



Biological Response to Inhaled 2007 Compliant Diesel Emissions

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THE HEALTH HAZARDS AND RISKS OF DIESEL EMISSIONS HAVE BEEN DIFFICULT, INTERESTING, AND LONGSTANDING ISSUES

- Ubiquitous exposure and plausible hazards
- Health research back to at least the 1950s
- Tremendous attention since the late 1970s
 - **Limited parallel research attention to gasoline emissions**
- Generally poor understanding of emissions among health researchers
- Large epidemiological database showing associations with occupations
 - **Limited by poor documentation of actual exposures**
- Clear, but largely unusable cancer signal from animals (mechanism due to overload of particles at unrealistic doses)
- Diverse range of non-cancer effects in humans and animals from experimental exposures
- **Very little information on impacts of emission reduction strategies**
- **No information on 2007 or 2010-compliant emissions**

CANCER HAZARD

Kotin et al., *Indust. Health* 11:113, 1955 (USC)

Solvent extracts of diesel and gasoline emissions caused tumors in mouse skin painting assay

Related to source, operating condition, and aromatic hydrocarbons

Mutagenicity

- Interest heightened in 1970s with application of Ames test

Reverse mutations in *Salmonella* bacteria

Many labs studied many variables through 1980s (huge literature)

Biodirected fractionation pointed toward nitro-aromatic compounds

Carcinogenicity

- Several large-scale rodent inhalation studies conducted during 1980s

U.S., Germany, Switzerland, Japan

Results generally consistent across studies

Extreme exposures increased lung tumors in rats

No increase in mice or Syrian hamsters

- Led to recognition of “particle overload” phenomenon

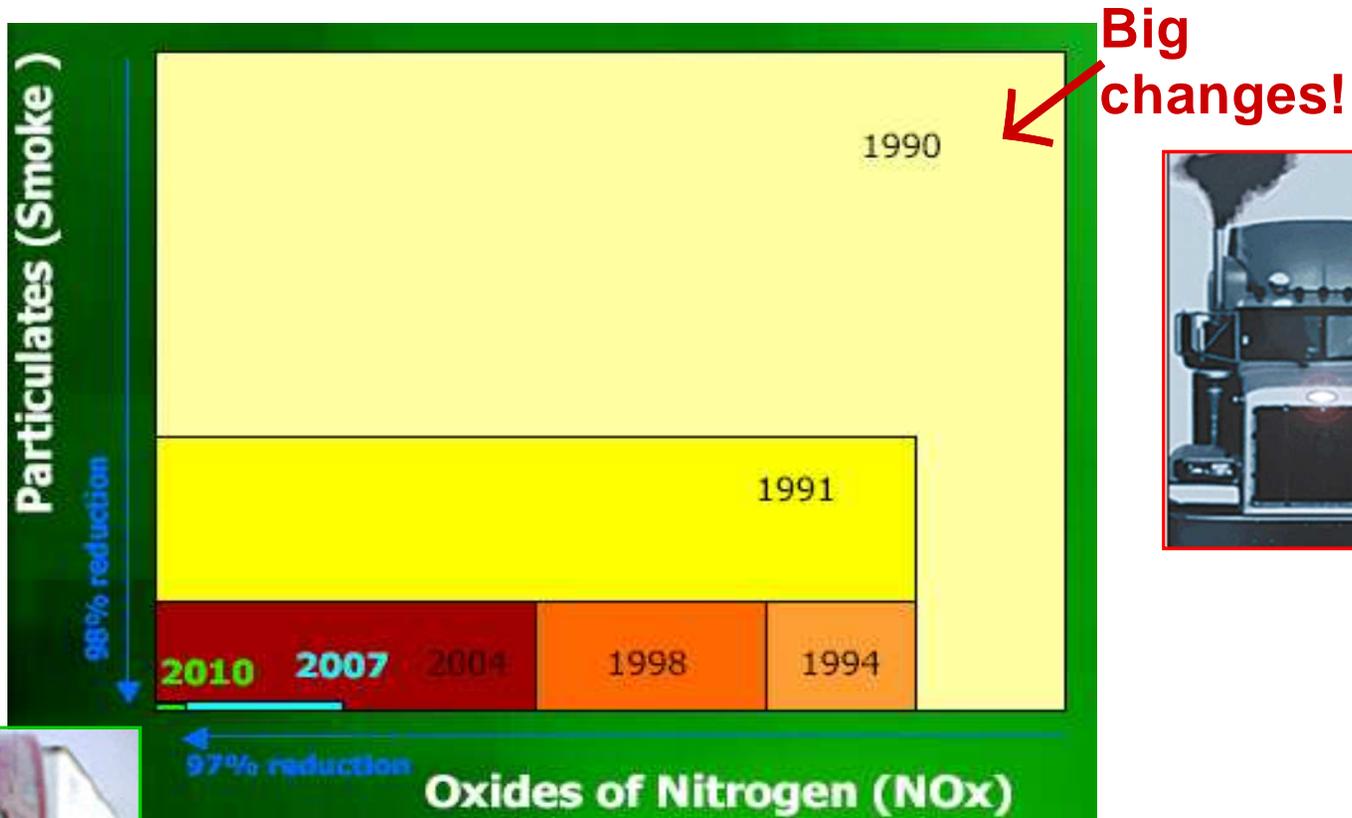
OPERATING CONDITION AFFECTS MUTAGENICITY

- 1980 GM 5.7L certification fuel, Shell 10w-30 oil
- Engine on test stand operated on Federal Test Procedure (FTP, US-72)
- Filter mass extracted in dichloromethane and tested in Ames TA98 –S9

	<u>Revertants/μg</u> <u>Extract</u>	<u>Revertants/Second</u>
Full FTP Cycle	1.4	1,000
0-55 mph acceleration	5.3	12,000
55 mph cruise	5.1	10,000
30 mph cruise	1.2	1,000
0-30 mph acceleration hot	2.7	5,000
0-30 mph acceleration cold	2.0	2,000
Idle	2.0	1,000

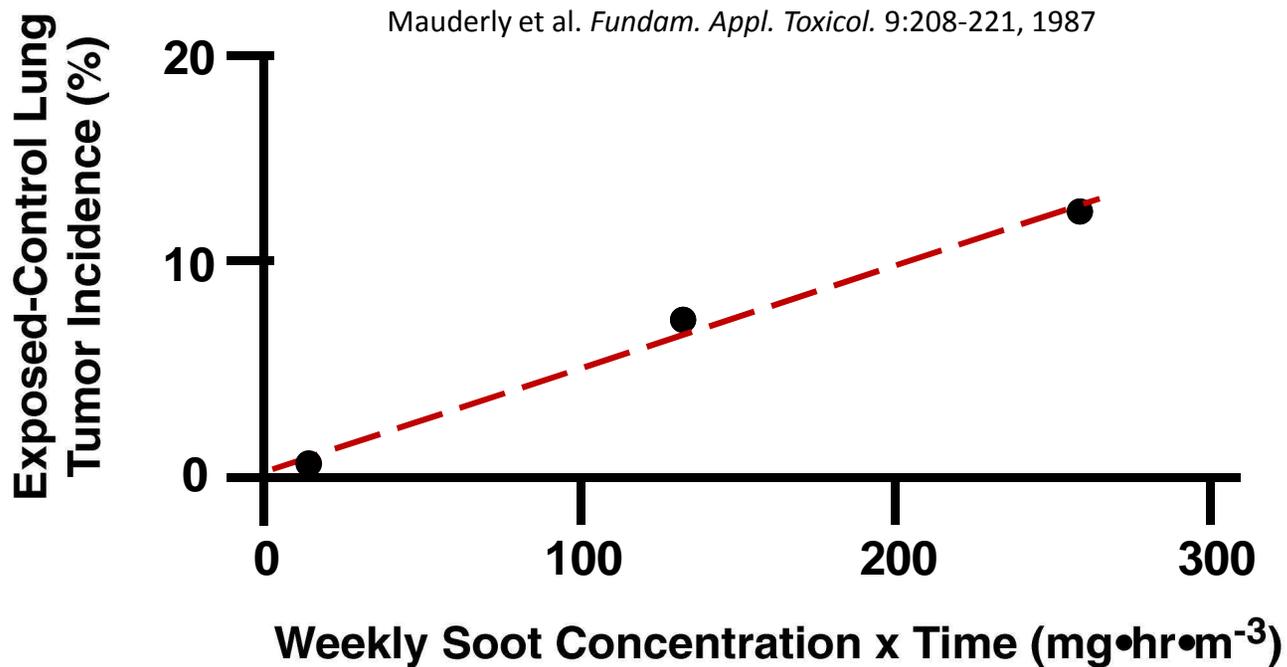
[Bechtold et al., *Fundam. Appl. Toxicol.* 4: 370, 1984]

OLD DIESEL ≠ NEW DIESEL



A LESSON FROM DIESEL ANIMAL CANCER RESEARCH: LUNG TUMORS IN RATS

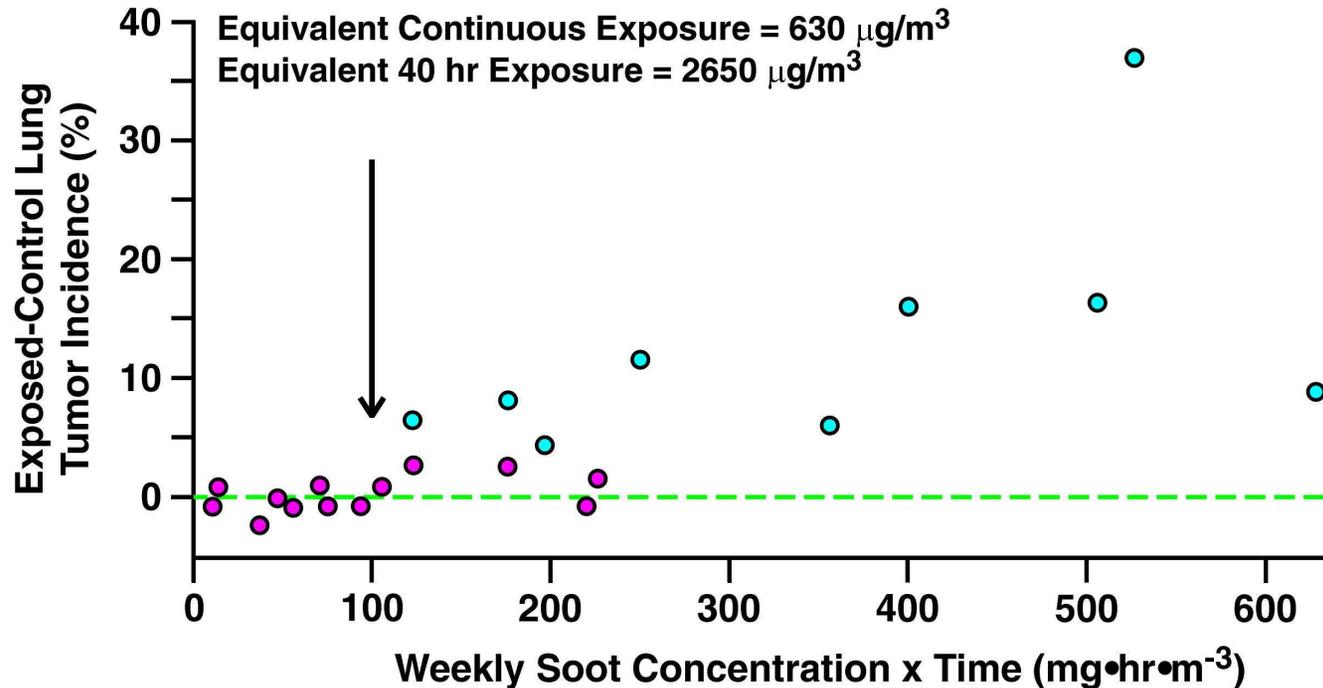
Rats exposed 7 hr/day, 5 days/wk x 30 mo to whole diesel exhaust



Some projected risks for humans exposed at much lower levels from linear extrapolation of these results

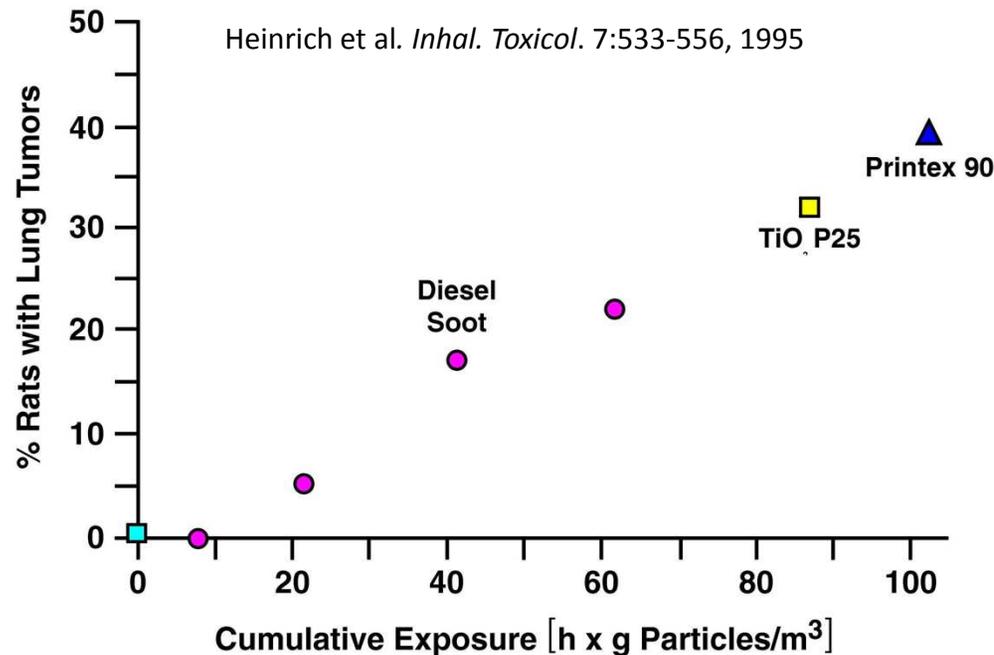
THE EVIDENCE IN ANIMAL STUDIES SUGGESTED THAT LOWER DOSES DID NOT CAUSE EXCESS TUMORS IN LIFETIME STUDIES

Mauderly, in *Environmental Toxicants*, Lippmann Ed., Wiley, 2000



Increased tumors resulted only from exposures that caused preceding chronic-active inflammation and progressive epithelial hyperplasia-metaplasia

THE LUNG “OVERLOADING” CONCEPT EVOLVED



Other poorly-soluble, respirable PM fell on the same exposure-response line in rats

Mutagen-free carbon blacks gave same result in two studies (LRR & Fraunhofer)

The same exposures were not tumorigenic in normal test strains of mice

Patterns of particle retention in the lung differ between rats and humans

Consensus evolved that results from “overloaded” rats should not be used to estimate risks to humans

- Threshold
 - Nonspecific
 - Species-specific
- } Not reliable for estimating human risk



Advanced Collaborative Emissions Study Phase 3B

RFP 06-1 primary (null) hypothesis:

Emissions ... will have very low pollutant levels and will not cause an increase in tumor formation or substantial toxic effects in rats and mice at the highest concentration of exhaust that can be used ... compared to animals exposed to clean air, although some biological effects may occur.

CORE BIOSCREENING STUDY DESIGN

Chronic Carcinogenicity Bioassay of Wistar Han Rats:

- **Expose 288/group 16 hr/day, 5 days/wk for 24-30 months**
- **3 dilutions of whole emissions + clean air controls**
- **166/group committed to carcinogenesis bioassay**
- **122/group allocated for interim evaluations at 1, 3, 12, & 24 months**

Pulmonary function (3, 12, & 24 mo)

Lung lavage, lung tissue & cell proliferation

Hematology & serum chemistry (3, 12, & 24 mo)

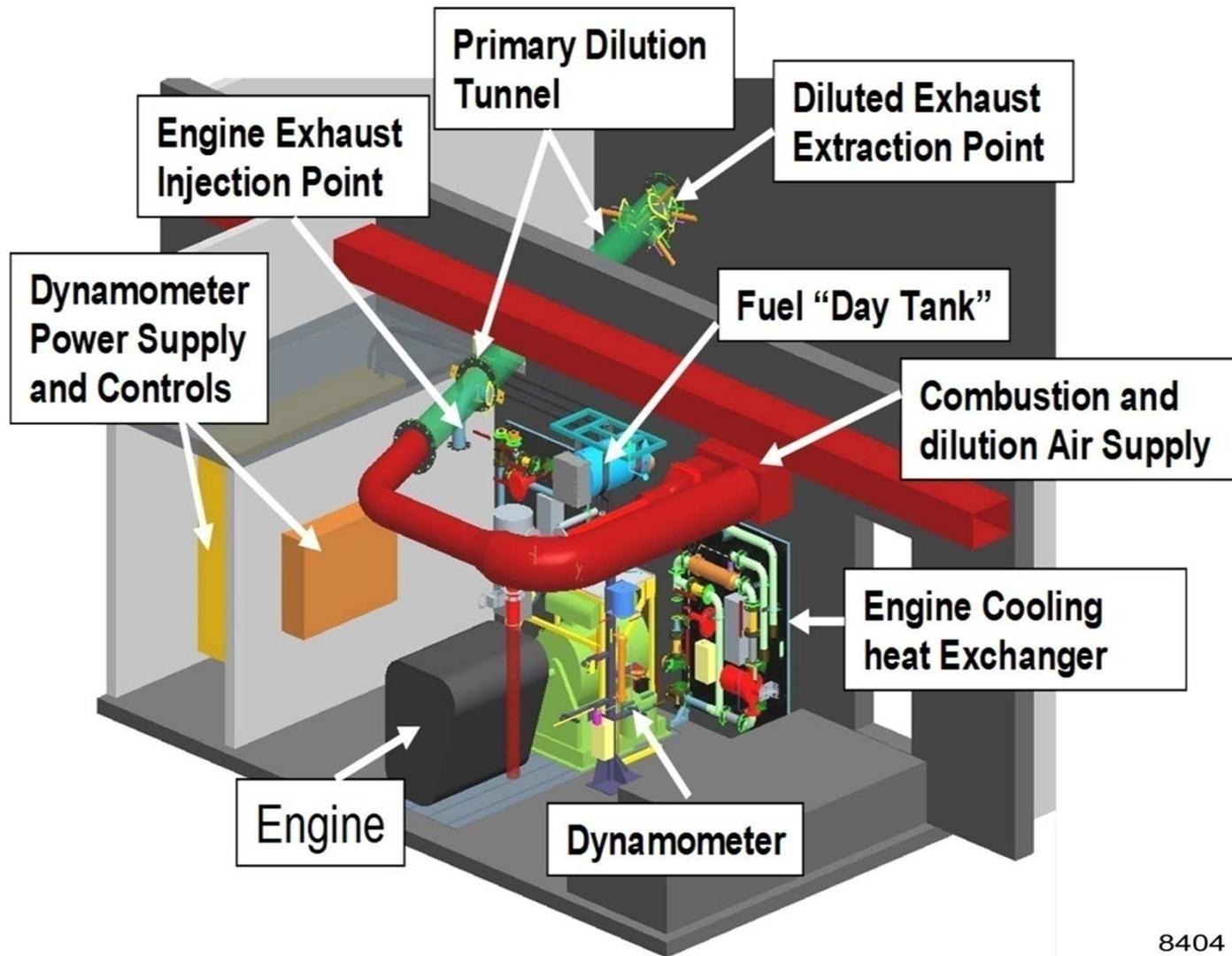
Histopathology

BIOSCREENING STUDY IN MICE

Subchronic Inhalation Study of C57BL/6 Mice:

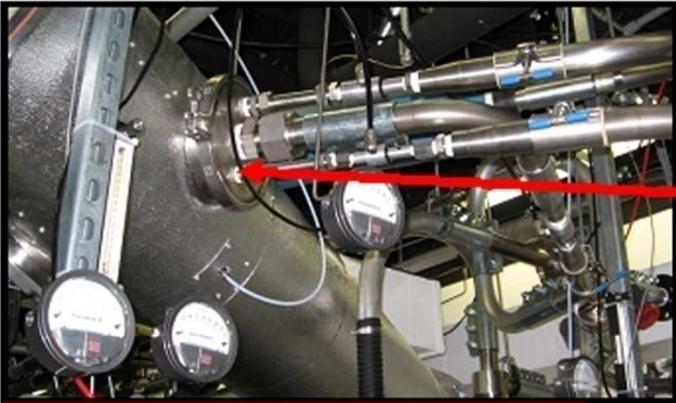
- **Expose 120 mice/group 16 hr/day, 5 days/wk for 13 weeks**
Same control and treatment groups as for rats
- **40 mice/group allocated for evaluation at 1 and 3 months**
Bronchoalveolar lavage Serum chemistry
Cell proliferation Histopathology
Hematology
- **80 mice/group allocated for ancillary health response assays**
Blood and tissue collections

EXHAUST GENERATION



8404

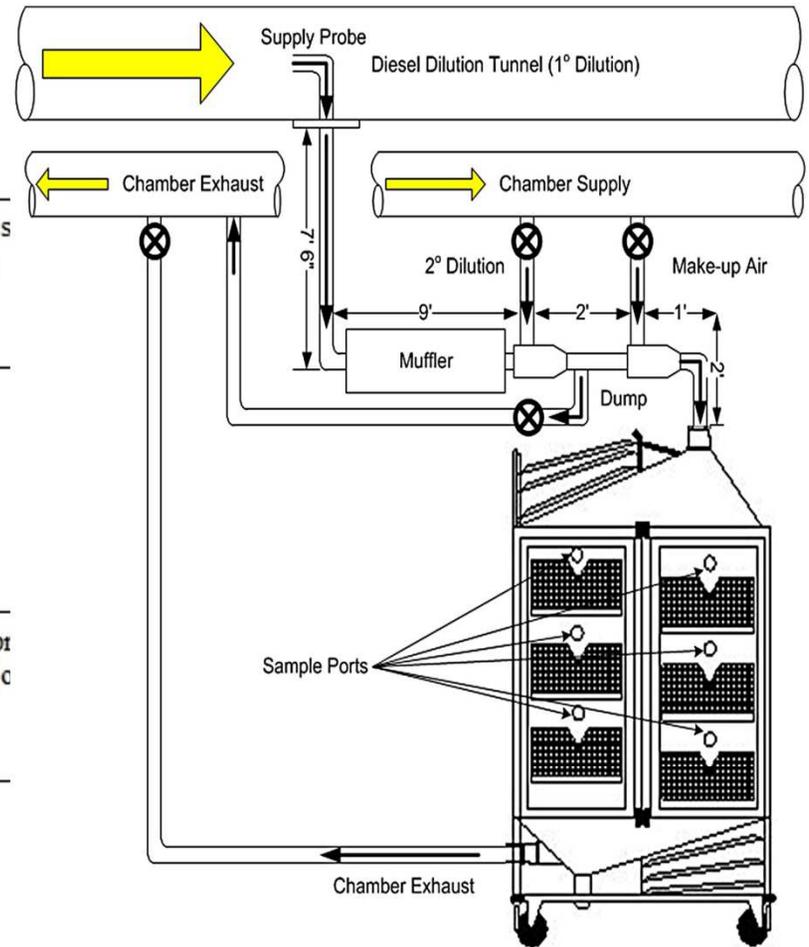
EXPOSURE SYSTEM



Exposure system proximal tunnel take-off point



Exposure system proximal inside tunnel take-off point



Drawing not to scale:

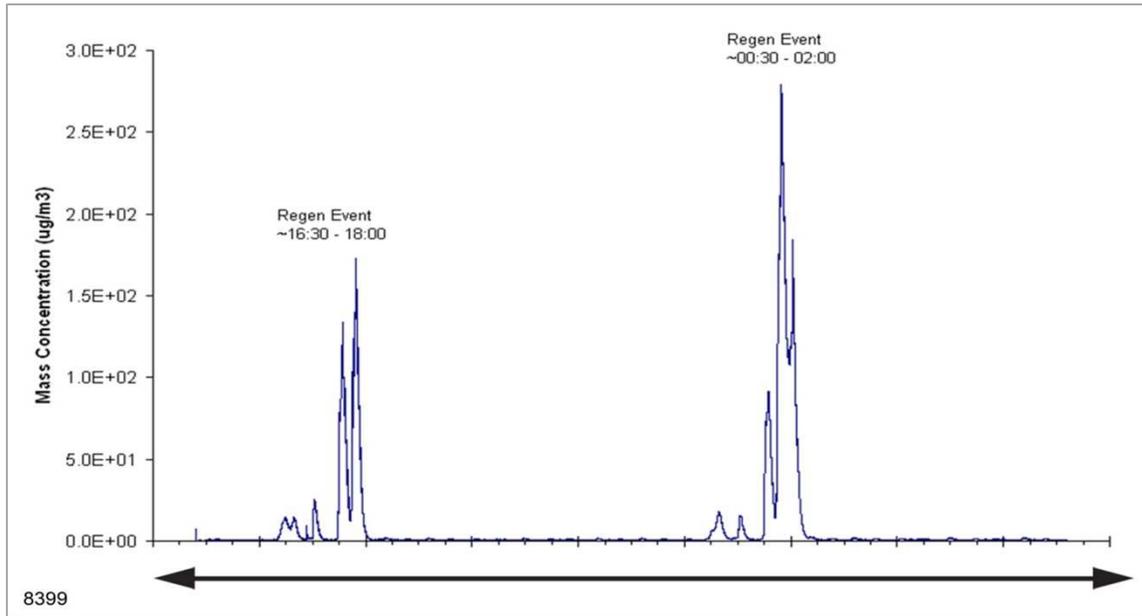
Delivery Line Dimensions:
 Tunnel to Muffler - 7' 6"
 Muffler to Dilution/Dump - 9'
 Dilution/Dump to Make-up - 2'
 Make-up to Chamber - 3'

Exposure Chamber

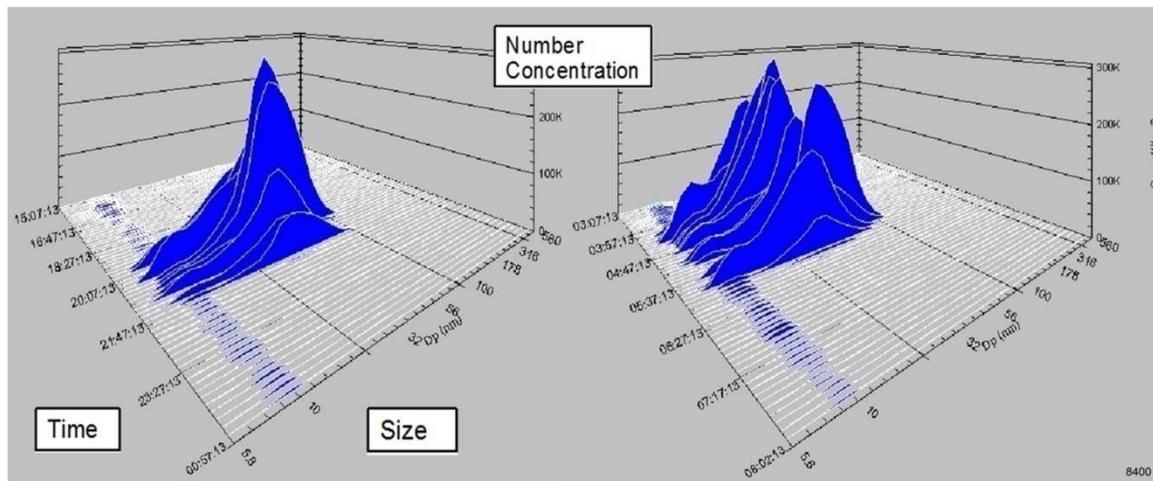
AVERAGE EXPOSURE CONCENTRATIONS: 12 MONTHS

Gases:	High		Mid		Low	
	Mean	Stdev	Mean	Stdev	Mean	Stdev
NO ₂ (ppm)	4.2	0.5	0.91	0.11	0.109	0.013
NO (ppm)	5.8	1.1	1.40	0.23	0.293	0.160
NO _x (ppm)	9.9	1.4	2.30	0.29	0.402	0.159
CO (ppm)	6.8	2.9	n/a	n/a	n/a	n/a
THC (ppm)	0.5	0.4	n/a	n/a	n/a	n/a
SO ₂	23.9	4.4				
PM (µg/m ³):						
Chamber						
Inlet (filter)	9	5	3	3	2	1
Chamber						
(filter)	27	10	31	20	21	12

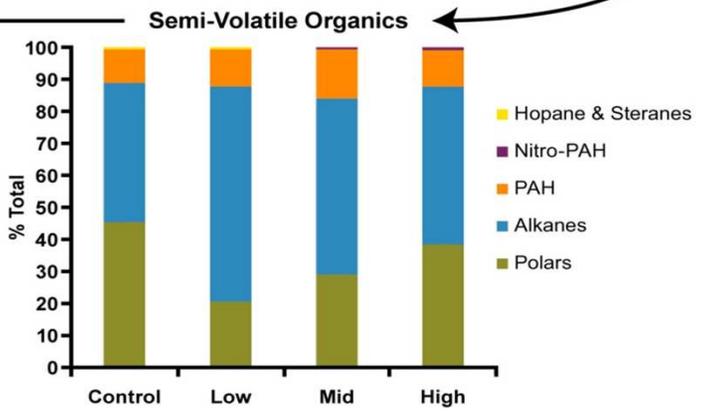
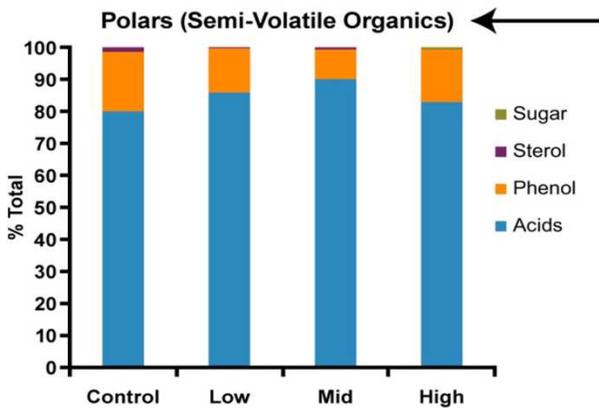
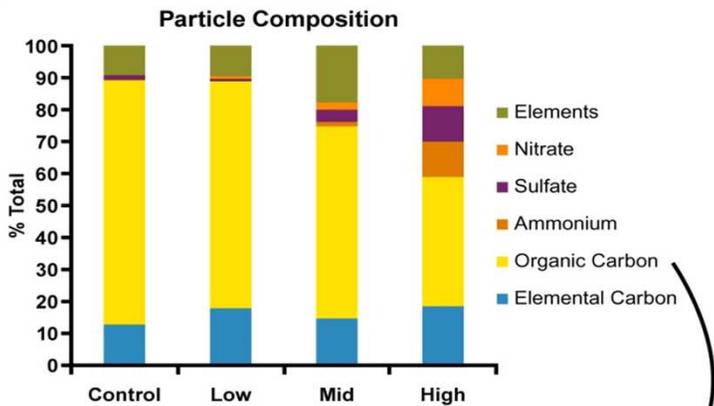
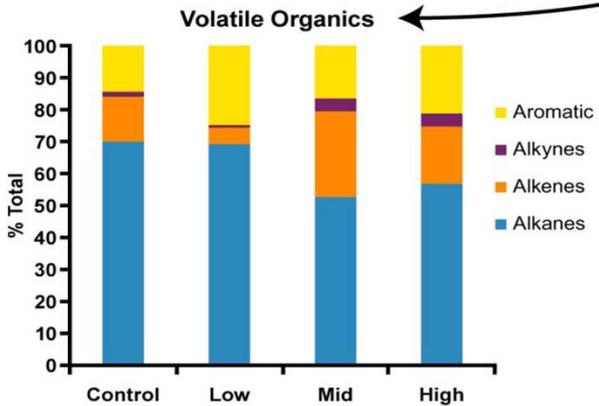
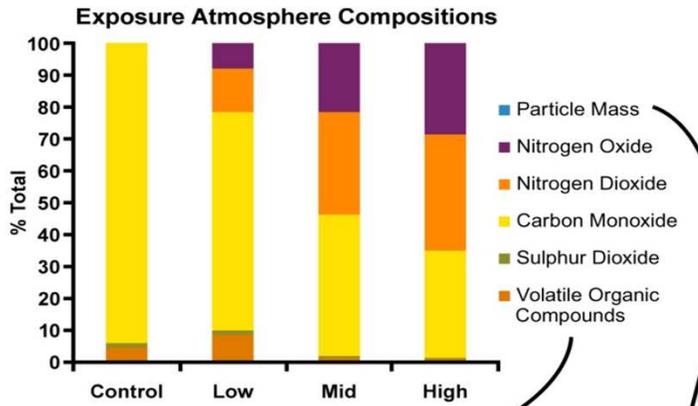
ATMOSPHERE COMPOSITION



Real-time particle mass



Real-time particle number



STATISTICAL APPROACH

ANOVA

- Experimental group, gender, group:gender interactions
 - If no significant gender difference, genders pooled
 - Dunnet's multiple comparison procedure used
 - Log transformations done on heteroscedastic data
 - Significance evaluated at $p=0.01$ and $p=0.05$

Biological Response in Rats

BIOLOGICAL RESPONSE INDICATORS

Hematology
Red Blood Cell Count
Hemoglobin
Hematocrit
Mean Corpuscular Volume
Mean Corpuscular Hemoglobin Concentration
Mean Corpuscular Hemoglobin
Platelet Count
Percent Reticulocytes
White Blood Cell Count and Absolute Differential
White Blood Cell Count
Neutrophils
Lymphocytes
Monocytes
Eosinophils
Basophils
Large Unstained Cells
Coagulation
Partial Thromboplastin Time
Prothrombin Time

Serum Chemistry
Alanine Aminotransferase (Alanine Transaminase)-Serum
Albumin
Aspartate Aminotransferase (Aspartate Transaminase)-Serum
Bilirubin (Total)
Blood Urea Nitrogen
Calcium
Chloride (Serum)
Cholesterol (Total)
Creatinine (Serum)
Glucose
Gamma Glutamyltransferase
Alkaline Phosphatase
Phosphates
Potassium (Serum)
Protein (Total)
Sodium (Serum)
Triglycerides
Calculated Variables and Ratios
Albumin/Globulin
Blood Urea Nitrogen/Creatinine
Globulin

BIOLOGICAL RESPONSE INDICATORS

Lung Lavage
Lactate dehydrogenase activity
Protein
Albumin
Hemoglobin
Alkaline Phosphatase
Total cell counts/differentials
Total antioxidant capacity
Sodium (Serum)
Triglycerides
Lung Tissue
IL-1 β
TNF α
MIP-2
KC
IL-6
Oxidized/Reduced Glutathione
Heme oxygenase-1
8-Hydroxy-Guanosine
Cell proliferation

Pulmonary Function (Rats only)
Quasistatic Chord Compliance
CO Diffusing Capacity/Alveolar Volume
Forced Expiratory Flow
Mean Mid Expiratory Flow
Quasistatic vital capacity
Forced Vital Capacity
Other
Clinical Observations
Mortality
Body Weight
Organ Weights
Tissue Histopathology

Findings: Rodent 1, 3 and 12 Month Sacrifices

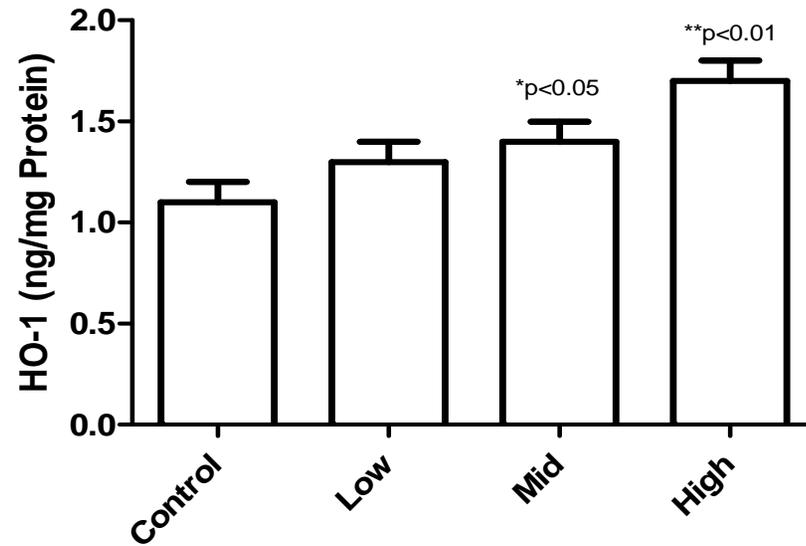
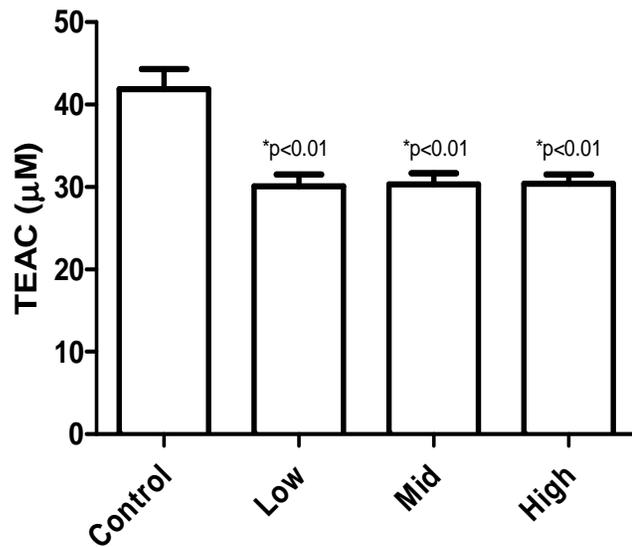
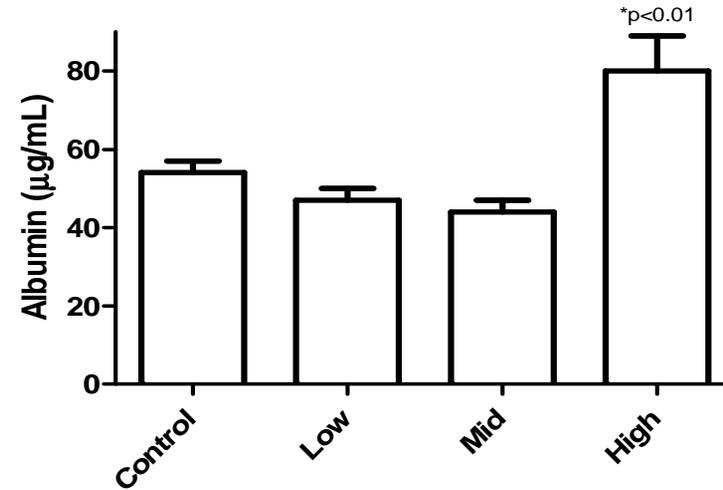
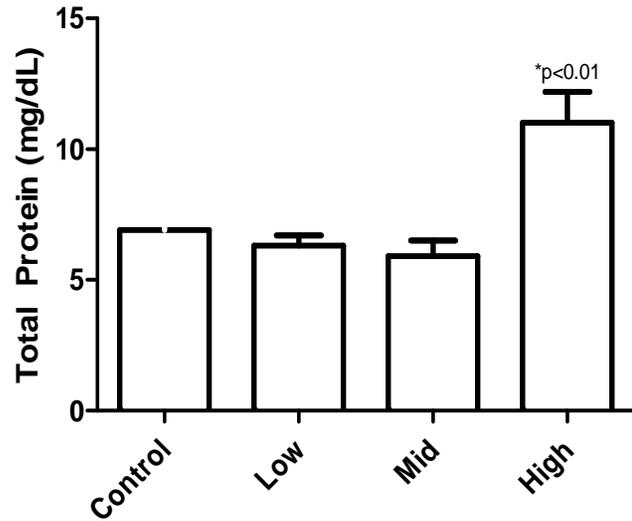
The majority of the analyses showed no difference between diesel exhaust exposure and clean air control.

Histopathology analysis revealed mild/minimal exposure-related hyperplasia in the rats after 3 months of exposure, but not in mice. The hyperplasia increased at 12 months, but was still considered mild/minimal severity.

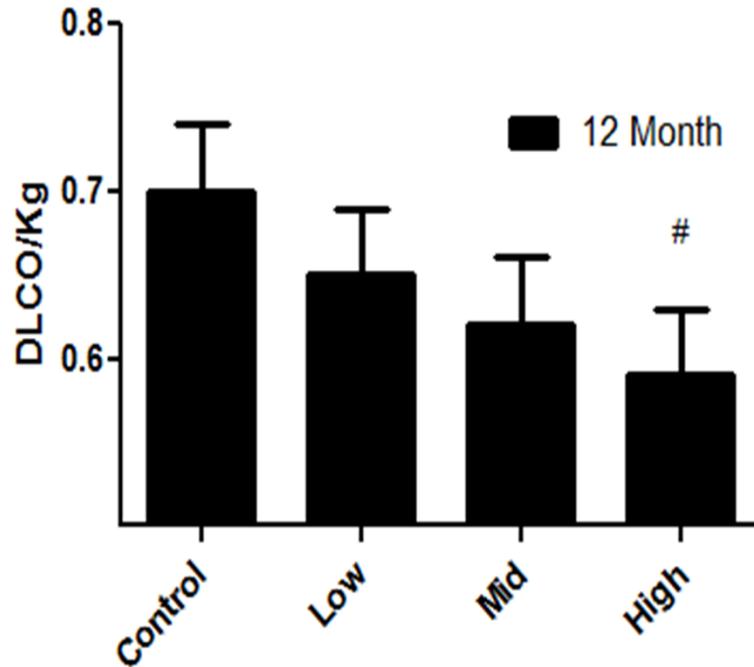
Statistically significant findings were noted for several indicators of pulmonary stress and inflammation in rats and mice (fewer findings in mice).

Pulmonary function assessments in rats showed slight differences in exposed rats compared with control after 3 months of exposure.

INFLAMMATORY/OXIDANT RESPONSE IN RATS

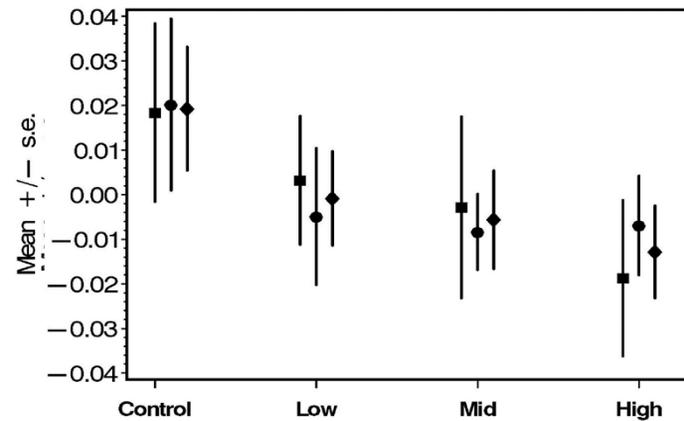


RESPIRATORY FUNCTION IN RATS



Significant ($p < 0.05$) trend observed for DLCO g at 3 and 12 months
Only reached significance when genders were combined in analysis

No statistically significant findings for Other parameters at 12 months (FVC and MMEF decreased slightly at 3 months only).



HISTOPATHOLOGY IN MALE RATS AT 3 AND 12 MONTHS

Incidence and Types of Findings Note all findings considered minimal/mild severity (~1 on 1-4 scale)

Males 3 Month

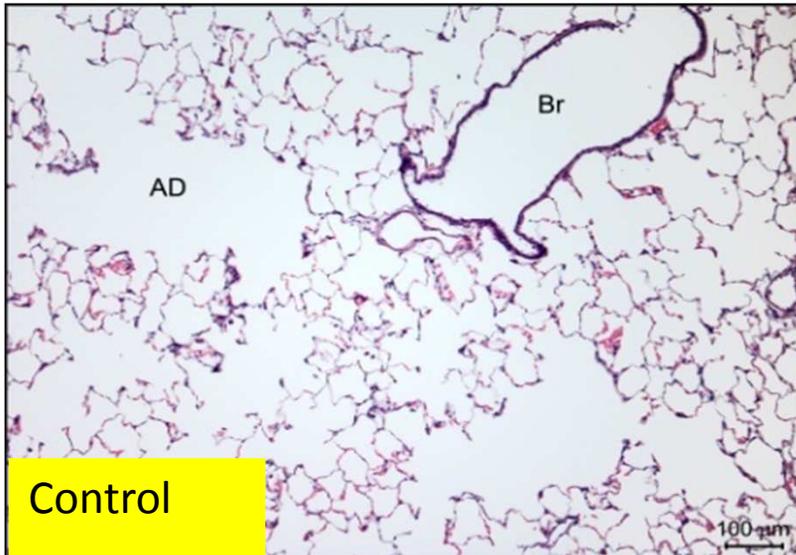
Lung	Control	Low	Mid	High
Hyperplasia Epithelium Periacinar	0/10	0/10	0/10	10/10
Accumulation Macrophage	0/10	0/10	0/10	3/10
Fibrosis Interstitial	0/10	0/10	0/10	4/10

Males 12 Month

Lung	Control	Low	Mid	High
Hyperplasia Epithelium Periacinar	0/10	0/10	0/10	10/10
Accumulation Macrophage	0/10	0/10	0/10	Not evaluated yet
Fibrosis Interstitial	0/10	0/10	0/10	10/10

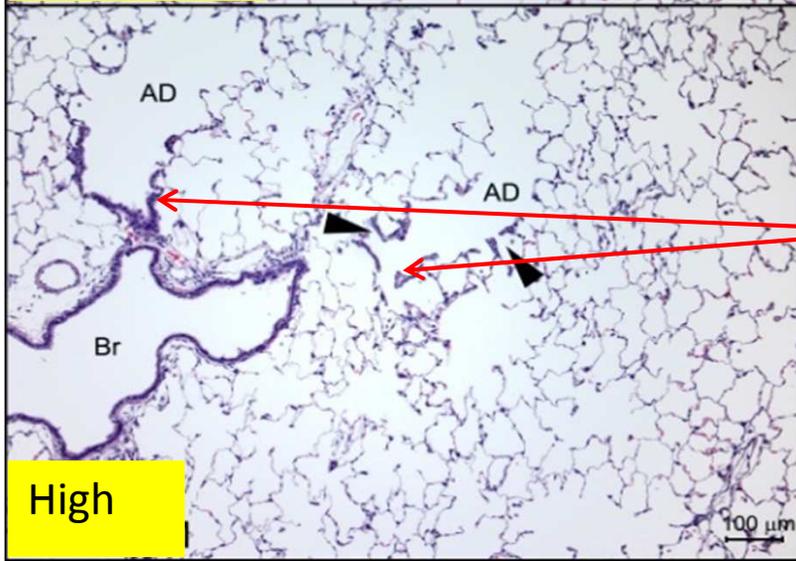
Initial read shows similar incidence trend at 12 months for Hyperplasia. However increased fibrotic tissue found.

HISTOPATHOLOGY IN RATS AT 3 MONTHS



Epithelial hyperplasia observed at high exposure level (associated with alveolar ducts)

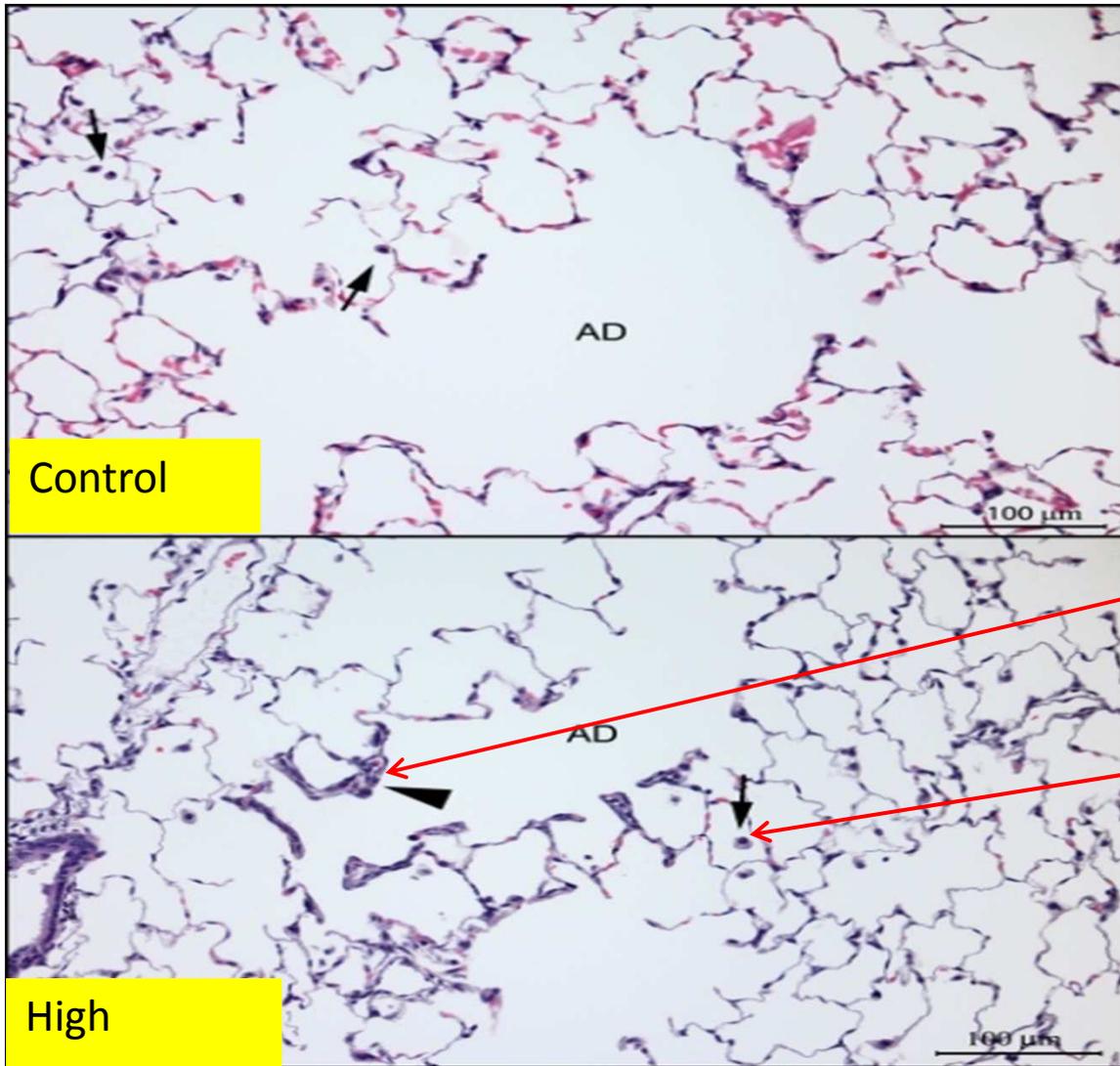
Findings generally mild



Thickening of alveolar duct septae

AD = Alveolar Duct; Br = Bronchiole

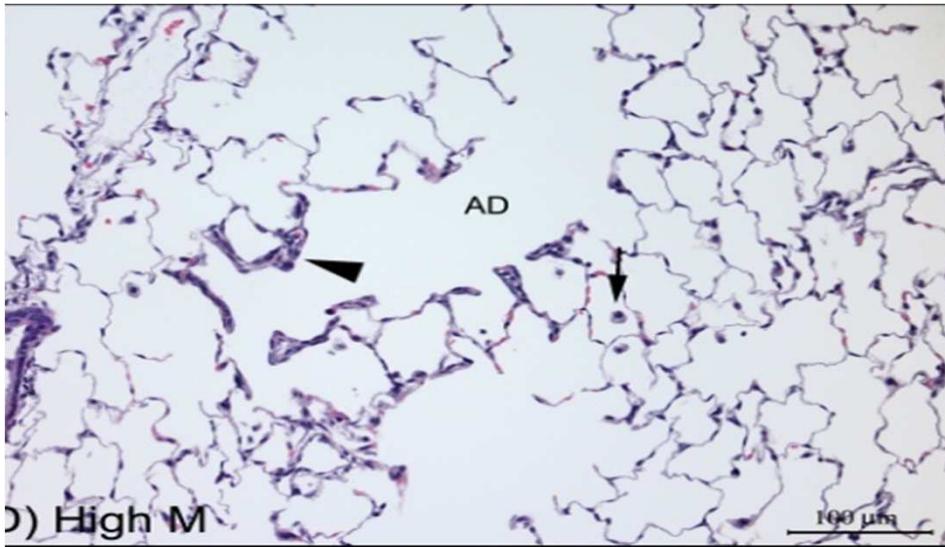
Histopathology in Rats at 3 Months: Higher Power View of Previous Slide



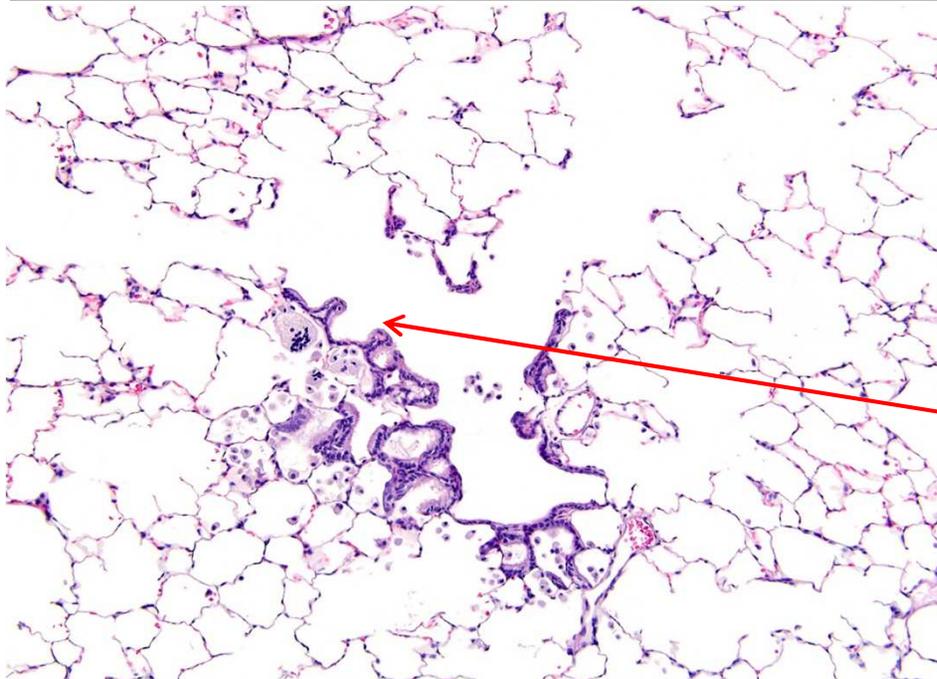
Thickening of alveolar duct septae

Macrophage

HISTOPATHOLOGY IN RATS AT 3 AND 12 MONTHS:



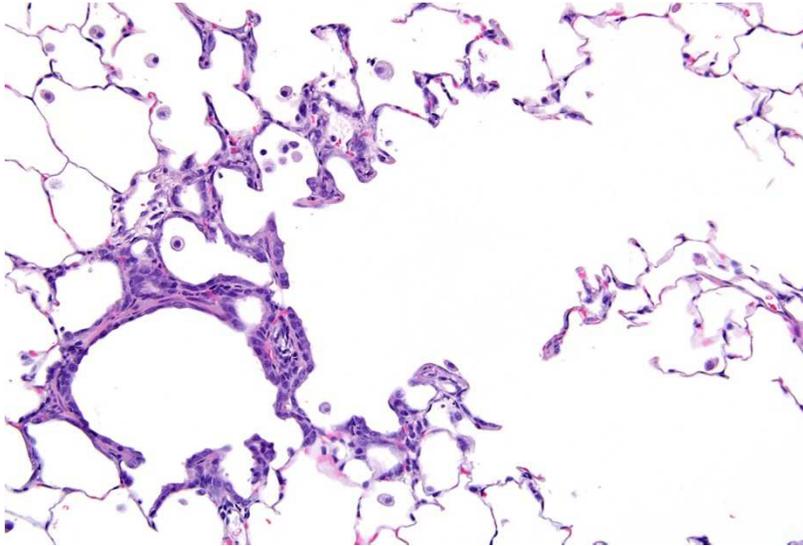
3 months



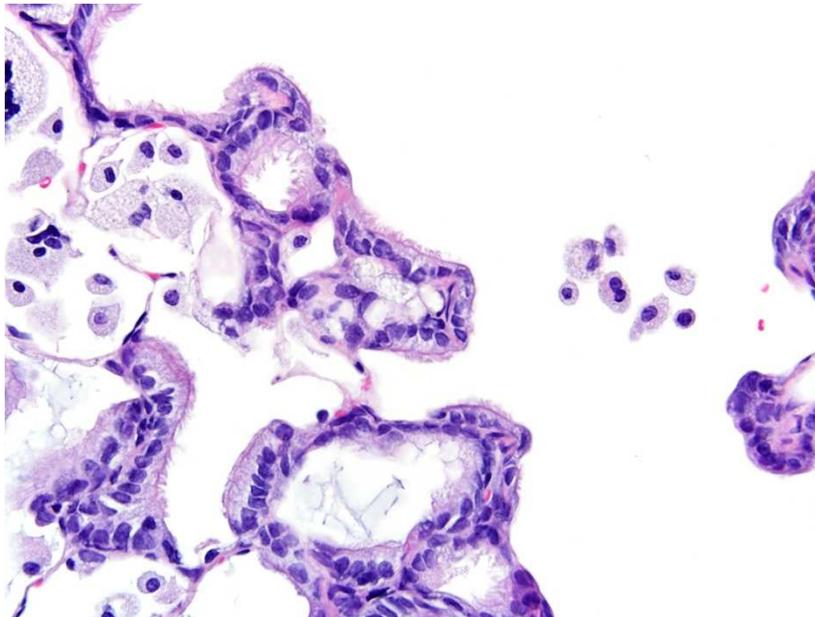
12 months

Enhanced epithelial hyperplasia at 12 months. Only observed at high level, but in both sexes.

HISTOPATHOLOGY IN RATS AT 12 MONTHS:



Thickened septae with slight increase in fibrous tissue



Alveolar duct area showing bronchiolization. Note cuboidal ciliated cells in alveolar region.

Role of NO₂ in Observed Effects?

When HEI designed the study, it was expected that at the high concentration (16 hr/day 4.2 ppm NO₂) some NO₂-related effects may be observed. This was based on results of previous studies, including:

HEI Study (Mauderly et al., 1989) F344 rats exposed (7hr/day, 5 days/week) to 9.5 ppm NO₂

Pulmonary function, histopathology, and, immune response assessed after 12, 18, 24 mo (1820, 2730, 3640 hr) of exposure

Findings: NO₂ caused epithelial hyperplasia, thickening of walls of terminal bronchioles, inflammation, and oxidative stress. There was little effect on respiratory function.

Effects at 12 mo not significantly different than at 24 months

How do the NO₂ “doses” compare at 12 mo?

Mauderly et al:17,290 ppm-hr.

ACES: 17,472 ppm-hr

SUMMARY

- **2007 compliant diesel exhaust studied in the ACES program shows low particle emissions, primarily during the regeneration of the diesel particle trap. The emissions have low volatile and semivolatile organics, and an increase in the proportion of NO₂ compared with older technologies.**
- **Exposures produced mild to no response in the mice and minimal inflammatory, tissue remodeling and respiratory function changes in rats.**
 - **Tissue injury was considered minimal (1 on scale of 1-4)**
 - **Statistically significant findings observed primarily at high level**
 - **Respiratory function effects were trends only, and only significant when genders were pooled to enhance statistical power**
- **Remainder of study under way**

Study Schedule

72	6/13/11	Rats reach 72% survival
73	6/20/11	Female rats reach 400g – move to R16 cages
120	5/14/12	Pulmonary function block D
121	5/21/12	Pulmonary function block E, 104 wk sacrifice block D (16, 16 = 32)
122	5/28/12	Pulmonary function block F, 104 wk sacrifice block E (12, 12 = 24)
123	6/4/12	104 wk sacrifice block F (12, 12 = 24)
138	9/17/12	Rats reach 60% survival
147	11/19/12	Terminal (30 mo) sacrifice block D
148	11/26/12	Terminal (30 mo) sacrifice block E
149	12/3/12	Terminal (30 mo) sacrifice block F
175	6/3/13	Submit draft comprehensive Final report on all results from Phase 3B
186	8/19/13	Estimate complete final revision of final report

Bioassay Team

Jake McDonald	LRRI	Principal Investigator and Exposure Operations
Judy Chow	DRI	Analytical Chemistry
Nancy Crowley	LRRI	Database Manager
Melanie Doyle-Eisele	LRRI	Study Director
Jennifer Roberts	LRRI	Quality Assurance
Andrew Gigliotti	LRRI	Necropsy, Histology, Histopathology
Joe Mauderly	LRRI	Advisor and Pulmonary Function
Rodney Miller	EPL	Histopathology
JeanClare Seagrave	LRRI	Bronchopulmonary Lavage & Cell Proliferation
Steve Seilkop	SKS	Biostatistician
Cheryl DiCarlo	LRRI	Attending Veterinarian & Animal Care
Barbara Zielinska	DRI	Analytical Chemistry