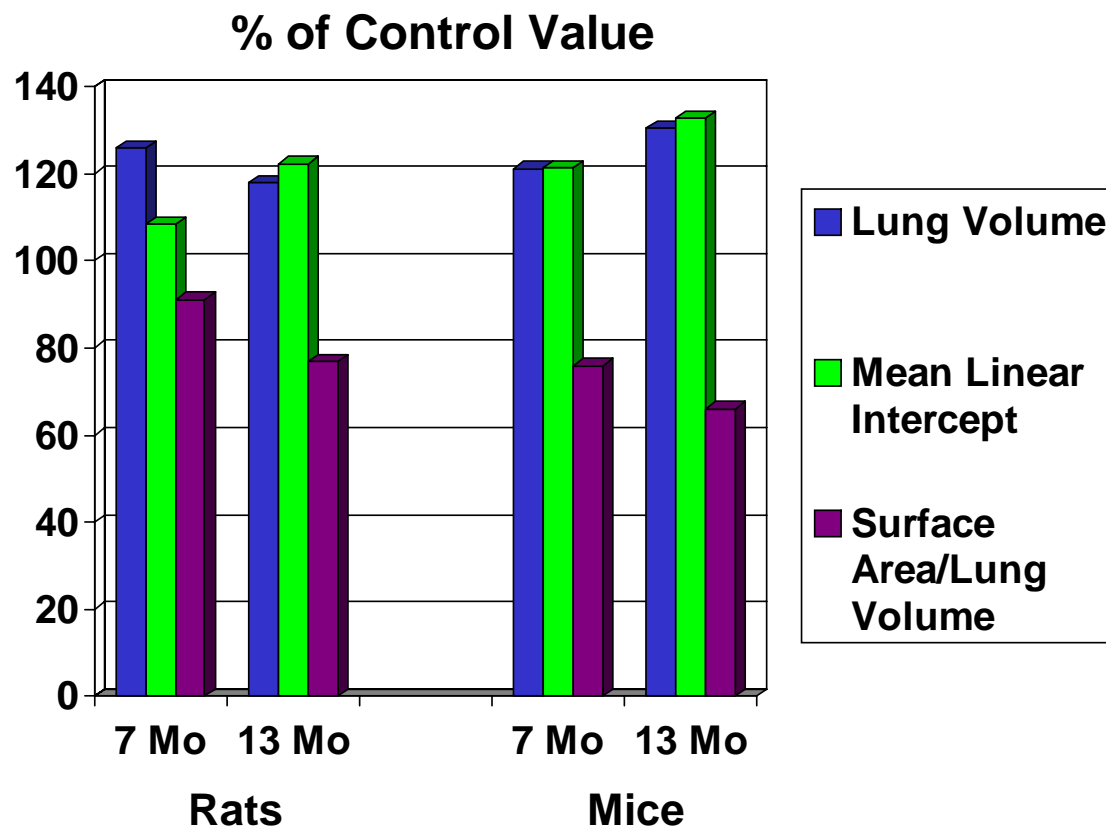
Keratinization (K)

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

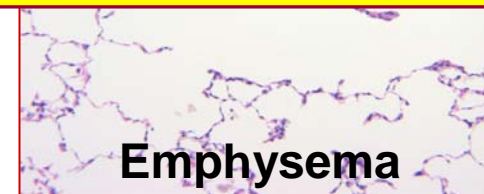
RODENT MODELS OF COPD FROM SUBCHRONIC EXPOSURE TO MAINSTREAM CIGARETTE SMOKE

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/m³ (2R1)
- Emphysema by histopathology and morphometry



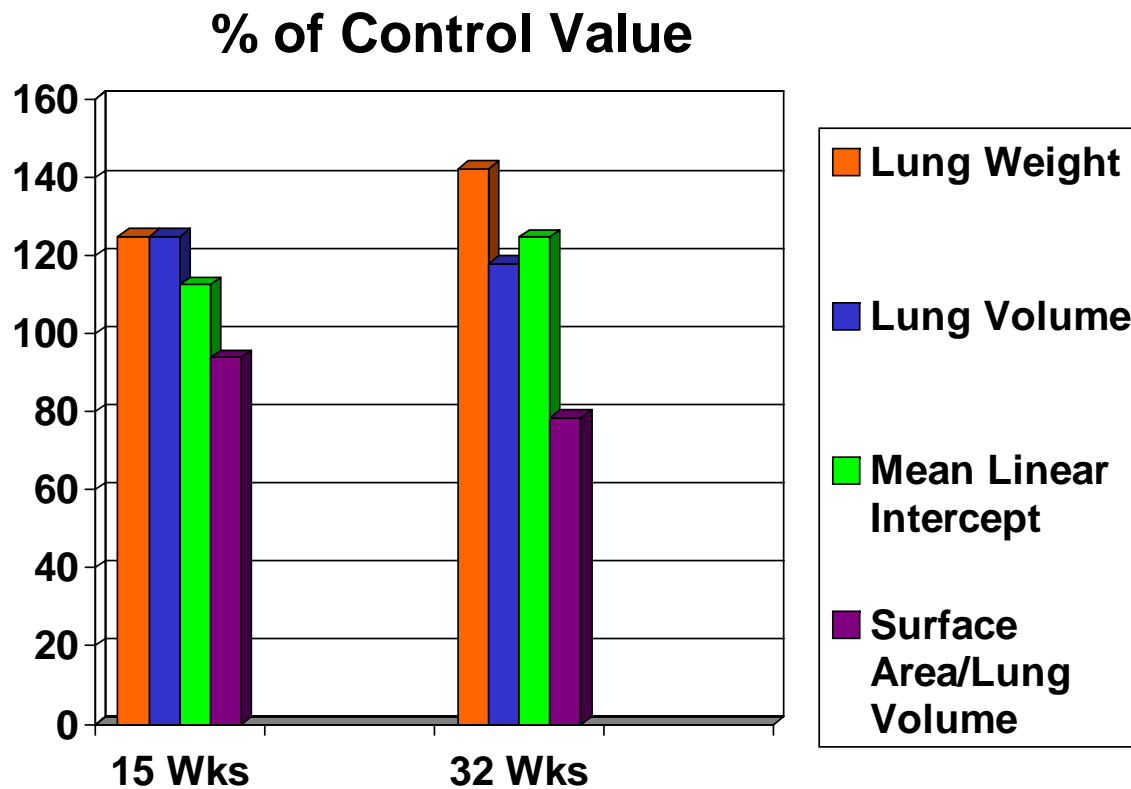
- **Emphysema in both species**
- **Some progression between 7 and 13 mo**
- **Slightly greater response in mice**



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/m³ (2R1)
- Emphysema by histopathology and morphometry

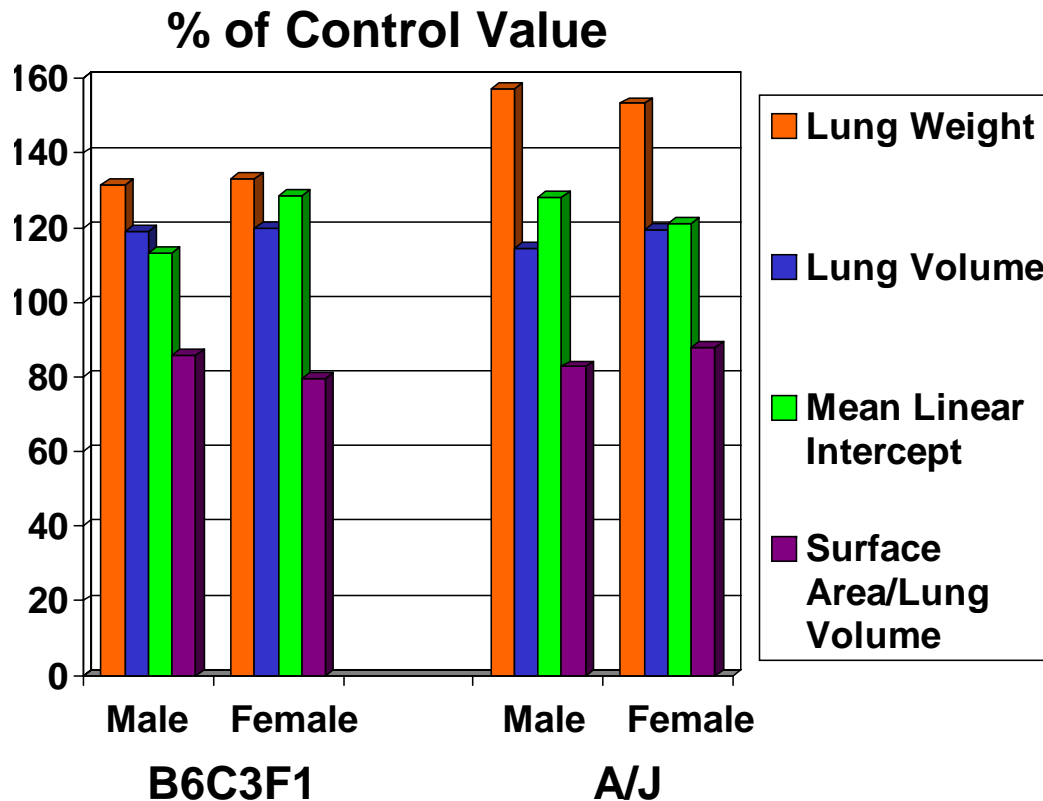


- **15 weeks produces evidence of emphysema**
- **Modest progression of all changes except lung volume**

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/m³ (2R1)
- Emphysema by histopathology and morphometry



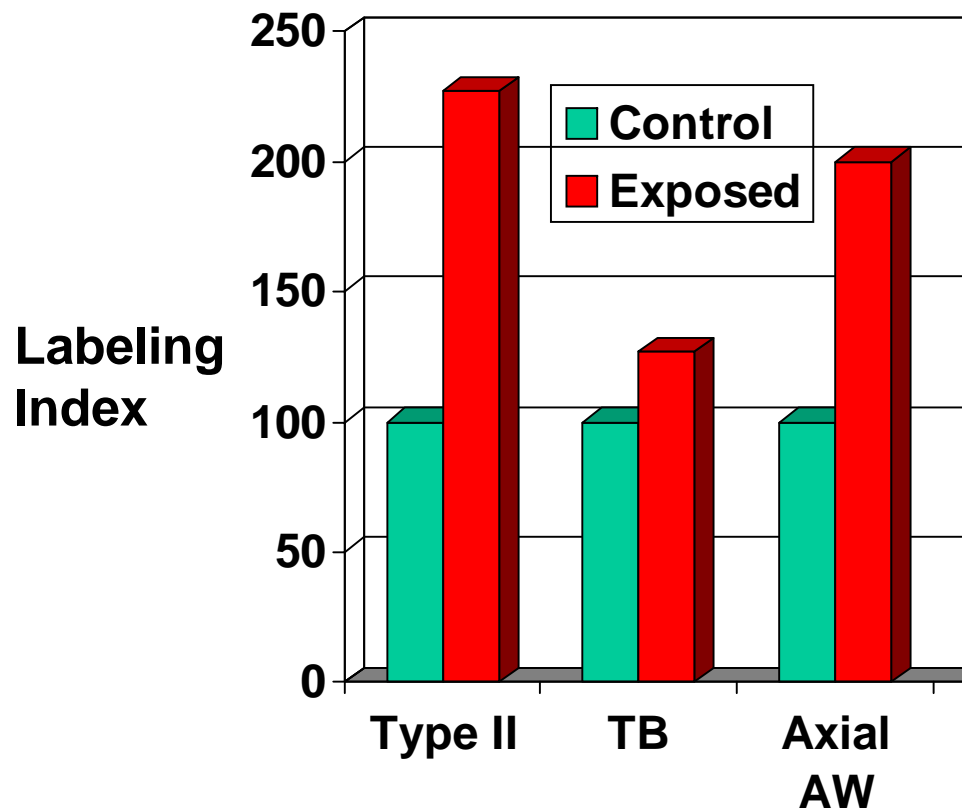
- Overall, neither strain nor gender made a large difference
- A/J had greater change in lung weight
- Authors cited A/J as more responsive on basis of Lm/BW, which was largely a function of BW

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

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/m³ (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**

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/m³ (2R1)
3. Exposed young for 4 wks to FA or SS 6 hr x 5 d/wk at 5 mg TPM/m³
4. Exposed young for 6 wks to FA, Ovalbumin (aerosol 6 hr x 5 d/wk at 5 mg/m³), 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**

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/m³ (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/m³
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

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/m³ (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 normal 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)

RODENT MODELS OF IMMUNOLOGICAL EFFECTS

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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**

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

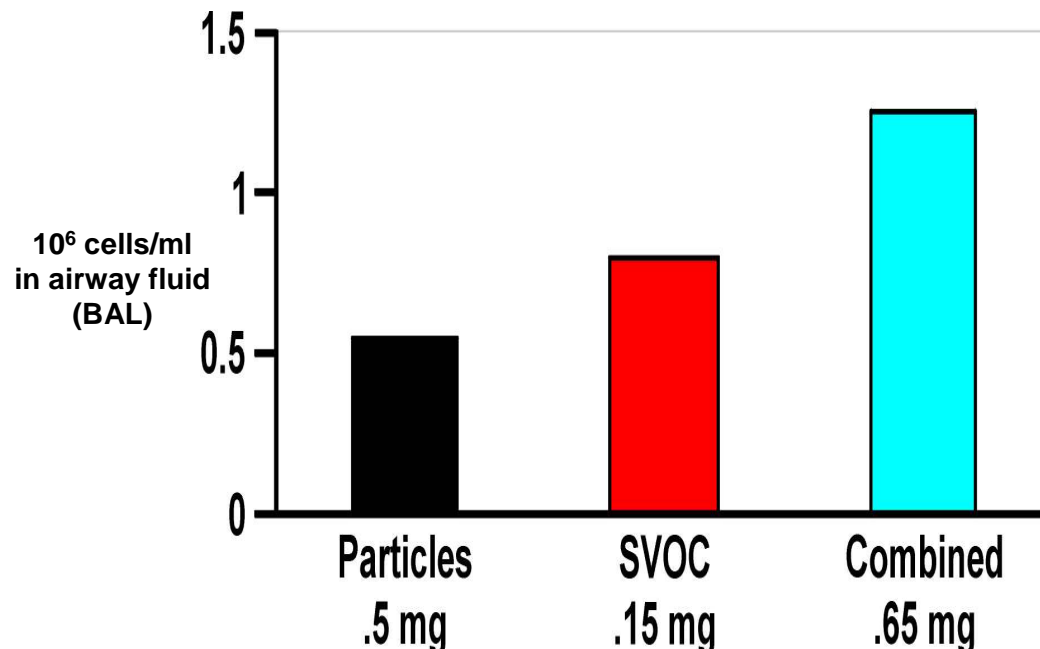
Apply multivariate analyses to data from different mixtures studied using identical protocols ?

OK – if you have such a database

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

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:

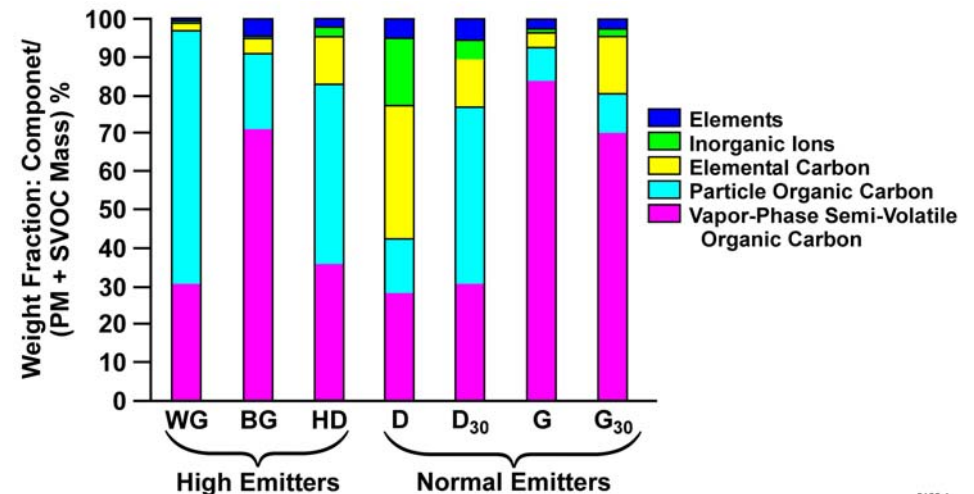
Ames mutagenicity

Inflammation after instillation into rat lungs

Samples

Gasoline (5)	G
Gasoline 30°	G ₃₀
White smoker gas.	WG
Black smoker gas.	BG
Diesel (3)	D
Diesel 30°	D ₃₀
High-emitter diesel	HD

Composition



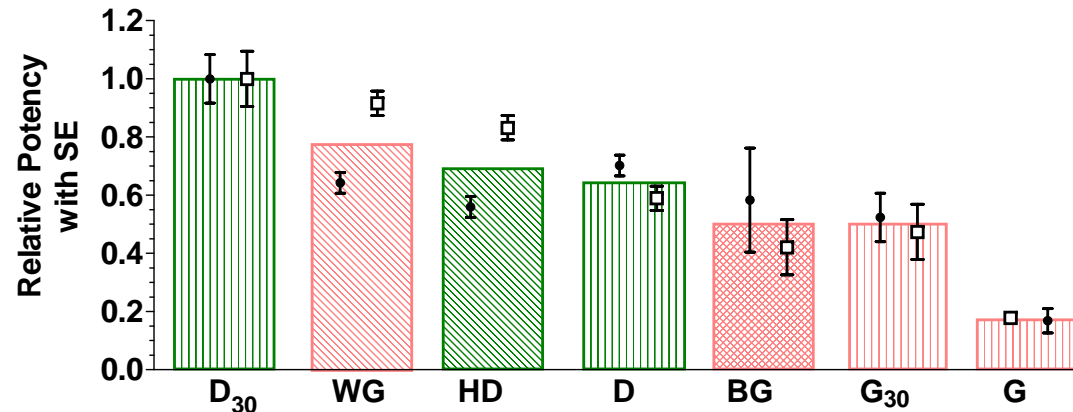
5185-1a

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

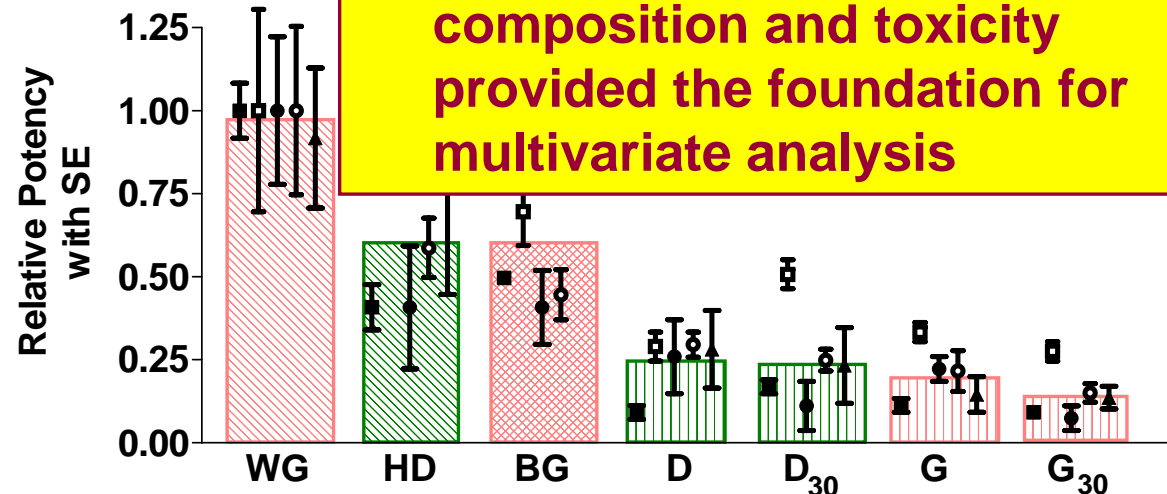
[Zielinska et al., *J. Air Waste Man. Assoc.* 54: 1138-1150, 2004]

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

Ames Mutagenicity
(TA 100 ± S9)



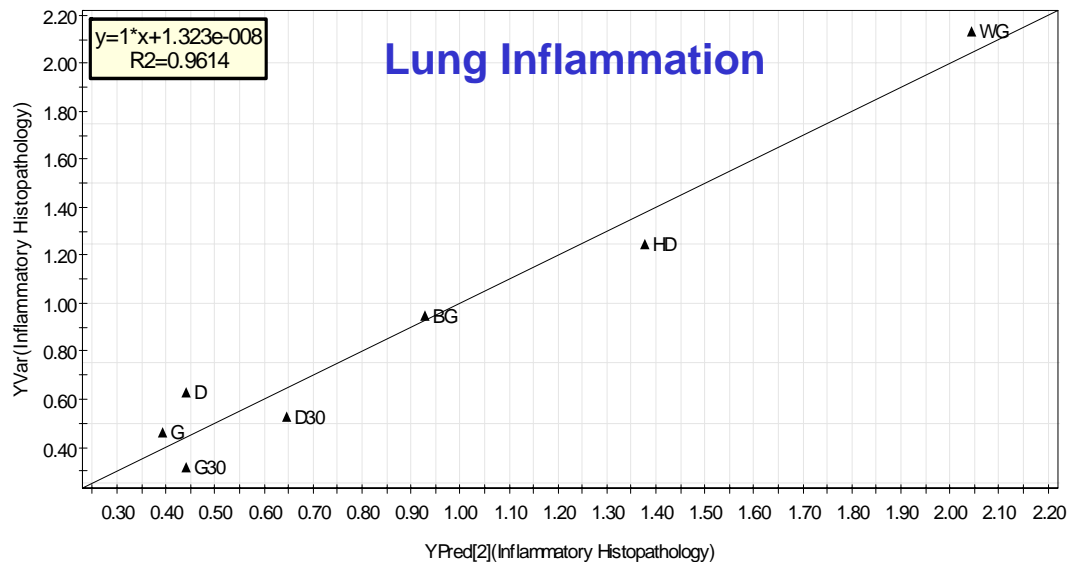
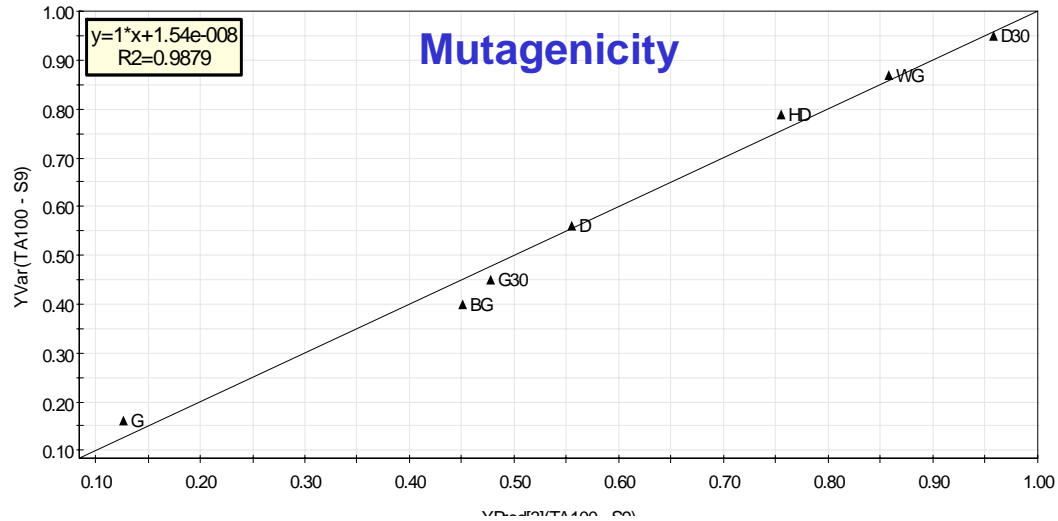
Lung Inflammation
(5 parameters)



● Substantial differences in both composition and toxicity provided the foundation for multivariate analysis

- Total leukocytes
- PMN
- Macrophages
- Histopathology
- ▲ MIP-2

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 mutagenicity (*of course*)
- Hopanes and Stearanes co-varied most closely with inflammation

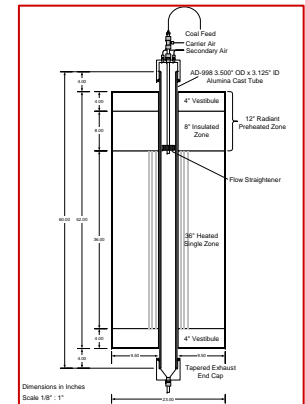
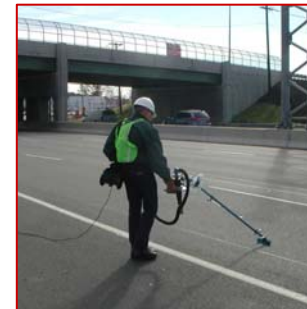
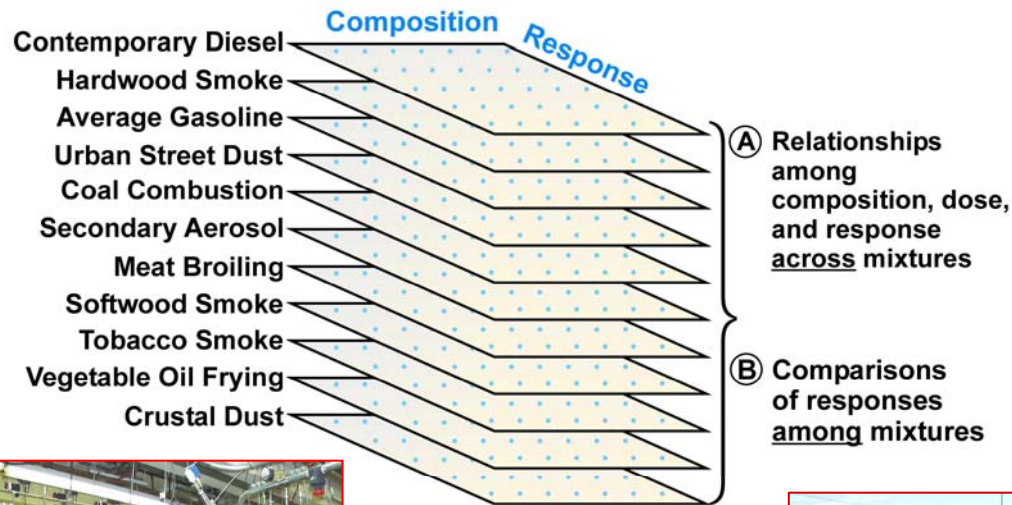
IDENTIFYING PUTATIVE CAUSAL COMPONENTS AMONG INHALED ENVIRONMENTAL MIXTURES

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

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)

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

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)